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Honored Life Member
John B. Glude

John Glude was born August 2, 1918 to William and Florence Glud of Silverdale, Washington. William Glud worked at the Bremerton Naval Shipyard and Florence worked as an elementary school teacher. Growing up in the Silverdale area with his brother Clarence, John got his first taste of aquaculture when his father had two ponds on the property where trout were raised. The fact that he was able to be involved with raising trout got him interested in aquaculture, which he continued to pursue and later became known for.

Along with his skills related to farming, building, and raising fish, he helped his father create an innovative system for generating electricity by taking advantage of the changing elevation of the stream entering their property. A wooden sluice carried water from the stream to the pond. There the water was released, falling into cups on the paddle wheel they had constructed. Through a series of axles and gears, enough force was created to generate all the electricity they needed. They were the first in the area to have electric lights.

John attended grade school and high school in Silverdale from which he graduated in 1935 as class salutatorian at the age of 16. He then entered the University of Washington received his Bachelor of Science degree in Fisheries in 1939, with a major in Fisheries and a minor in Engineering. He then got his first job with the Washington Department of Fisheries (WDF), but left briefly during World War II. He put his education in engineering to work by taking a job as a naval architect draftsman at the Tacoma Naval Shipyard. His work was considered vital to the war effort. After the war was over, he returned to his position as a Fishery Biologist with the WDF. He was then involved with research to determine the effects of polluted waters from pulp mills on oysters. This work formed the basis for regulatory actions to reduce sulfite liquor waste from pulp mills, which set the stage for assisting in the preservation of the valuable oyster resources in the state of Washington.

After the war, John was sent to Japan to inspect sea oysters for export to the United States to prevent contamination and/or introduction of undesirable organisms to the west coast oyster growing areas. Cedric Lindsay was a colleague of John's with the WDF who also went along with him on some of the early trips to Japan to inspect seed oysters for importation to the United States. These trips ultimately led to major seed importation to the Pacific coast of the United States and thus maintained the major oyster fisheries until recent years when seed shipment from Japan was no longer necessary. There John learned a great deal about the various Japanese methods for culturing oysters and other species which he brought back to the United States to share with growers.

In 1948, John was offered a position at the Woods Hole Oceanographic Institute in Woods Hole, Massachusetts. The main emphasis during that time was research on the abundance and survival of soft shell clams throughout the entire U.S. east coast. The research project was moved the following year to a former fish hatchery at Boothbay Harbor, Maine. He continued his research on softshell clam populations and other species found in the region. During this time, he became lab director of the Boothbay Harbor facility and instituted further research on artificial propagation of clams and other species. Some of the earlier efforts were relative to wild catches of soft shell clams, but were also concerned with hatchery setting. He also studied the effects of green crab predation and their control. The research during those years on the soft shell clam was ground-breaking and is still referred to extensively.

John continued his career in the federal government with the U.S. Fish and Wildlife Service and the subsequent National Marine Fisheries Services (NMFS) under the National Oceanographic and Atmospheric Administration (NOAA). He later became director of the NMFS Laboratory in Annapolis, Maryland where he moved in 1956 with his wife Jean, daughter Nancy and son Terry. This laboratory was primarily engaged in research to develop methods for farming shellfish (main emphasis on oysters). With the lab's closure, John was offered a position in the national headquarters of NMFS in Washington, D.C. While there, he was in charge of the shellfish research branch of the NMFS and responsible for seven regional laboratories. At that time, he developed the first National Aquaculture Plan through NOAA.

During President Kennedy's Administration, John was appointed to lead a team of fisheries experts to assist Ireland in improving their fisheries resources. He spent one year on this project and recommended many changes to the Irish Department of Fisheries which were implemented to improve the economic situation.

Yearning to return to the northwest and the state of Washington, John accepted a position of Assistant Regional Director of the Northwest Region of the National Marine Fisheries Service, which was headquartered in Seattle, Washington. His family moved back to the state where John not only oversaw federal fisheries research in the area but also pushed to promote and implement the NOAA National Aquaculture Plan.

Upon retirement in Seattle, John started the Glude Aquaculture Consultants. Much of his work involved aquaculture, and he was a primary consultant for numerous aquaculture projects in various countries. He continued to encourage aquaculture enterprises and eventually organized a program in Puerto Rico to test the applicability of known culture methods for fresh water prawns. John also acted as a consultant for the United Nations Food and Agriculture Organization where he headed a team of scientists to determine how to increase fishery resources and revenues in a number of developing countries. One project was entitled "The South Pacific Fisheries Investigation" through which recommendations were provided for best approaches to increase fisheries activities for the regions.

John has been a life member of several professional organizations. He served as Vice President and President of the World Aquaculture Society in 1977 and 1978, respectively. He also served for two years as president of the National Shellfisheries Association in 1963–65. Along with this service to the societies, he has published over 100 scientific papers related to his many areas of shellfish research. John is retired now, but he is most certainly a pioneer in the fields of aquaculture and fisheries, best known for his research on clam and oyster culture. An avid sportsman, his love for fly-fishing and duck hunting is well recognized. I have been on many duck hunting trips with John and his brother-in-law Dick Steele in Dabob Bay, Hood canal in Washington State and know of his obsession with bird hunting. John now resides at 6101 River Crescent Drive, Annapolis, MD 21401.

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GROWTH OF THE NORTHERN QUAHOG, *MERCENARIA MERCENARIA*, IN AN EXPERIMENTAL-SCALE UPWELLER

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ABSTRACT Upwellers have proven to be extremely effective as bivalve nursery units and their use is steadily increasing in North America. The re-analysis of previous work by others suggests an asymptotic relationship between growth (% volume increase per day) and chlorophyll-*a* effective flow rate (the amount food flowing past a unit biomass of northern quahogs, μg per minute per liter of northern quahog volume). An experiment field study was conducted to define the relationship between food flow and bivalve stocking density. Furthermore, this study was designed to investigate other significant environmental parameters influencing bivalve growth in an experimental-scale upweller system.

Northern quahog, *Mercenaria mercenaria* (Linné), seed were grown from ~2 (longest axis) to ~13 mm in an experimental-scale floating upweller from June 21 to August 19, 1999 (four separate experimental periods) in Point Judith Pond, Wakefield, Rhode Island. Flow rates and stocking densities were varied in order to produce a chlorophyll-*a* effective flow rate range of 360 to 1,500 $\mu g \cdot min^{-1} \cdot l^{-1}$, and growth and environmental parameters were measured semiweekly. During the first two-week experiment (June 21 to July 7) an asymptotic relationship was observed between growth (% increase/day) and chlorophyll-*a* effective flow rate. A significant difference in growth was found between the treatments. The difference in the functional relationship between experiments 1 and 3 was possibly related to lower DO values, which reduced differential growth in experiment 3. In experiment 1, the low-biomass treatments grew faster than the high-biomass treatments. A significant difference in growth between treatments was also observed in experiment 3, although the asymptotic relationship was less pronounced. In experiment 3, the high-biomass replicates grew faster than the low-biomass replicates. Experiments 1 and 3 both experienced similar environmental conditions; however, experiment 1 encountered higher morning dissolved oxygen (DO) levels. In addition, the within experiment variability in experiment 3 was much less than the variability in experiment 1; therefore, accentuating growth differences in experiment 3. In both experiments 1 and 3 maximum growth occurred near treatment 2 in a range of chlorophyll-*a* effective flow rates of 550 to 650 $\mu g \cdot min^{-1} \cdot l^{-1}$. In experiments, 2 and 4 there were no significant differences in growth between treatments.

Growth appeared to be limited by low oxygen. In order to eliminate the effect of food limitation on growth, the upper third of the replicates (the fastest growing animals) were used to calculate the relative growth rate (RGR) during the two-month experiment. Growth was linearly correlated with morning-dissolved oxygen ($R^2 = 0.42$) and with chlorophyll-*a* ($R^2 = 0.35$). The critical DO threshold for growth in upwellers appears to be 5 ppm, below which growth is adversely affected. During this study, morning DO levels were less than 50 % saturated, indicating the potential for DO levels to be increased. Future research should investigate methods for elevating DO levels in upwellers.

KEY WORDS: northern quahog, *Mercenaria mercenaria*, upweller, growth

INTRODUCTION

Over the last decade, the use of upwellers as bivalve nursery units has increased dramatically in North America (Manzi & Castagna 1989). A number of studies have explored the relationships between flow rate, stocking density, and growth in upwellers (Hadley et al. 1999; Baldwin et al. 1995; Malinowski & Siddall 1989; Malinowski 1988; Manzi & Hadley 1988; Manzi et al. 1986; Manzi 1985; Hadley & Manzi 1984; Manzi et al. 1984; Bayes 1981; Claus 1981; Manzi & Whetstone 1981; Rodhouse & O'Kelly 1981). The majority of research on upwellers has focused on the northern quahog, *Mercenaria mercenaria*, because of its significant aquaculture potential. In particular, the northern quahog grows well at high densities, has adapted to a variety of geographic sites along the northeast coast, and has a lucrative market.

Manzi et al. (1986) described a qualitative relationship between flow rate, stocking density, and growth in an experimental-scale upweller. In their experiment, stocking densities were varied while flow rates were held constant. The flow rate was converted to an effective flow rate by multiplying the amount of food (μg/l of chlorophyll-*a*) by the flow rate (l/min). The amount of food passing by a unit biomass of clams was defined as the chlorophyll-*a* effective flow rate ($\mu g \cdot min^{-1} \cdot kg^{-1}$). During a period of optimal northern quahog growth in the fall 1982 the authors found that a maximum biomass increase of 267% (over 30 days) occurred at the highest chlorophyll-*a* effective flow rate of 1,929 $\mu g \cdot min^{-1} \cdot kg^{-1}$ and the most efficient growth (213%) occurred at an intermediate chlorophyll-*a* effective flow rate of 476 $\mu g \cdot min^{-1} \cdot kg^{-1}$. If growth (% increase/day) is plotted as a function of chlorophyll-*a* effective flow rate, the data is represented by an asymptotic relationship; in particular, as the chlorophyll-*a* effective flow rate increases, growth increases steeply and then levels off with increasing chlorophyll-*a* effective flow rates (Fig. 1). Efficiency in this upweller system refers to economically optimizing both upweller space (density) and pumping capacity (flow). Theoretically, growth will be optimized at some percentage of the maximum growth rate; as indicated in Figure 1, 80 to 90% of the maximum growth rate equates to a chlorophyll-*a* effective flow rate range of 470 to 700 $\mu g \cdot min^{-1} \cdot kg^{-1}$.

Manzi et al. (1986) concluded that food supply was the primary limitation in their upweller system. Their data suggests that to obtain unlimited growth, northern quahog seed needed to remove approximately 150 $\mu g \cdot min^{-1} \cdot kg^{-1}$. The investigators deduce that northern quahog growth was reduced if more than 20% of the ambient chlorophyll-*a* concentration (μg/l) was removed as water passed by the bivalves. Consequently, to supply the necessary ration of 150 $\mu g \cdot min^{-1} \cdot kg^{-1}$ without exceeding 20% removal, food must be supplied to the bivalves at a rate of 750 $\mu g \cdot min^{-1} \cdot kg^{-1}$. Malinowski and Siddall (1989) confirmed that ambient chlorophyll-*a* concentrations were reduced by ~20% through an initial

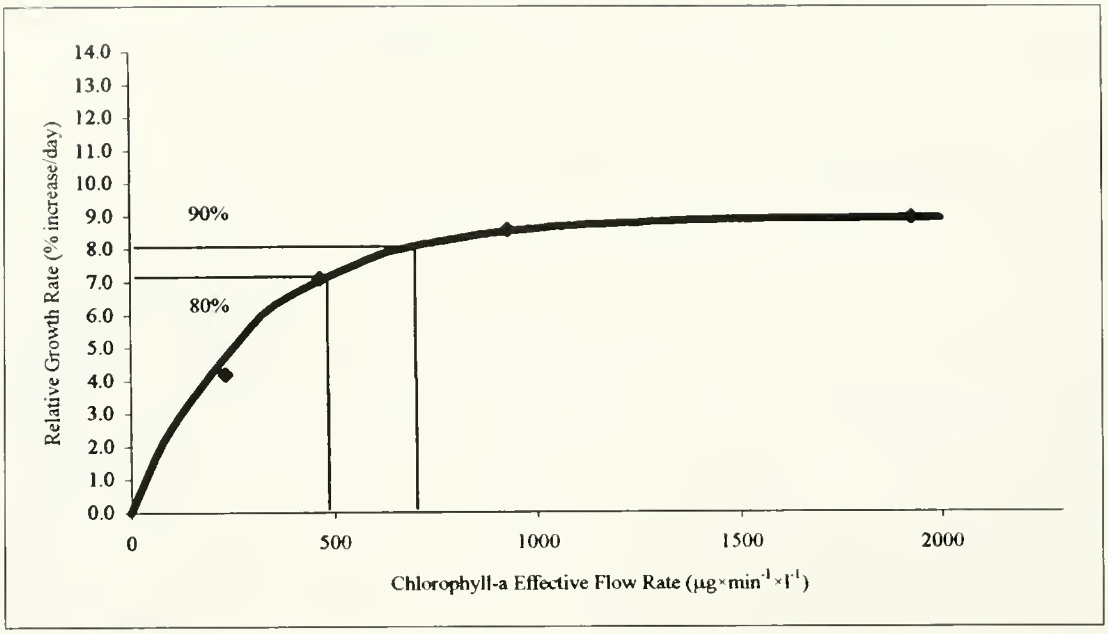

Figure 1. Growth (% increase/day) of northern quahogs as a function of chlorophyll-*a* effective flow rate re-plotted from Manzi et al. (1986).

silo of northern quahogs at similar stocking densities. However, they found that after water passed through an initial group of northern quahogs it could then support an additional equivalent biomass of northern quahogs at the same growth rate. They conclude that to achieve maximum growth of northern quahogs in an upweller it is necessary to pass more water through the animals than can actually be filtered; therefore, the low rate of chlorophyll-*a* removal reported by Manzi et al. (1986) may reflect a large amount of unused water through the system. The authors hypothesize that this surplus water may be a physical requirement of the system where minimum flow rates are required to create uniform flows through the seedbed, remove waste products, maintain water quality, and maintain a minimum concentration of chlorophyll-*a*.

Manzi et al. (1986) found that food was the primary limitation in their upweller system, while Malinowski and Siddall (1989) concluded that flow rate was the primary limitation. Malinowski and Siddall (1989) also speculate on the importance of environmental conditions, specifically water quality, but they fail to characterize these parameters in their system. Growth and survival of the northern quahog is clearly influenced by the surrounding environment. Northern quahog adults and juveniles can survive in water temperatures from 1 to 33°C, but grow optimally at 23°C (Stanley 1985; Stanley & Dewitt 1983). Northern quahogs can tolerate salinities between 10 and 35‰ (Stanley & Dewitt 1983) for short periods, but prefer to inhabit waters greater than 20‰ (Castagna & Kraeuter 1981). Northern quahogs have been known to endure oxygen concentrations below 1 mg O_2/l (Stanley & Dewitt 1983) for more than three weeks; however, growth is significantly reduced and an oxygen debt is incurred when oxygen concentrations fall below 5 mg O_2/l (Stanley & Dewitt 1983; Hamwi 1969).

Although there is a general disagreement as to the limiting parameter for growth in upwellers, in the literature growth is clearly related to both system operating parameters (flow rate and stocking density) and environmental conditions at the site (temperature and dissolved oxygen).

Given the environmental conditions in the study area, an experimental field study was conducted as to:

(1) Define a relationship between food flow, bivalve stocking density, and growth so as to determine the chlorophyll-*a* effective flow rate that optimizes growth; and
(2) Determine the most significant limiting parameter for bivalve growth in the upweller system.

The experiment monitored growth of northern quahog seed in an experimental-scale floating upweller at three ranges of nominal chlorophyll-*a* effective flow rates of 350, 600 and 1,400 $\mu g \cdot min^{-1} \cdot l^{-1}$. In addition, environmental conditions were monitored at the site.

MATERIALS AND METHODS

Experimental Design

Growth of northern quahog seed was studied over an 8-week period in an experimental-scale floating upweller system located in a nutrient-rich estuary. At the beginning of the experiment the ambient chlorophyll-*a* concentration (μg/l) was measured at the site, and flow rates and stocking densities were adjusted to achieve three nominal ranges of chlorophyll-*a* effective flow rates, including a low (~350 $\mu g \cdot min^{-1} \cdot l^{-1}$), medium (~600 $\mu g \cdot min^{-1} \cdot l^{-1}$), and high range (~1,200 $\mu g \cdot min^{-1} \cdot l^{-1}$). Each combination of effective flow rate (μg/min) and northern quahog biomass (l) or chlorophyll-*a* effective flow rate ($\mu g \cdot min^{-1} \cdot l^{-1}$) represents a treatment, as shown in Table 1. The average chlorophyll-*a* concentration during the time period was 11.70 ± 2.06 μg/l (S.E.) and the flow rates were set at 4 l/min, 6 l/min, and 8 l/min resulting in three effective flow rates of 38.36 μg/min, 57.84 μg/min, and 77.12 μg/min. The northern quahog seed were initially stocked at a biomass of 0.055 l (density of 0.3 l/cm^2) and 0.109 l (density of 0.6 l/cm^2) resulting in the desired range of chlorophyll-*a* effective flow rates. The experiment was a two (density) by three (effective flow rate) factorial design with six treatments of chlorophyll-*a* effective flow rates. Each treatment was replicated in triplicate resulting in 18 observations (silos).

TABLE 1.

Chlorophyll-*a* effective flow rates and their corresponding treatment.

Effective Flow Rate (μg/min)	Initial Stocking Biomass (liter)	
	Low	High
Low	Treatment 4	Treatment 1
Medium	Treatment 5	Treatment 2
High	Treatment 6	Treatment 3

Site Location

The experiment was conducted at Ram Point Marina, Inc., at the head of Point Judith Pond, Wakefield, Rhode Island (41°25.57′N; 71°29.87′W) (Fig. 2). Ram Point Marina, Inc. is located on a spit between Silver Spring Cove and the Upper Pond. The site was selected to take advantage of the relatively high and consistent phytoplankton biomass (chlorophyll-*a* concentrations >10 μg/l, Rheault 1995) during the summer months. The experimental-scale floating upweller was situated at the northernmost corner of the marina to ensure a water depth >1.4 meters at mean low tide.

Upweller System

An experimental-scale floating upweller was designed and constructed to ensure the control of flow through each silo. The upweller unit was 4.27-m long, 1.22-m wide, and 1.35 m deep. Two 25 cm inside diameter (ID) polyvinyl chloride (PVC) pipes were positioned at the base and ran the length of the unit forming a manifold. Ten 15-cm (ID) silos were plumbed into the top of each manifold. Flow was provided by a half horsepower axial flow

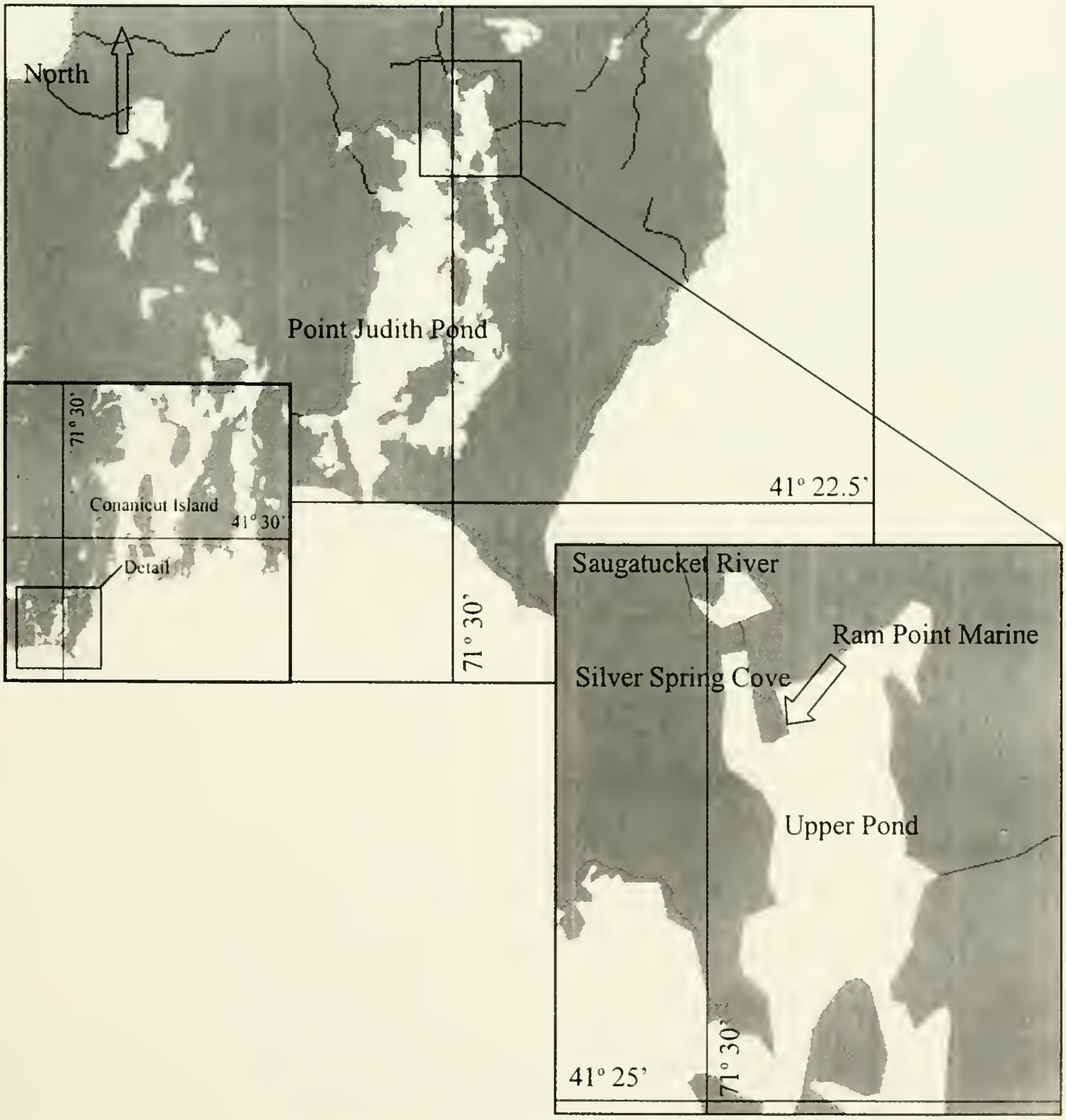

Figure 2. Location of the experimental-scale upweller at Ram Point Marina, Point Judith Pond, Wakefield, Rhode Island.

pump (Ice Eater, Power House) mounted in each manifold. Water was pumped along the manifold, flowed up each 15-cm silo, and exited through an 8-cm (ID) ball valve plumbed into the top of each silo. The seed were placed on a Nytex screen 0.5 m above the silo's base. When the unit was in operation, each ball valve lay approximately 8 cm above the water line. Flow through each silo was manipulated with the ball valve and was measured volumetrically with a graduated cylinder and a stopwatch.

Northern quahog seed (300,000 at 0.6 mm) were purchased from Bluepoints Company, Inc., West Sayville, New York. The seed were held in the upweller until they reached >2 mm (longest axis).

Data Collection

At the beginning of each experiment the seed were pulled from the unit, sieved, and randomly distributed throughout the 18 replicates at a biomass of 0.055 l (wet volume) and 0.109 l. In addition, the valve length of a random sample ($n = 75$) of seed was measured to the nearest 0.01 mm with vernier calipers. Five subsamples of northern quahogs were also taken to develop a relationship between wet volume (l) and wet weight (kg). Each experiment was terminated when the biomass in the slowest growing replicate doubled. This occurred approximately every two weeks during the summer. At the termination of each two-week experiment, the valve length of a random sample ($n = 25$) of northern quahogs from each replicate was determined. Four two-week experiments were completed during the summer 1999.

The change in volume of each silo was measured semiweekly resulting in 3- to 4-day growth intervals. Semiweekly flow rates to each silo were also measured in the morning or late at night to minimize wave activity. Care was taken to ensure that the upweller unit was not altered during measurements and flows were adjusted accordingly.

Chlorophyll-*a* (Chl-*a*), particulate organic matter (POM), temperature, salinity, and dissolved oxygen (DO) were measured semiweekly from an empty silo. With the start of the second two-week experiment (July 7) all environmental parameters were taken in the morning, midday, and evening to quantify daily fluctuations at the site. Discrete chlorophyll-*a* samples ($n = 3$) were taken with a syringe. Samples were pre-filtered with a 150 μm Nytex screen to remove particulates that bivalves are unable to filter (Defossez & Hawkins 1997). Samples (10 ml) were forced through a 25-mm diameter Whatman GF/F filter contained in a 25 mm Swinnex filter holder. The procedure for chlorophyll-*a* analysis is slightly modified from the standard procedure outlined in Strickland and Parsons (1972). Filters were dissolved in acetone for 24 hours and read on a Turner Designs fluorometer (Model 10-005R, Turner Designs, Inc., Sunnyvale, CA). All samples were corrected for phaeophytin-a. One-liter samples were also taken ($n = 2$) for POM analysis. The samples were pre-filtered on a 150 μm Nytex screen and later analyzed in the laboratory. In the laboratory, samples were vacuum pumped through a pre-ashed 47 mm Whatman GF/F filter (normal pore size 0.1 μm), rinsed with isotonic ammonium formate, and dried in an oven at 110°C for 24 to 48 hours. Filters were then ashed for >6 hours at 450°C in a muffle furnace. Filters were weighed on an Ohaus electronic balance (Model AS120) to the nearest 0.1 mg. Temperature, salinity were measured with an YSI (Model 30) probe and oxygen was measured with an YSI (Model 55) probe. The oxygen probe was calibrated prior to each measurement.

When measuring the change in volume of northern quahog seed, each silo and screen was cleaned with freshwater. Once a week the remainder of the upweller manifold was cleaned by a diver to ensure consistent flow through the system.

Data Analysis

The chlorophyll-*a* effective flow rate ($\mu g \cdot min^{-1} \cdot l^{-1}$) for each replicate was calculated as the product of the average chlorophyll-*a* concentration (μg/l) during the period and the flow rate (l/min) to the replicate all divided by the average biomass (l) of the replicate during the same period. This study characterized growth as the relative growth rate (RGR) and was calculated as:

$$RGR = \{[(Volume_{(final)} - Volume_{(initial)})/Volume_{(initial)}] \times 100\}/\#\text{ of days}$$

where volume is measured in liters. RGR is expressed as a percentage increase per day (% increase/day). POM (mg/l) was calculated as the difference between total suspended particulate matter (SPM) and particulate inorganic matter (PIM).

The Effect of Food Limitation on Growth

To elucidate differences in growth between treatments the total RGR (% volume increase) was divided by the longest period available, the length of each experiment. Since the RGR (% increase/day) measures the change in volume over each two-week experiment, the average chlorophyll-*a* concentration and average treatment biomass during the time was used to calculate treatment chlorophyll-*a* effective flow rates. Prior to ANOVA analysis, the RGR (% increase/day) was arcsine transformed (Sokal & Rohlf 1995). Within each experiment, one-way ANOVAs were performed for each two-week experiment with the average RGR (% increase/day) as the dependent variable and treatment as the independent variable. Differences between treatment means were elucidated with the Tukey Honestly Significant Difference (HSD) test. When the one-way ANOVA proved significant, a within experiment two-way ANOVA was performed to further investigate the effective flow rate and density as independent variables. Again, the Tukey HSD test was used to verify differences in means. The strength of the relationship was characterized by the standard omega-squared (ω^2), when appropriate. The ω^2 was calculated as

$$\omega^2 = [SS_{Effect} - df_{S/A}\,(MS_{S/A})]/SS_T + MS_{S/A}$$

where SS_{Effect} is the sum of squares of the effect, $df_{S/A}$ is the degrees of freedom for the error term, $MS_{S/A}$ is the mean squares for the error term, and SS_T is the sum of squares total.

A between experiment one-way ANOVA was performed to elucidate growth differences between the two-week experiments. The Tukey HSD test quantified differences between means.

The Effect of Environmental Characteristics on Growth

In order to illustrate the effect of environmental parameters on northern quahog growth, growth was characterized at the finest possible scale. In particular, RGR was calculated for each 3 to 4 day period (growth interval) between semiweekly volume determinations. The daily value of each environmental parameter was averaged over the concurrent growth interval.

To eliminate the effect of food limitation on growth, RGR of the upper third of the replicates (upper 1/3 RGR) was calculated. A linear regression analysis (SAS Institute, Inc.) of the upper one-third RGR was performed with temperature, salinity, chlorophyll-*a*, and dissolved oxygen to determine which independent variables were significant in determining growth. Dissolved oxygen concentrations were converted to percent saturation based on temperature and salinity measurements (Benson & Krause 1984). A step-wise linear regression analysis was also performed to elucidate the most significant parameter(s) for predicting growth in the experiment.

RESULTS

The Effect of Food Limitation on Growth

There was no observed mortality during the course of the two-month experiment. Calculated mortality was extremely variable in experiments 1 and 2 because counts per ml were not replicated. In addition, counts were not made before and after sieving. In experiments 3 and 4, counts per ml were replicated ($n = 3$) and counts were made before and after sieving. Mortality was calculated to be 111 ± 3% (S.E.) and 99 ± 2% (S.E.) respectively. The first experiment began on June 21 and ended on July 7, 1999 (16 days) and the northern quahogs grew from 3.11 ± 0.06 mm (S.E.) to 3.95 ± 0.05 mm (S.E.). The average chlorophyll-*a* concentration was 16.42 ± 2.25 μg/l (S.E.) and the average treatment biomass ranged from 165.8 to 85.5 ml. The chlorophyll-*a* effective flow rate ranged from 420 to 1,445 $\mu g \cdot min^{-1} \cdot l^{-1}$ roughly correlating with a RGR from 4.76 to 9.32% increase/day. As the chlorophyll-*a* effective flow rate increased, the RGR increased until ~650 $\mu g \cdot min^{-1} \cdot l^{-1}$ at which point growth leveled off (Fig. 3a). Growth, as measured by RGR, was subjected to a one-way ANOVA with six levels of treatment. This was found to be statistically significant ($F(5, 11) = 5.48$, $P < 0.05$). The strength of the relationship was 0.57 as indexed by the standard omega-squared (ω^2). The Tukey HSD test indicated that the mean RGR for treatment 1 ($M = 5.54$) was significantly lower than the means for treatment 4 ($M = 8.14$), 5 ($M = 8.41$), and 6 ($M = 9.26$). To investigate the effect of effective flow rate and biomass on growth, a two-way ANOVA was performed with three levels of seston flux and two levels of volume. Both effective flow rate ($F(2, 11) = 5.13$, $P < 0.05$) and biomass ($F(1, 11) = 13.36$, $P < 0.05$) were statistically significant. The strength of the relationship (ω^2) was 0.21 and 0.31, respectively. The interaction between effective flow rate and biomass was found ordinal; therefore, the main effects were examined by the Tukey HSD test. The Tukey HSD test indicated that the low-biomass treatments ($M = 8.60$) grew faster than the high-biomass treatments ($M = 6.99$).

The second experiment began on July 7 and ended on July 22, 1999 (15 days) and the northern quahogs grew from 3.46 ± 0.11 mm (S.E.) to 6.28 ± 0.07 mm (S.E.). The average chlorophyll-*a* concentration was 11.83 ± 1.16 μg/l (S.E.) and the average treatment biomass ranged from 205.6 ml to 91.6 ml. The chlorophyll-*a* effective flow rate ranged from 231 to 977 $\mu g \cdot min^{-1} \cdot l^{-1}$ roughly correlating with a RGR from 9.58 to 12.73 % increase/day (Fig. 3b). RGR was consistently high within the chlorophyll-*a* effective flow rate range specified. RGR was subjected to a one-way ANOVA and there was no statistical difference between treatments ($F(5, 11) = 1.48$, $P > 0.05$).

The third experiment began on July 22 and ended on August 5, 1999 (14 days) and the northern quahogs grew from 7.04 ± 0.11 mm (S.E.) to 9.96 ± 0.07 mm (S.E.). The average chlorophyll-*a* concentration was 18.55 ± 2.12 μg/l (S.E.) and the average treatment biomass ranged from 184.8 to 84.6 ml. The chlorophyll-*a* effective flow rate ranged from 411 to 1,720 $\mu g \cdot min^{-1} \cdot l^{-1}$ roughly corresponding to a RGR from 7.79 to 10.09 % increase/day (Fig. 3c). The RGR increased slightly with an increase in the chlorophyll-*a* effective flow rate until ~610 $\mu g \cdot min^{-1} \cdot l^{-1}$, at which point growth decreased and leveled off. RGR was subjected to a one-way ANOVA and was found to be statistically significant ($F(5, 11) = 7.13$, $P < 0.05$). The strength of the relationship was 0.64 as indexed by the ω^2. The Tukey HSD test indicated that the mean RGR for treatment 2 ($M = 9.76$) was significantly higher than the means for treatment 4 ($M = 7.97$), 5 ($M = 8.62$), and 6 ($M = 8.38$). In addition, the mean RGR for treatment 4 ($M = 7.97$) was significantly lower than the mean for treatment 3 ($M =$

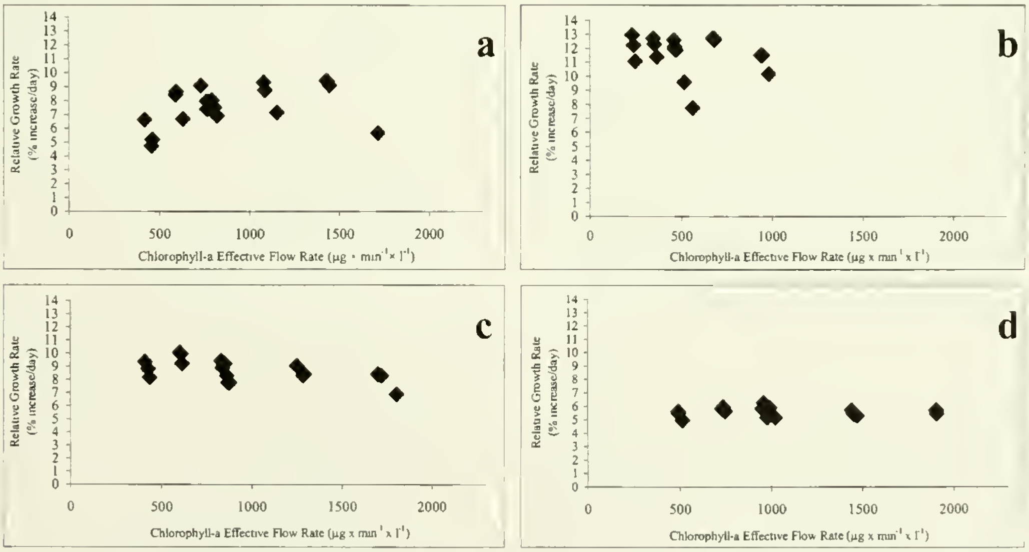

Figure 3. Growth (% increase/day) as a function of chlorophyll-*a* effective flow rate for (a) experiment 1 (June 21 to July 7, 1999 with an average chlorophyll-*a* concentration of 16.42 ± 2.25 μg/l S.E.); (b) experiment 2 (July 7 to July 22, 1999 with an average chlorophyll-*a* concentration of 11.83 ± 1.16 μg/l S.E.); (c) experiment 3 (July 22 to August 5, 1999 with an average chlorophyll-*a* concentration of 18.55 ± 2.12 μg/l S.E.); and, (d) experiment 4 (August 5 to August 19, 1999 with an average chlorophyll-*a* concentration of 17.91 ± 3.17 μg/l S.E.).

9.20). A two-way ANOVA found both effective flow rate (F (2, 11) = 5.99, $P < 0.05$) and biomass (F (1, 11) = 22.33, $P < 0.05$) were statistically significant. The strength of the relationship (ω^2) was 0.21 and 0.45, respectively. The interaction between effective flow rate and biomass was found ordinal; therefore, the main effects were examined by the Tukey HSD test. The Tukey HSD test indicated that the high-biomass treatments (M = 9.25) grew faster than the low-biomass treatments (M = 8.32). The Tukey HSD test also found that the replicates with an effective flow rate of 111.3 μg/min (M = 9.19) grew faster than the replicates with an effective flow rate of 74.2 μg/min (M = 8.87).

The fourth experiment began on August 5 and ended on August 19, 1999 (14 days) and the northern quahogs grew from 9.37 ± 0.12 mm (S.E.) to 11.47 ± 0.08 mm (S.E.). The average chlorophyll-*a* concentration was 17.91 ± 3.17 μg/l (S.E.) and the average treatment biomass ranged from 147.4 to 73.4 ml. The chlorophyll-*a* effective flow rate ranged from 491 to 1,905 $\mu g \cdot min^{-1} \cdot l^{-1}$ roughly corresponding to a RGR from 4.98 to 5.96% increase/day (Fig. 3d). RGR was consistently low within the chlorophyll-*a* effective flow-rate range specified. RGR was subjected to a one-way ANOVA and there was no statistical difference between treatments (F (5, 11) = 0.76, $P > 0.05$).

A one-way ANOVA was performed to compare the RGR between the two-week experiments. This was statistically significant (F (3, 64) = 135.34, $P < 0.05$) with an ω^2 of 0.80. The Tukey HSD test indicated that there was a significant difference between all the mean RGRs, with growth highest in experiment 2 (M = 11.89) and decreasing in experiments 3 (M = 8.81), 1 (M = 7.71), and 4 (M = 5.57).

The Effect of Environmental Characteristics on Growth

The upper one-third RGR varied considerably during the course of the experiment from a high of 10.37 ± 0.43% increase/day (S.E.) on June 22 to a low of 5.03 ± 0.41% increase/day (S.E.) on June 29 (Fig. 4a). During the course of the two-month experiment, RGR decreased sharply (June 22 to June 29), then increased (June 29 to July 17), and then gradually decreased (July 17 to August 19).

Temperature during the experiment varied from 21.4 to 27.3°C (Fig. 4b). Other than a brief drop in temperature in mid July due to a rainstorm, temperature was consistent during the experiment. A linear regression analysis indicated that temperature was not significant in determining growth as indicated by the upper one-third RGR (F (1, 15) = 0.58, $P > 0.05$). Similarly, salinity during the experiment was relatively consistent ranging from 21.4 to 29.9‰ (Fig. 4c). Linear regression analysis determined that salinity was not significant in predicting the upper one-third RGR (F (1, 15) = 0.70, $P > 0.05$). The seston concentration, as indexed by the chlorophyll-*a* concentration, varied substantially during the course of the experiment from peaks of 21.2 ± 1.23 μg/l (S.E.) on June 28 and 22.8 ± 0.86 μg/l (S.E.) on July 3, to a nadir of 9.5 ± 0.09 μg/l (S.E.) on July 13 (Fig. 4d). Generally, as chlorophyll-*a* concentrations increased growth decreased. Similarly, as chlorophyll-*a* concentrations decreased growth increased. Linear regression analysis indicated that this trend was significant (F (1, 15) = 7.46, $P < 0.05$) with a correlation coefficient (R^2) of 0.35 (Fig. 5a). There was a minimal amount of variability between chlorophyll-*a* measurements as exemplified by the low standard errors between measurements. The chlorophyll-*a* concentration during the day, however, varied considerably as indicated by the high standard errors between morning, midday, and evening chlorophyll-*a* determinations. Morning DO was used to quantify the effect of limiting dissolved oxygen on growth as morning DO values were consistently the lowest oxygen values experienced by the northern quahogs. Morning DO values ranged from a high of 8.00 ppm on June 22 to a low of 3.37 ppm on July 8 (Fig. 4e). Morning DO values correlate with the upper one-third RGR during the two-month experiment. As morning DO values decreased growth decreased; conversely, as morning DO values increased growth increased. A linear regression analysis indicated that this trend was significant (F (1, 15) = 10.13, $P < 0.05$) with a correlation coefficient (R^2) of 0.42 (Fig. 5b). Morning dissolved oxygen (ppm) tracked the morning percent saturation of DO. During periods of low morning DO (4–3 ppm), % saturation approached 50% and during periods of high morning DO (7–8 ppm), % saturation ranged from 80 to 100%.

Growth was best characterized with morning DO and chlorophyll-*a* (F (2, 15) = 8.22, $P < 0.05$) resulting in an R^2 of 0.56. As the chlorophyll-*a* concentration increased morning DO decreased and as the chlorophyll-*a* concentration decreased morning DO increased (Figs. 4d and e).

DISCUSSION

The Effect of Food Limitation on Growth

The one-way ANOVAs found significant differences in growth between treatments in experiments 1 and 3, but none in experiments 2 and 4. Experiments 1 and 3 were characterized by relatively high morning DO values, while experiments 2 and 4 experienced relatively low morning DO values. According to the reevaluation of the data (Fig. 1) presented in Manzi et al. (1986), growth as a function of the chlorophyll-*a* effective flow rate (the amount of food passing by a unit biomass of clams) should follow an asymptotic function. Specifically, growth should increase from the origin (zero growth and zero chlorophyll-*a* effective flow rate) with increasing chlorophyll-*a* effective flow rates until a particular point where growth asymptotes or even decreases.

In the first experiment, growth (% increase/day) followed the relationship presented in Figure 1. Growth increased as the chlorophyll-*a* effective flow rate increased until ~650 $\mu g \cdot min^{-1} \cdot l^{-1}$ at which point growth reached an asymptote. The one-way ANOVA found a significant difference in growth between the treatments with a relatively strong relationship as indexed by the standard omega-squared (ω^2 = 0.57). Furthermore, treatment 1 grew significantly slower than treatments 4, 5, and 6. Treatments 4, 5, and 6 represent the asymptote of the function where growth asymptotes regardless of an increase in the chlorophyll-*a* effective flow rate. In addition, treatments 4, 5, and 6 were those with a low initial stocking density of 0.3 l/cm^2. In order to further investigate the effect of effective flow rate and biomass on growth a two-way ANOVA was performed. There were significant differences between growth with the levels of effective flow rate and biomass. In particular, the low-biomass replicates (treatments 4, 5, and 6) grew faster than the high-biomass replicates (treatments 1, 2, and 3).

In the third experiment, the one-way ANOVA also indicated a significant difference in growth between the treatments with an even stronger relationship (ω^2 = 0.64). The functional relationship between growth and chlorophyll-*a* effective flow rate was different from that postulated in Figure 1. Growth increased slightly with increasing chlorophyll-*a* effective flow rate, but then decreased slightly, reaching an asymptote above 1,000 $\mu g \cdot min^{-1} \cdot l^{-1}$.

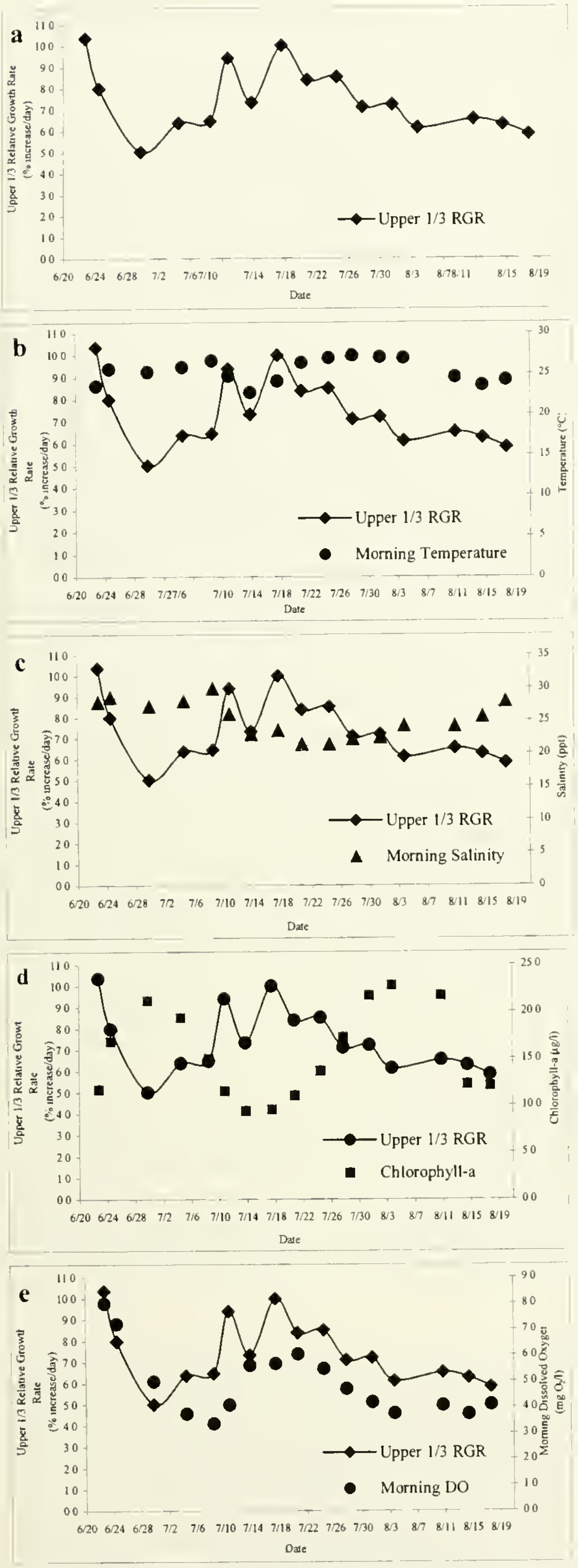

Figure 4. Relative growth rate (% increase/day) for the upper third of replicates (a) from June 21 through August 19, 1999 with (b) morning temperature (°C); (c) morning salinity (‰); (d) morning chlorophyll-*a* (µg/l); and, (e) morning dissolved oxygen (ppm).

This trend is supported by the Tukey HSD test, which indicated that treatment 2 grew significantly faster than treatments 4, 5, and 6. In addition, treatment 3 grew significantly faster than treatment 4. Since treatment 3 and treatment 4 have nearly the same chlorophyll-*a* effective flow rate, a significant difference in growth indicates an effect of biomass on growth, with the higher biomass treatment growing faster that the lower biomass treatment. The two-way ANOVA also found a significant effect of biomass on growth with the high-biomass replicates growing faster that the low-biomass replicates. The two-way ANOVA also indicated a significant effect of seston flux on growth with the replicates with the intermediate effective flow rate of 111.3 µg/min growing faster that the replicates with the lower effective flow rate of 74.2 µg/min.

Experiments 1 and 3 both experienced initially high morning DO values that decreased during the course of the experiment. Experiment 1 had higher initial morning DO values (8–7 ppm) than experiment 3 (5.5–6 ppm). The chlorophyll-*a* concentration also increased substantially during both experiments, with values peaking at 21.19 ± 1.23 µg/l (S.E.) in experiment 1 and 22.83 ± 0.86 µg/l (S.E.) in experiment 3. In addition, both experiments experienced the same range of chlorophyll-*a* effective flow rates. The difference in the relationship between growth and chlorophyll-*a* effective flow rate in experiment 1 and experiment 3 is probably the result of a number of factors. First, since experiment 3 did not experience the initially high morning DO levels observed in experiment 1, the treatments might not have had a chance to separate or grow differentially. Second, the spread in replicates in experiment 3 was considerably smaller than that in experiment 1; therefore, small growth differences between treatments in experiment 3 are essentially accentuated. In other words, the statistical difference between treatments in experiment 3 is a result of the relatively small within replicate variability. In both experiments 1 and 3, maximum growth occurred near treatment 2 in a range of chlorophyll-*a* effective flow rates of 550 to 650 $\mu g \cdot min^{-1} \cdot l^{-1}$. In order to verify this result, growth should be investigated within the chlorophyll-*a* effective flow rate range of 0 to 500 $\mu g \cdot min^{-1} \cdot l^{-1}$.

The benefit of defining a relationship between growth and the amount of food passing by a unit biomass of animals (chlorophyll-*a* effective flow rate) is apparent in the application to other growers. The relationship can be easily applied to upwellers in a variety of locations, provided optimal environmental conditions persist. An aquaculture extension agent could characterize the water conditions at a site to determine that the minimum water quality standards are met, such as temperature, salinity, and dissolved oxygen. The agent could then measure the amount of chlorophyll-*a* and estimate the average food concentration at the site. With this estimate, the grower could determine the biomass and effective flow rate needed to optimize growth in the upweller.

The Effect of Environmental Characteristics on Growth

When environmental conditions were suitable for northern quahog growth, especially in the beginning of experiment 1, the effect of food limitation on growth was apparent. When environmental conditions were less than optimal, as in experiments 2 and 4, growth appears constant over a wide range of chlorophyll-*a* effective flow rates. In other words, growth was not controlled by food limitation, but some other factor. To quantify the effect of environmental conditions on growth, the upper one-third of replicates, the fastest growing northern quahogs, were used to determine

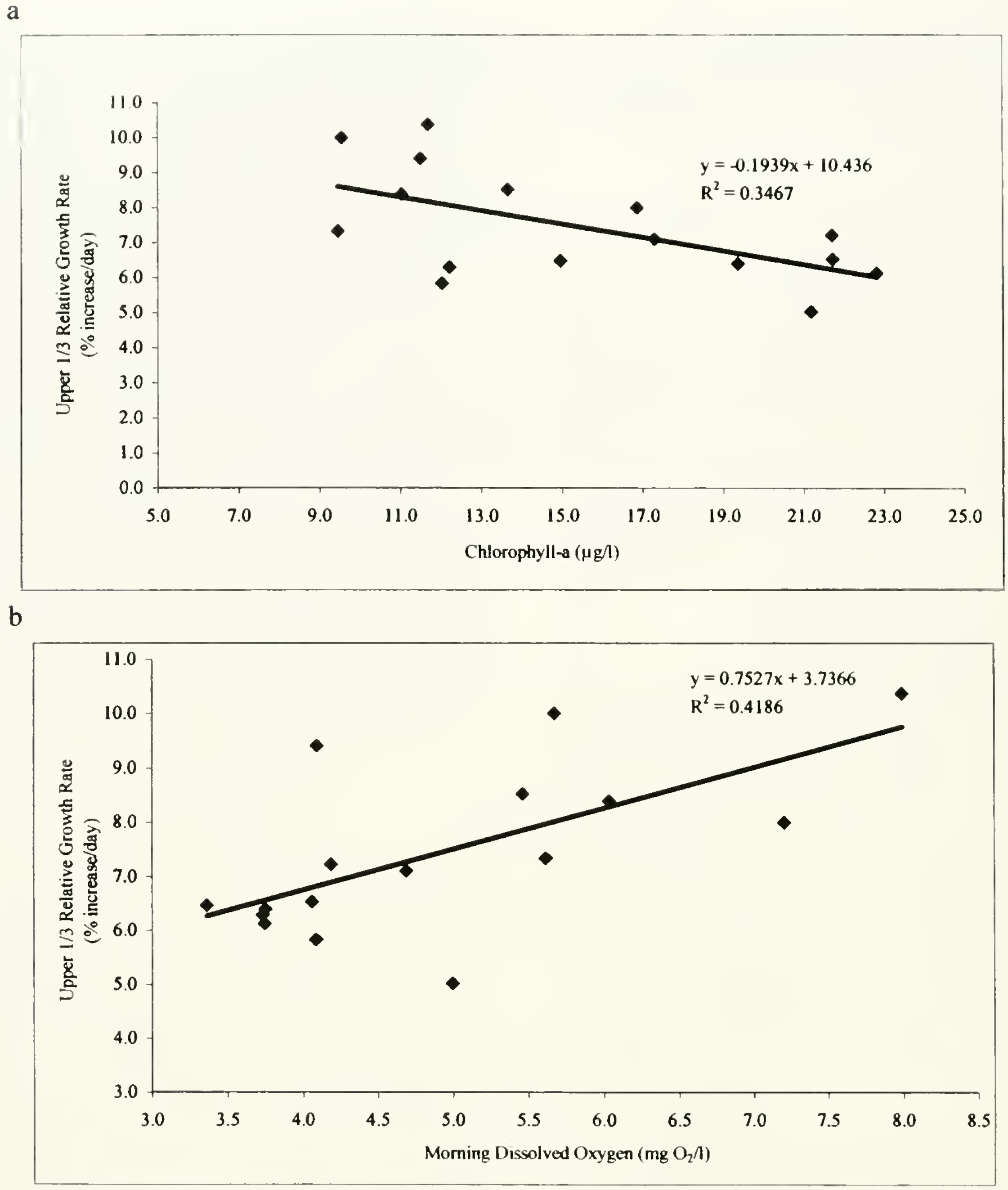

Figure 5. Linear regression of the relative growth rate (% increase/day) for the upper third of replicates from June 21 through August 19, 1999 versus (a) chlorophyll-*a* (µg/l) and (b) morning dissolved oxygen (ppm).

growth. By eliminating the slowest two-thirds replicates, the effect of food limitation on growth was minimized; therefore, differences in growth were constrained by the environmental conditions at the time.

Manzi et al. (1986) concluded that food limited growth in their experimental-scale upweller. Although there were signs of food limitation on growth in experiments 1 and 3, growth in experiments 2 and 4 were controlled by other factors. Malinowski and Siddall (1989) determined that the flow rate limited growth in their upweller system. They surmise that flow through the upweller had to be above a critical threshold in order to create a uniform flow (distribute food evenly among the clam seed), maintain water quality, remove wastes, and provide a sufficient chlorophyll-*a* concentration to the northern quahogs. Although Malinowski and Siddall (1989) were unable to quantify the effect of water quality on growth, they eluded to the importance of environmental conditions on growth.

Over the course of the two-month experiment, growth was positively correlated with morning DO and negatively correlated with chlorophyll-*a*. In late June and early July, the experimental site at Point Judith Pond experienced a pronounced algae bloom. The bloom was evident as an increase and peak in the chlorophyll-*a* concentration (Fig. 4d). There was a clear relationship between chlorophyll-*a* and morning DO, specifically as the chlorophyll-*a* concentration increased, morning DO levels decreased (Figs. 4d and e). The decrease in morning DO was a result of a combination of algae decomposition and algae respiration. At night, the algae were constantly respiring, converting captured energy into simple sugars, an oxygen consuming and carbon dioxide producing process. The algae were also continually dying off and decomposing, again an oxygen consuming process. A second algae bloom in the upper pond was apparent in mid August. Again, the same relationship between chlorophyll-*a* and morning DO was apparent. In late July, the chlorophyll-*a* concentration decreased substantially and morning DO levels increased. This decrease in chlorophyll-*a* was most likely a result of zooplankton grazing described by Bengtson (1982). Alternatively, the decrease in chlorophyll-*a* could have been caused by a crash or die off of a particular species of algae. The cyclic pattern of algae in the upper pond could be further verified by quantifying the species of algae present as well as the amount of zooplankton at the study site.

The relationship between primary productivity (chlorophyll-*a* concentrations) and low dissolved oxygen in shallow coastal and estuarine areas has received considerable attention, as this phenomenon appears to be increasing on a global scale. Diaz and Rosenberg (1995) reviewed the diversity of research characterizing low DO events in the Chesapeake Bay. They found that the cause and ecological consequences of these events varied from tributary to tributary.

Regardless of the specific controlling mechanisms, an increase in algae biomass caused a distinct decrease in morning DO (<5 ppm) resulting in depressed clam growth. The relationship between low DO and depressed clam growth has not been characterized in the literature; however, a number of researchers have successfully characterized changes in stress levels (Sparks & Strayer 1998) and predator behavior (Taylor & Eggleston 2000; Tallqvist 2001) of bivalves exposed to low oxygen concentrations. Hamwi (1969) determined that *Mercenaria mercenaria* were able to maintain a constant rate of respiration with decreasing oxygen levels until 5 ppm. The northern quahog is a classic oxygen regulator (Hamwi 1969). As the oxygen concentration decreases, bivalves can increase their rate of oxygen consumption through two mechanisms: (1) increasing their pumping rate; or (2) increasing their percentage of oxygen utilization. Hamwi (1969) determined that the pumping rate of northern quahogs remained constant with decreasing oxygen concentrations; however, northern quahogs were able to regulate O_2 consumption by increasing the percentage of oxygen utilized. When oxygen levels reached 5 ppm or below, Hamwi (1969) found that oxygen uptake in northern quahogs decreased continuously and an oxygen debt was incurred. Once conditions were favorable, the oxygen debt was rapidly repaid in a matter of hours and northern quahogs were able to function normally.

Although juvenile northern quahogs can survive in oxygen concentrations below 1 ppm for up to three weeks (Stanley & Dewitt 1989), 5 ppm is the critical threshold for northern quahog growth. There have been a number of studies that have investigated the effect of low oxygen levels on survival and tolerance, yet none have investigated the effect of low oxygen levels on growth. Based on the work completed by Hamwi (1969), 5 ppm is the critical threshold for northern quahog growth. When oxygen concentrations fall below 5 ppm, the northern quahogs cannot maintain sufficient oxygen uptake and incur an oxygen debt. In essence, the northern quahogs shut down and stop growing until oxygen levels rise above this critical threshold.

The results of this study stress the importance of sufficient oxygen concentrations for northern quahog growth in upweller systems. A number of methods could be used to ensure optimal oxygen levels in an upweller. The upweller could be moved to a site that experiences lower chlorophyll-*a* values and higher morning DO values, but food for the northern quahog would be compromised. Alternatively, the oxygen concentration in the upweller could be increased. During periods of low morning DO (<4 ppm), the % saturation was below 60; therefore, during periods of low morning DO, oxygen concentrations have the potential of being increased. Future research should investigate the most cost effective and efficient method of increasing dissolved oxygen levels in this upweller as well as in the more traditional passive flow upwellers. With optimal DO levels, the effect of food limitation on growth can be further defined and replicated.

SUMMARY AND CONCLUSIONS

The hypothesized relationship between growth and chlorophyll-*a* effective flow rate was only apparent during the first two-week experiment (experiment 1). Although there were significant differences in growth between treatments in the third two-week experiment (experiment 3), these differences were most likely the result of small within sample variability. For the remainder of the experiment, northern quahog growth was limited by environmental conditions. Specifically, the relative growth rate of the upper one-third of the replicates was positively correlated with morning-dissolved oxygen ($R^2 = 0.42$) and negatively correlated with chlorophyll-*a* ($R^2 = 0.35$). The critical dissolved oxygen threshold for northern quahog growth in the experimental-scale upweller appeared to be 5 ppm, below which growth was adversely affected. Future research should investigate the most effective method for elevating DO levels in commercial floating upwellers.

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FLOW CYTOMETRIC MEASUREMENT OF HEMOCYTE VIABILITY AND PHAGOCYTIC ACTIVITY IN THE CLAM, *RUDITAPES PHILIPPINARUM*

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ABSTRACT The assessment of blood cell viability and defense abilities is a major concern in the study of pathological processes. In this work, we devised and validated flow cytometric assays to measure viability and phagocytic activity of hemocytes from the clam *Ruditapes philippinarum*, a species susceptible to the bacteria-caused Brown Ring Disease (BRD). Validated assays were subsequently used to measure hemocyte parameters following experimental contamination with BRD's etiologic agent *Vibrio tapetis*. Results show that clams that developed BRD symptoms had lower phagocytic rates and a higher percentage of dead hemocytes than those that did not. *In vitro* interactions between hemocytes and *V. tapetis* demonstrated that clam hemocytes are able to phagocytose formalin-fixed *V. tapetis*, but at lower rates than latex beads. Live *V. tapetis* were able to kill clam hemocytes *in vitro*. The *in vitro* assay also showed that phagocytosis increased with increasing temperature from 8° to 21°C. This work demonstrated the efficiency of flow cytometry for measuring molluscan blood cell activities during host/pathogen interactions and points the way for further experiments using this analytical tool.

KEY WORDS: bivalve, bacteria, phagocytosis, hemocyte viability, flow cytometry, *Ruditapes philippinarum*

INTRODUCTION

Changes in overall hemocyte activity have been observed in parasitized bivalves or those experimentally exposed to pathogens (Nottage & Birkbeck 1990; La Peyre et al. 1995; Anderson 1996; Garreis et al. 1996; Allam et al. 2000a, 2000b). Techniques used to investigate these changes generally have serious drawbacks. Microscopy is subjective and involves a considerable expenditure of time, especially when large numbers of samples need to be evaluated. Spectrophotometric methods are based on the measurement of activity in the whole sample and not on a cell-by-cell basis. Flow cytometry, however, has proven to be extremely useful in overcoming these problems. In the field of molluscan research, flow cytometry has been used to quantify phagocytosis (Alvarez et al. 1989; Brousseau et al. 2000; Allam et al. 2001; Fournier et al. 2001) and hemocyte viability (Ashton-Alcox & Ford 1998; Ashton-Alcox et al. 2000; Fournier et al. 2001).

In flow cytometry, particles are passed single-file through a laser beam. The light scattered by the particles indicates their size and internal complexity. Fluorescence, whether autofluorescence or from a fluorescent tag, is measured by specific detectors. Flow cytometric phagocytosis measurements usually involve the use of fluorescent particles that are detectable by the flow cytometer even after phagocytosis has taken place (Alvarez et al. 1989; Brousseau et al. 2000). Specific fluorescent dyes that indicate membrane integrity and permeability, intracellular redox potential, or enzymatic activity are available and can indicate cell viability using flow cytometry (Combrier et al. 1989).

Although molluscan hemocytes have been studied by flow cytometry before, validation of the results is rarely reported. In this work, we refined and verified by microscopy, two flow cytometry-based assays to measure the phagocytic activity and the viability of hemocytes in the clam *Ruditapes philippinarum*. These allowed us to investigate hemocyte activity in clams experimentally infected with *Vibrio tapetis*, the bacterial agent of Brown Ring Disease (BRD) in Europe (Paillard et al. 1994). We further studied the effect of *in vitro* contact between the pathogenic bacterium and hemocytes on hemocyte viability, and compared the uptake of *V. tapetis* with that of similarly sized fluorescent beads. We also investigated the effect of temperature on phagocytosis because temperature is strongly associated with the prevalence and intensity of BRD in nature and under experimental conditions (Paillard et al. 1994; Allam 1998).

MATERIALS AND METHODS

Experimental Animals

Ruditapes philippinarum were obtained from 2 locations: southern Puget Sound, Washington, USA (length = 39.4 ± 0.46 mm, mean ± SEM), and the Bay of Brest, Brittany, France (length = 37.7 ± 0.65 mm). Clams were shipped overnight to the laboratory where they were immediately placed in quarantine, aerated 35-L standing-water tanks (about 35 clams per tank at 13°C and 34 ppt), in which they were kept during the remainder of the study. Clams were fed daily throughout the experiments using a mixture of cultured algae. All the experiments reported here were conducted using clams from the USA, where BRD has never been reported, except for the infection experiment, which was done using French clams.

Clams affected with BRD were obtained by challenging them with *V. tapetis* as previously described (Allam et al. 2000a). Briefly, a 0.5 ml (5×10^7 bacteria) aliquot of a suspension made with exponentially growing *V. tapetis* (ATCC 4600, strain P16) was inoculated into the pallial cavity of each experimental clam. Control clams were inoculated with the same volume of sterile seawater. After 4 weeks of incubation under the conditions described above, hemolymph was collected and processed for phagocytosis and viability assays. The clams were then shucked and BRD development on shells was recorded (Paillard & Maes 1994; Allam et al. 2000a).

Hemolymph Sampling

Animals and hemolymph samples were kept on ice during processing to avoid hemocyte clumping. Hemolymph was withdrawn from the posterior adductor muscle as described by Auffret and

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Oubella (1995). Samples were immediately divided into aliquots and diluted in different solutions as described below.

Design and Validation of the Phagocytic Assay

In vitro Incubation of Hemocytes with Beads

Fluorescent latex beads, 2.02 μm in diameter (Fluoresbrite Calibration grade, Polysciences, USA), were dispersed by expelling them through a 26-gauge needle and diluting them with filtered seawater (FSW) to give a final concentration of 6×10^6 beads ml^{-1}. Two hundred microliters of this suspension were placed in each well of a 24-well microplate, which was centrifuged for 10 min at 200 × g at room temperature (~21°C) to form a uniform monolayer of beads on the bottom of each well. Hemolymph samples were immediately diluted with ice-cold sterile seawater (SSW) to give a final concentration of 5 to 7×10^5 cells ml^{-1} and 200-μl aliquots of these suspensions were added to each well to give approximately a 1:10, cell:bead ratio. Following a 30-minute incubation in thermostatic chambers (Minifrige II®, Boekel Industries, Inc., Philadelphia, PA) adjusted to 21°C, conditions determined to be optimal during preliminary assays, the cytoskeleton-inhibitor Cytochalasin B (10 μg ml^{-1}, final concentration) was added to each experimental well to stop hemocyte activity. Attached cells were released by trypsinization (0.4% trypsin in 1% EDTA-saline solution for 10 min) followed by gentle sonication for one minute at room temperature (RT). Microscopic observation verified that this procedure detached cells from wells and also released non-ingested particles from the surfaces of hemocytes. Formalin was then added for a final concentration of 3% to fix the sample, which was transferred to a microfuge tube where it was held on ice until processed, within an hour, by flow cytometry. Because the flow cytometric profile for hemocytes from each clam was unique (see later), it was necessary to have a control (i.e., no phagocytosis) profile for each individual. Thus, a control well was established for each clam in which Cytochalasin B was added at the beginning of the incubation period to prevent phagocytosis. After the incubation, the control wells received the trypsin and formalin treatments as described above.

Flow Cytometry

Flow cytometry was performed on a Coulter EPICS C equipped with an argon laser and operated at a wavelength of 488 nm. Gains and photomultiplier high voltage settings were adjusted to include all cell and bead particles. Forward light scatter (FLS) and green fluorescence (GFL) list mode data were collected. A total of 10,000 particles were counted for each sample. The percent phagocytosis was calculated for each clam by bitmapping (electronic outlining) each of three particle types: (1) free beads; (2) non-bead-associated cells; and (3) bead-associated cells (Fig. 1). The bitmaps had been established previously by running beads alone, then non-bead-associated cells plus free beads, and then samples with phagocytosed beads. For each clam, the sum of counts in bitmaps 2 and 3 represented the total hemocytes in the flow cytometry sample. In each sample, the percent phagocytosis was computed as the ratio of bead-associated hemocytes to total hemocytes × 100. For each clam, the percent phagocytosis was calculated as the difference between the percent phagocytosis in the test wells and the percent phagocytosis in the control well. The percent phagocytosis was always below 0.5% in the control wells. For each sample, mean fluorescence intensity (channel number) was calculated within each bitmap.

Assay Validation

Epifluorescence microscopy was used to compare and correlate the percent phagocytosis results obtained from flow cytometry. Bead-associated and non-bead-associated hemocytes were counted using a Zeiss ICM 405 microscope equipped with a standard FITC filter set. A minimum of 250 cells was counted in each sample. This comparison included control samples with added Cytochalasin B. In addition, the reproducibility of the assay was tested by establishing duplicate experimental wells for each clam and comparing flow cytometric results for the replicates using correlation analysis. Finally, to determine the effect of storage on the percent phagocytosis measurement, samples were collected as described above, processed by flow cytometry, stored in glass tubes at 4°C, and re-analyzed after 3 and 7 days.

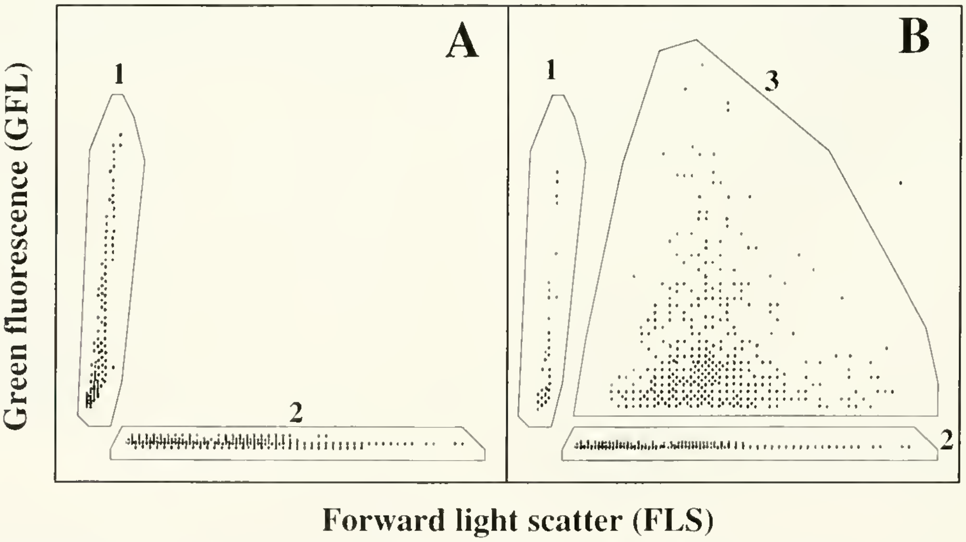

Figure 1. Flow cytometry bivariate plots showing the bitmaps used to calculate bead-associated and non-bead-associated cells in control (A) and test (B) mixtures. Bitmap 1: beads alone, Bitmap 2: non-bead-associated cells, Bitmap 3: bead-associated cells.

Design and Validation of the Viability Assay

In vitro Incubation of Hemocytes with the Fluorescent Vital Stain

The percentage of dead cells was assessed using the fluorescent nucleic acid stain ethidium homodimer-1 (EHD) previously used by Ashton-Alcox and Ford (1998). EHD binds to nucleic acids by intercalation. It does not permeate cells with intact membranes; thus only dead cells, or those with damaged membranes, become fluorescent. Hemolymph samples were diluted in cold Alsever's solution (1:10, v:v) immediately after collection. Then, 2 μM EHD (Molecular Probes, Eugene, Oregon, USA), dissolved in DMSO according to manufacturer's directions, was added. The mixture was incubated at room temperature for 30 minutes. An unstained control sample was made for each clam.

Flow Cytometry

Flow cytometry was used to collect light scatter parameters and log red fluorescence (LRFL) signals for at least 5000 cells. The percentage of dead cells was determined by setting a cursor at the upper limit of the LRFL signal for the unstained control, which was used as the "zero" channel for the stained cells in the parallel treated sample (Fig. 2). The percentage of dead cells was calculated as the ratio of cells above the "zero" channel to total hemocytes × 100.

Assay Validation

Fluorescence microscopy was used to compare and correlate the hemocyte viability results obtained from flow cytometry. Fluorescent and non-fluorescent hemocytes were counted using an epifluorescence microscope as described above. A minimum of 200 cells was counted in each sample. The reliability of the assay was also tested by using hemocytes that had been killed by immersion in boiling water for 5 min. Hemocyte mortality was confirmed microscopically, after EHD uptake, to be 100%, whereas untreated cells were more than 96% viable. Five mixtures of untreated and heat-killed hemocytes were made using 0%, 25%, 50%, 75%, and 100% heat-killed hemocytes. The percentage of viable and non-viable hemocytes was measured flow cytometrically as described above.

Effect of Temperature on Phagocytosis

Because BRD development is partially controlled by temperature, the phagocytosis assay was used to quantify the effect of temperature on phagocytosis by *R. philippinarum* hemocytes *in vitro*. Hemolymph was withdrawn from clams and immediately diluted with ice-cold SSW to give a final concentration of about 5 to 7 × 10^5 cells ml^{-1}. For each clam, 6 test wells and one control well of hemocytes and beads were established. Two replicates were incubated at 8°C, two at 13°C, and two at 21°C in the Mini-frige II® temperature chambers. These temperatures were selected because they are associated with the development of significantly different BRD prevalences and intensities in experimentally challenged clams (Allam 1998). The single control well was incubated at 21°C since preliminary studies showed no evidence of phagocytosis in controls at any tested temperature. All samples were processed for flow cytometry as described above.

In vitro Interactions Between Hemocytes and *V. tapetis*

The first experiment was devised to study the uptake of fluorescently labeled *V. tapetis* by hemocytes. Exponentially growing *V. tapetis*, cultured on marine agar were suspended in phosphate-buffered saline (PBS) to obtain about 10^9 cfu ml^{-1}. The bacteria were then fixed in 2% formalin, washed 3 times with PBS, and resuspended in 1 ml PBS (pH 7.4) containing 1 mg FITC (Sigma). The mixture was incubated for 30 min at RT, washed twice in PBS, and finally resuspended in sterile seawater. Labeled bacteria were then placed in each well of a 24-well microplate, centrifuged to form a uniform layer on the bottom of each well (10 min, 500 × g, 21°C) and used for measuring the phagocytic activity of hemocytes as described above. Six wells were established for each

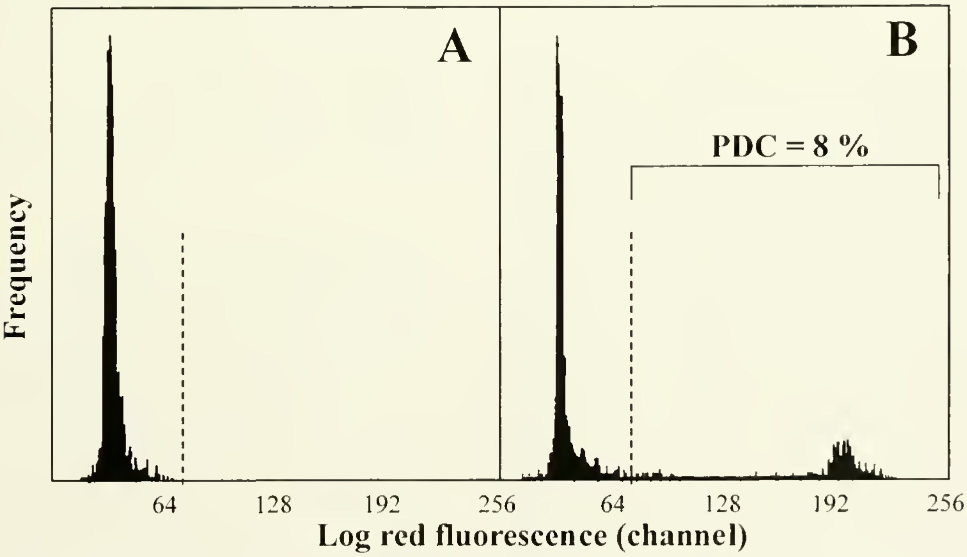

Figure 2. Distribution of log red fluorescence (LRFL) in unstained control and sample stained with ethidium homodimer. A cursor was set at the upper limit of the LRFL signal for unstained control, which was used as the "zero" channel for the stained cells within the sample. PDC: Percent Dead Cells.

clam using the same hemocyte:test particle ratio (1:10): 3 incubated with labeled bacteria (2 test, and 1 control well with Cytochalasin B added), and 3 incubated with standard fluorescent beads (2 test, and 1 control well).

The second experiment investigated the effect of live *V. tapetis* on hemocyte viability. Hemolymph samples were diluted with cold Alsever's solution (1:10, v:v) immediately after collection. Exponentially growing *V. tapetis* were suspended and diluted in sterile Alsever's solution to obtain about 10^7 cfu ml^{-1}. One milliliter of this suspension was transferred to a sterile plastic tube and mixed with 1 ml of diluted hemolymph to give approximately a 1:50, cell:bacteria ratio. Ten μg ml^{-1} of Cytochalasin B was immediately added to the mixtures to prevent phagocytosis. They were then incubated with mild agitation on a rocker plate for 2 h at room temperature. A duplicate tube containing only sterile Alsever's solution and Cytochalasin B was prepared for each clam and was used as a control preparation. After incubation, EHD was added and the percentage of dead hemocytes measured using the flow cytometer as described previously.

Statistics

Percent phagocytosis and viability values were arcsine transformed before the use of statistical tests, however tables show means and standard errors of non-transformed values. Correlation analysis was used in the validation of the phagocytosis and viability assays. Correlation analysis, as well as repeated measures ANOVA, was also used to test the effect of storage time on the percent phagocytosis. Mean values in clams with (symptomatic) and without (asymptomatic) BRD symptoms were compared using a Student's *t*-test. This test was also used to compare the *in vitro* effect of *V. tapetis* on hemocyte viability. Differences were considered significant at $\alpha = 0.05$.

RESULTS

Assay Validation

Phagocytosis

The percent phagocytosis of beads calculated by flow cytometry (19.9 ± 2.5) was significantly ($P = 0.039$) lower than that calculated by fluorescent microscopy (26.4 ± 2.6), but the two methods were highly correlated (N = 24, $r^2 = 0.81$, $P < 0.0001$). Microscopic observation confirmed that the beads associated with hemocytes were internalized. The percent phagocytosis in duplicate wells was also highly and significantly correlated (N = 72, $r^2 = 0.67$, $P < 0.0001$); consequently, two wells per individual was considered sufficient replication in all subsequent experiments. Although there appeared to be a slight decrease in the percent phagocytosis in samples stored for seven days at 4°C (Table 1), a repeated measures ANOVA showed no statistically significant effect of time. Nevertheless, all further flow cytometric samples were processed within 24 hours because there was some microscopic evidence of an increase in broken cells and membrane fragments in stored samples. Fluorescence intensity (channel number) was equal to 5.7 ± 0.3 in non-phagocytic cells (mean ± SEM), 12.6 ± 4.1 in free beads, and 27.4 ± 7.3 in phagocytic cells. No significant changes in fluorescence intensity were observed in preserved samples.

TABLE 1.

Effects of storage on flow cytometric counts of percent phagocytosis in *R. philippinarum*. Samples were processed at the day of collection and re-analyzed after 3 and 7 days of storage at 4°C. Means, standard errors of the means, and correlation coefficients are presented ($N = 24$ clams with 2 replicates each). No significant differences were observed among means (repeated measures ANOVA) which were highly correlated ($P < 0.0001$).

	Day 0	Day 3	Day 7
Mean ± SEM	22.3 ± 1.3	18.8 ± 0.9	19.9 ± 1.1
Day 0	1	0.81	0.85
Day 3		1	0.77
Day 7			1

Viability

The percentage of dead hemocytes calculated by flow cytometry (3.7 ± 1.1) was not significantly different from values measured microscopically (4.2 ± 1.0, $P = 0.69$), and the two methods were highly correlated (N = 10, $r^2 = 0.92$, $P < 0.001$). About 99.9% of heat-killed hemocytes were located within the upper channels on the LRFL histograms, and thus detected by the flow cytometer as dead cells. Flow cytometric estimates indicated that the hemocyte mixture made without addition of heat-killed hemocytes contained 3.8% dead cells, while those containing 25, 50 and 75% heat-killed cells contained 28.1, 52.0 and 75.6% dead hemocytes, respectively.

Disease Effects on Phagocytosis and Hemocyte Viability

Flow cytometric measurements showed a somewhat lower percentage of phagocytic hemocytes (7.2 ± 1.0%, N = 21) compared with those from asymptomatic animals (10.4 ± 1.3%, N = 23, $P = 0.044$) and no difference in fluorescence intensity among phagocytic hemocytes (33.2 ± 9.0 for diseased and 25.1 ± 5.1 for asymptomatic clams, $P = 0.420$). Symptomatic clams had a significantly higher ($P = 0.0006$) percentage of dead hemocytes (13.4 ± 1.0%, N = 54) when compared with asymptomatic clams (9.2 ± 0.5%, N = 20).

Temperature Effects on Phagocytosis

The percent phagocytosis was clearly related to the temperature at which the hemocyte-bead mixture was held (Table 2). This

TABLE 2.

Effect of temperature on *in vitro* phagocytosis of beads by hemocytes from *R. philippinarum* ($N = 24$ clams with 2 replicates each). For each parameter, letters (a, b and c; or x and y) represent significant differences among different temperatures (ANOVA, $P < 0.05$). Fluorescence intensity refers to the peak fluorescent channel of those hemocytes that had phagocytosed beads.

	8°C	13°C	21°C
% Phagocytosis			
Mean ± SEM	7.9 ± 0.7^{a}	14.9 ± 1.0^{b}	21.1 ± 1.2^{c}
Range	2.7–19.3	4.4–25.5	12.5–41.6
Fluorescence Intensity			
Mean ± SEM	15.1 ± 3.0^{x}	24.0 ± 4.4^{y}	29.6 ± 8.3^{y}
Range	10–21	18–33	19–38

percentage at 21°C was about 21%, at 13°C, it was 15%, and at 8°C, it was 8%. Similarly, fluorescence intensity within phagocytic cells was also dependent upon the incubation temperature. This was about 30 and 24 in mixtures incubated at 21 and 13°C, respectively, and only 15 in those held at 8°C (Table 2).

Uptake of V. **tapetis** *versus Beads*

Clam hemocytes phagocytosed fluorescent latex beads at a significantly higher rate (22.3 ± 1.3%) than they did FITC-labeled *V. tapetis* (12.8 ± 2.9%, $P = 0.002$, N = 24).

Hemocyte Killing by **V. tapetis**

The percentage of dead cells increased significantly after incubation of hemocytes with the pathogenic bacterium, *V. tapetis*. In control mixtures, the percentage of dead hemocytes was 6.8 ± 2.2 while it reached 20.4 ± 5.9% in mixtures with *V. tapetis* added (Student's *t*-test, $P = 0.029$, $N = 11$).

DISCUSSION

Flow cytometry has been used in several previous studies to quantify phagocytosis and hemocyte viability in marine bivalves (Alvarez et al. 1989; Ashton-Alcox & Ford 1998; Brousseau et al. 2000; Fournier et al. 2001). However, none of these studies reported validating the methodology by comparing the flow cytometric results against standard microscopic measurements. In this study, we quantified phagocytosis and hemocyte viability in individual clams by both flow cytometry and microscopy. The high correlation between the two methods shows that flow cytometry is not only a rapid and versatile method for analyzing these two important parameters, but that the results are very comparable to more traditional methods for assaying marine bivalve hemocytes.

Notwithstanding the good correlation between flow cytometry and microscopy, differences in means between the two methods may be significant. Some of this disparity undoubtedly comes from uncertainty in drawing bitmaps to delineate presumed flow cytometer particle groupings. In our study, the fact that microscopy estimated a higher percent phagocytosis may be the result of the inclusion of some cell debris in samples analyzed by flow cytometry. Large, bead-free debris could fall above the noise discriminator for forward light scatter and be counted as non-phagocytosing cells, which would decrease the calculated percent phagocytosis. Indeed, microscopic observation revealed the presence of a limited quantity of such cell debris, which increased when samples were stored for several days. Such debris was not generated in the viability assay since the experimental protocol did not involve the addition of trypsin and the mild sonication used in the phagocytosis assay to detach adhered cells. Probably for this reason, the percentages of dead cells obtained using flow cytometry were not different from those measured microscopically.

Critical to phagocytic assays is the detachment of cells from the incubation vials and the separation of non-ingested particles from the phagocytic cells. Trypsinization is generally used to detach cells (Alvarez et al. 1989) while a variety of methods have been developed to discriminate non-ingested particles (De Boer et al. 1996; Mortensen & Glette 1996; Lopez-Cortes et al. 1999). We found that a single procedure, trypsinization in the presence of EDTA followed by gentle sonication, was quite effective in both detachment and separation steps. The centrifugation step over a sucrose gradient (Alvarez et al. 1989) is not required here to separate non-bound beads from phagocytic cells since the forward light scatter allows easy differentiation between these particles based on size.

In this study, fluorescent latex beads were used as standard experimental particles for phagocytosis, as in the work of Alvarez et al. (1989) and Brousseau et al. (2000) who studied phagocytosis by hemocytes in *Crassostrea virginica* and *Mya arenaria*, respectively. These commercially available, fluorescent beads are extremely convenient for reproducible flow cytometric work due to their standardized sizes and fluorescence, qualities that are difficult to establish by labeling natural microorganisms. It must be recognized, of course, that latex beads may not evoke the same response from hemocytes as would foreign cells. Indeed, the present work demonstrated that the uptake of labeled *V. tapetis* was significantly lower than that of beads, despite precautions taken to ensure the same experimental conditions and hemocyte-to-particle ratios. Differences between the two test particles may be the result of specific interactions between hemocytes and beads or bacteria involving recognition factors that retard the uptake of formalin-fixed bacteria. Using microscopy, Lopez-Cortes et al. (1999) studied the phagocytic activity of *R. philippinarum* against *V. tapetis* and noted that the uptake of bacteria depended on the *V. tapetis* strain used. They also noted that viable *V. tapetis* were more efficiently phagocytosed than were the formalin-fixed bacteria and concluded that this was related to the presence of "specific recognition molecules" on the outer membrane of *V. tapetis* that combine with hemocyte receptors and that might be altered during fixation. Most work using labeled bacteria as test particles has been done after fixation of the microorganisms, which stabilizes the tag intensity because it prevents cell division or other processes that could alter intensity. Nevertheless, with appropriate controls, the use of live tagged bacteria should be included in cytometric assays to investigate the role of bacterial epitopes in recognition processes.

The development of BRD was associated with a significant decrease in phagocytic activity by hemocytes and an increase in the percentage of dead hemocytes. The lower phagocytic activity in diseased clams could be related to the percentage of dead hemocytes, since dead or moribund hemocytes are not capable of phagocytosis. Previous work has shown that clams with BRD have a high percentage of dead cells in the hemolymph compared to healthy animals and that this percentage increases with the development of the disease (Allam et al. 2000a, 2000b). The loss of phagocytic capacity and the death of hemocytes may result from deterioration of the physiological condition in severely infected clams (Plana et al. 1996), or from direct killing of hemocytes by *V. tapetis*. Indeed, results presented here demonstrate that *V. tapetis* is able to kill clam hemocytes *in vitro*.

It is often of interest to know the number of particles a phagocyte has ingested (phagocytic index) as well as the proportion of phagocytosing cells. The peak fluorescence intensity in the hemocytes that did ingest beads provided an index for the relative number of beads ingested by each cell. From this, it can be concluded that although clams with BRD symptoms had a smaller proportion of phagocytic hemocytes compared to asymptomatic clams, there was no difference in the number of beads each cell ingested.

Our results show a positive correlation between temperature and both percent phagocytosis and the phagocytic index of latex beads and agree with previous studies of other marine bivalves (Feng & Feng 1974; Foley & Cheng 1975; Alvarez et al. 1989; Tripp 1992; Chu & La Peyre 1993). It is relevant that the development of BRD seems to be at least partially controlled by water temperature (Paillard et al. 1997; Allam 1998). Laboratory experiments have shown high prevalence and intensity of the disease when clams are incubated at 8°C and 13°C compared to clams incubated at 21°C (Allam 1998). Conversely, there are improved repair processes (recalcification) at 21°C as compared to the lower temperatures. It is possible that the low prevalence of BRD at 21°C is related to better performance of the clam's defense system, including phagocytosis. The pathogen may also be less virulent at this temperature, although growth of *V. tapetis* is not inhibited at 21°C (Maes 1992; Paillard et al., unpublished).

In summary, we have described and validated flow cytometric methods to measure the phagocytic activity and viability of hemocytes from the clam, *Ruditapes philippinarum*. Additional types of cytometric measurements will undoubtedly be adapted from vertebrate systems for use with molluscs and other invertebrates. For example, reactive oxygen species (ROS) generated by phagocytic cells represent a current concern of bivalve pathobiologists and ecotoxicologists (Winston et al. 1996; Bramble & Anderson 1997; Lambert & Nicolas 1998). The successful use of flow cytometry for ROS measurements in aquatic mammals (De Guise et al. 1995) and fish (Verburg van Kemenade et al. 1994) indicate that flow cytometric methods can also be adapted for this purpose in bivalves. As new cytometric assays come into use, the developmental protocol should include some type of verification against a method that is considered the standard for that assay.

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TRANSPLANTS OF INTERTIDAL SHELLFISH FOR ENHANCEMENT OF DEPLETED POPULATIONS: PRELIMINARY TRIALS WITH THE NEW ZEALAND LITTLE NECK CLAM

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ABSTRACT The New Zealand little neck clam, *Austrovenus stutchburyi* (Wood 1828), is widely distributed in sheltered intertidal habitats around New Zealand and has long been harvested by recreational and traditional fishers. Clam abundances have declined on many beaches due to excessive harvesting and habitat change, such as sedimentation and pollution.

The feasibility of transplanting clams as a method of shellfish enhancement was tested. Manipulative field experiments using tagged clams examined parameters likely to affect growth and survival of transplanted clams, including the size of seed used, density and shore level at which seed is planted out, and the season in which the transplant is undertaken.

Juvenile clams (10–18 mm) had a mean recovery rate of 30% after 1 y. Growth was highest for clams transplanted to low on the shore, but mortality was also highest for these clams. Optimal placement of juvenile seed for enhancement would be at mid-shore levels, where more clams were retained and reasonable growth still occurred. A much higher recovery rate (60%–90%) was achieved for adult clams (25–32 mm), and they were more likely to remain in the new area. These pilot trials demonstrate that transplant is feasible and would be particularly successful for adult clams.

KEY WORDS: New Zealand *Austrovenus stutchburyi*, clam, enhancement, transplant, growth, season, survival

INTRODUCTION

The commercial exploitation of soft shore bivalves in New Zealand has been at low levels, with the exception of the surf clam, *Paphies ventricosa* (Stace 1991). Other species, however, traditionally played an important part in recreational fisheries and in traditional Maori food gathering (Dobbinson et al. 1989; Turner 1997; Stace 1991). Although considered nationally abundant, many populations have become locally depleted (Browne & Pawley 1995; Pawley et al. 1996; Pawley et al. 1997; Morrison et al. 1999, and considerable anecdotal evidence). *Austrovenus stutchburyi*, also known as the New Zealand little neck clam (Belton 1986), is one of the most abundant of these soft shore bivalves, but populations are experiencing declines in some areas. In the Auckland region in particular, temporary closures have been put in place in an attempt to counter declining stocks. The causes of these declines and the subsequent need for stringent countermeasures have not been adequately identified or addressed, but have commonly been hypothesized as increased sedimentation, high levels of contaminants in sediment and water, and overharvesting. These causes are all associated with urban development in coastal areas.

Current management of shellfish populations in New Zealand is largely restricted to the imposition of daily bag limits and local bans, although these are often not well enforced (Kearney 1999). Natural recruitment may lead to the recovery of clam beds if harvesting bans continue and are enforced, but recovery may be unacceptably slow. This appears to be the case in Auckland, where beds have not returned to historical densities despite a 7-y harvesting ban (Morrison et al. 1999).

There are two main commercial approaches to shellfish enhancement which have been used in coastal environments around the world: (1) the spawning and rearing of spat in a hatchery, and (2) the collection of spat from the wild (Malouf 1989; Peterson et al. 1995; Marelli & Arnold 1996). Both are then followed by a period of on-growing (usually in a land-based facility) before transplantation to the site of enhancement. Another, more traditional technique is the use of "spawner transplants," which involves transplanting naturally established shellfish from locations where the species is abundant to "enhancement" sites (usually sites where numbers are very low). In Japan, clams and oysters have been transplanted to enhance stocks for centuries (Wada 1993). It is also a traditional management practice for hard clam (*Mercenaria mercenaria*) fisheries in the eastern states of the United States (Kassner & Malouf 1982). There are a number of earlier studies where European clams (*Cardium edule*) were taken from areas either of high spat fall or of high density and transplanted to areas of low density (Hancock 1969; Mason 1969). Recent transplants of adult scallops in the western Bogue Sound, North Carolina, resulted in enhanced recruitment of 568% compared to 3% for control sites (Peterson et al. 1996).

In New Zealand, enhancement techniques are not developed to the same level as those widely used overseas (Creese & Cole 1995). Although it is technically possible to rear New Zealand clams in the laboratory (Stephenson & Chanley 1979), reliable production of large numbers of spat has proved difficult, and no commercial or sizeable hatchery has yet been established to produce the quantities needed. Enhancement using adult clams had been suggested but not tested in New Zealand prior to the commencement of this research (Dobbinson et al. 1989).

Manipulation of several factors, shown separately to influence the survival of seed (juvenile or adult shellfish), can greatly improve the success of enhancement (Peterson et al. 1995). The literature on New Zealand clams and on international enhancement attempts identified several factors that might influence the transplant success of infaunal bivalves. The transplants of clams described in this paper manipulated not just one, but several factors that the literature suggested might be important in successful transplant of shellfish. Although the literature suggests that separately these factors might be important in restoration success, the interactions among them may be just as important as the individual factors themselves. A multifactorial design was used to detect all possible effects of density, size of seed, season, and shore level, including the interactions of these factors. Predation could not be feasibly incorporated into this already large experimental design, so this was investigated separately (authors' unpublished data).

In the long term, it would be hoped that transplanting clams would increase success of spawners and thereby recruitment. However, the primary objective of this experimental transplant was to assess the feasibility of the technique as a method for future enhancement, and to determine what factors might affect the success of transplants. In the long term, increased recruitment of clams or changes in overall community structure would be important variables by which to measure the outcome of enhancement. However, for this 1-y, small-scale project, it was decided that growth and survival of transplanted clams would be the most direct measures of the outcome of transplant.

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METHODS AND MATERIALS

Experiments were carried out in the Whangateau Harbour near Leigh in North Eastern New Zealand, with clams transplanted from Lews Bay to Point Wells (Fig. 1). Clams were collected from Lews Bay at low tide over 2 d, taken to the Leigh Marine Laboratory, and held in a saltwater flow-through system. Clams were double tagged, with aluminum pieces glued to the shell (Stewart & Creese 2000) and also with colored enamel paint to denote treatments.

At each of the control and experimental sites, the experiment was set up in a grid pattern with treatments (three replicates of each) assigned to plots (each 0.25 m^2) within the grid using an incomplete block design. There were a total of 15 plots at each site. Each plot was 1 m from any other plot and shared no border. *Austrovenus stutchburyi* are relatively sedentary and throughout the experiment no clams were observed to move between plots. When the clams were planted out, the sediment from each 0.25-m^2 plot was excavated and sieved through a 2-mm mesh sieve to remove any resident macrofauna and thereby standardize the initial state of sediments in the plots. To prevent desiccation and predation of newly transplanted clams before arrival of the incoming tide, clams were placed in the excavated holes and partially covered with sieved sediment. It was observed that, despite being covered initially, most clams would emerge and rebury themselves fully by the next low tide.

Lews Bay, from where clams were originally collected, served as a control site to enable the effect of translocating clams and site effects to be distinguished. Control clams were subjected to the same stress as those transplanted to Point Wells. Because clams are of a small size high on the shore, and are larger lower on the shore (Larcombe 1971), controls for each size class were only set up in the area from which those clams were collected. That is, small clams collected from high on the shore were returned to high on the shore, and large clams collected from low on the shore were returned to low on the shore (Fig. 2).

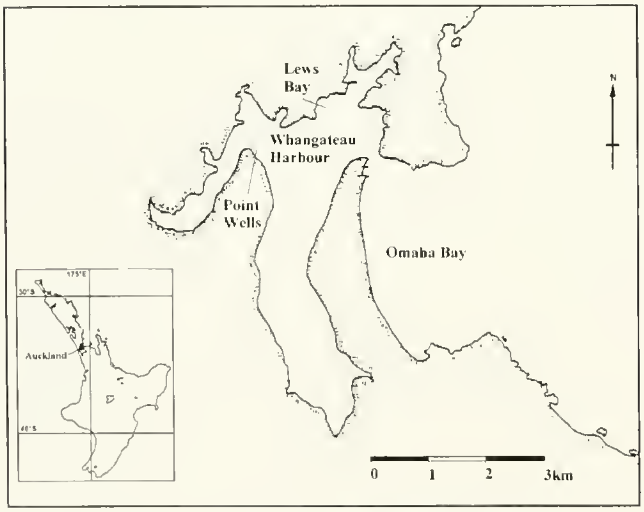

Figure 1. Location of study sites, Lews Bay and Point Wells in Whangateau Harbour, near Leigh in northeastern New Zealand.

To investigate the effect of shore level on growth and survival of transplanted clams, experiments at Point Wells were set up at two shore levels, mid (150 m from mean high water spring [MHWS]) and low (350 m from MHWS) (Fig. 2). Preliminary surveys of the site revealed a low abundance of clams at the high shore, consistent with other studies of clam distribution in the region (Larcombe 1971; Kearney 1999).

Within the transplant experiments, the effect of size was investigated by using two sizes of seed: "small" (10–18 mm shell length), which represented nonspawning clams, and "large" (25–32 mm), which represented an adult spawning size (Larcombe 1971). Two densities were compared to investigate whether it is better to space clams out or to pack them into smaller, more easily managed plots. The two densities compared were 200 clams/0.25 m^2 for the packed treatment and 50 clams/0.25 m^2 for the spaced treatment. Two transplant experiments were conducted, one commencing in March 1998 (summer transplant experiment) and one in September 1998 (winter transplant experiment), allowing comparison between two planting seasons.

A third trial (disturbance experiment) was established to assess the effect of disturbance from the bimonthly excavation of plots used in the two transplant experiments. At the low shore site at Point Wells, an additional three replicates of large clams at the 50/0.25 m^2 density were set up at the start of each experiment. These were excavated only at the end of the experiment, and growth and survival of clams were compared to those in plots disturbed every 2 mo.

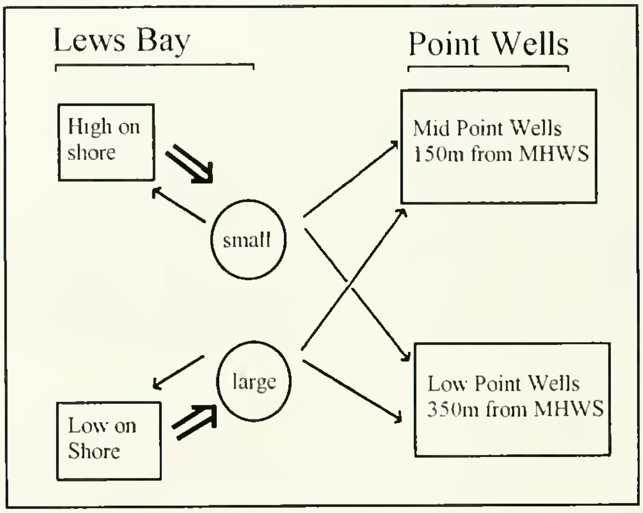
Lews Bay
Point Wells
High on shore
Mid Point Wells 150m from MHWS
small
large
Low on Shore
Low Point Wells 350m from MHWS

Figure 2. Small cockles were collected from high on the shore at Lews Bay and transplanted to mid and low shore levels at Point Wells. As a control, some small cockles were also returned to high on the shore at Lews Bay. Large cockles were collected from low on the shore at Lews Bay and transplanted to mid and low shore levels at Point Wells. As a control, some large cockles were also returned to low on the shore at Lews Bay.

Analysis

The effect of shore level was essentially a separate investigation at Point Wells. If those clams returned to Lews Bay had been planted out at mid-shore and low-shore levels as at Point Wells, this would not have served as a control, because clams would had to have been transplanted to different levels from which they were collected at Lews Bay. Because the "shore level" treatment was not balanced between Point Wells and Lews Bay, the two shore levels at Point Wells were treated as separate sites, additional to Lews Bay, for statistical analysis. The names "Mid Point Wells" and "Low Point Wells" continued to be used in order to retain their site identity.

Mortality

Weekly visits were made to each site and visual searches undertaken for any dead shells (cluckers), which remained on the surface. This was used as an estimate of apparent mortality (Arnold 1984; Dobbinson et al. 1989), to minimize disturbance from frequent digging.

Upon completion of all experiments (January 1999), plots were identified using the metal detector to locate the aluminum-tagged cockles (Stewart & Creese 2000). Plots had no external markings, which avoided interference with plots on public beaches. All clams were excavated and brought back to the laboratory. This enabled a direct measure of the number of clams remaining. Sediment in each plot was washed through a 2-mm sieve and tagged individuals were retained. A number of tagged cluckers were also retrieved during this process. The area (~1 m) immediately surrounding each plot was also sieved until no further tagged clams were retrieved in two successive sieves. In some instances, "finger ploughing" (James & Fairweather 1995) of the sediment was also used. When no additional clams were retrieved either through finger ploughing or sieving, the metal detector was used to locate any missed individuals. The detector was passed over the surface for a distance of up to 5 m away and when a "hit" was detected, the sediment was finger ploughed to retrieve the clam. This was repeated until no more clams were detected over the entire plot and surrounding area.

For each replicate plot, the number of clams in each of four categories was recorded; alive, missing, dead as undamaged empty valves, and dead as empty valves with observable shell damage.

Because the "summer transplant experiment" ran for 11 mo and the "winter transplant experiment" ran for 5 mo, a direct comparison of mortality could not be made between seasons. However, data for the first 5 mo of the summer transplant experiment were analyzed to provide a legitimate comparison between seasons. For mortality, this was done using only the number of shells retrieved during visual searches, and therefore it is a comparison of apparent rather than actual mortality. At the end of the experiment, survival (estimated from the number of live clams retrieved) was analyzed separately for the two seasons. Data were tested for normality and homogeneity (using Cochran's test) prior to performing analysis of variance (ANOVA).

Growth

Because clams were not numbered individually, growth was assessed by following changes in the modal length of size cohorts over time. Every 2 mo, a subsample of 20 clams was remeasured from each 0.25-m^2 plot. Subsampling avoided undue disturbance to plots. Plots and clams were relocated using the metal detector and clams were excavated, with care taken to minimize disturbance to those clams not measured. To ensure that a random sample was taken every time, a strip was randomly drawn through the plot and clams were sampled in this area. Comparisons of growth over the full experimental period could not be made between seasons. Interim calculations were made for growth after 5 mo for the summer transplant experiment.

RESULTS

Mortality

Apparent mortality of clams was estimated from the number of tagged cluckers retrieved after 5 mo for each season. A four-factor ANOVA was performed using the factors "season," "site," "size," and "density." There was no significant effect of season or density on apparent mortality ($P > 0.05$), nor any interaction involving these factors. The pattern of mortality between sites was not the same for large and small clams, giving a significant interaction ($P < 0.01$) between site and size. Mortality for large clams was highest at Mid Point Wells, but for small clams it was highest at Low Point Wells (Fig. 3). Survival was significantly different between large and small clams ($P < 0.01$), with mortality higher for large clams than for small ones (Fig. 3). In addition, a significant difference between sites ($P < 0.01$) was detected. Apparent mortality was lowest at Lews Bay, the site of origin for all transplanted clams (Fig. 3).

Survival was assessed from the number of clams retrieved at the end of both summer (after 11 mo), and winter (after 5 mo) transplants. The average survival was 75%–90% for large clams transplanted in winter, and greater than 60% for large clams trans-

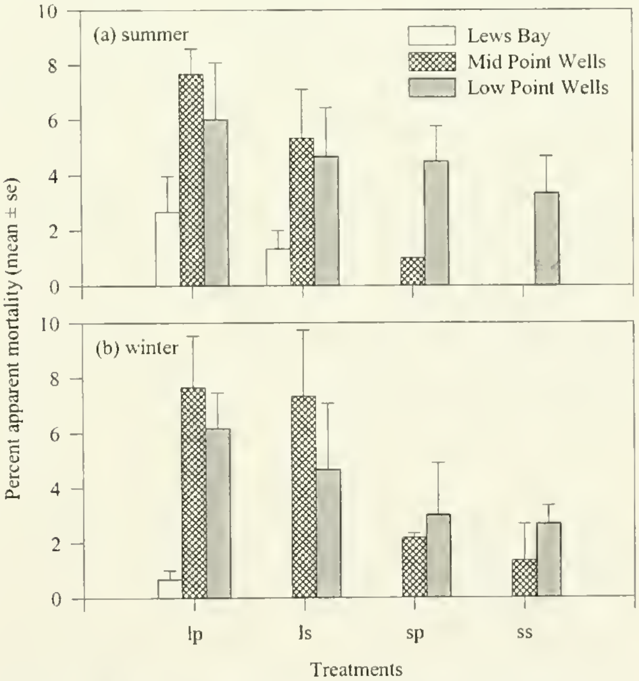

Figure 3. Mean percent apparent mortality, estimated from the retrieval of cluckers across treatments after 5 mo [from March to July for the summer transplant experiment (a) and September to January for the winter transplant experiment (b)]. $n = 3$ replicated plots at each site. lp = large cockles (25–32 mm shell length) at packed density (200/0.25 m^2), ls = large cockles at spaced density (50/0.25 m^2), sp and ss = small cockles (10–18 mm shell length) at packed and spaced densities, respectively.

planted in summer, with the exception of Lews Bay (Fig. 4). Even for small clams, average survival was greater than 30% at all sites except Low Point Wells in summer (Fig. 4). Survival at Lews Bay was confounded by storm events removing an entire shell bank containing the large clams in the winter transplant experiment, as well as many of the large clams in the summer transplant experiment. Therefore, data from Lews Bay were excluded from the analysis. Three-way ANOVAs were performed separately for each of the summer and winter transplants for the Mid Point Wells and Low Point Wells sites only, with the other factors being "size" and "density." With Lews Bay excluded, the two Point Wells sites effectively become "shore level" treatments. For the summer transplant experiment, both the "size × density" interaction ($P = 0.04$) and the "shore level × size" interaction ($P < 0.01$) were significant. Survival for large clams was similar between Mid Point Wells and Low Point Wells, but survival of small clams was lower at Low Point Wells (Fig. 4). Significantly more large clams were retrieved than small ones ($P < 0.01$), and overall survival of large and small clams was highest at Mid Point Wells ($P < 0.01$). The significant "density × size" interaction is due to fewer small clams in the spaced density treatment surviving than in the packed density treatment (Fig. 4). For large clams, survival was similar between density treatments (Fig. 4). Results for winter transplants are similar, but the "size × density" interaction was not significant ($P = 0.08$) (Fig. 4).

Because the storm events that affected experiments at Lews Bay affected only large clams planted low on the shore and not small clams planted higher on the shore, two-way ANOVAs on survival were run for small clams only. That is, the factor "size" was removed and only "site" (Lews Bay included) and "density" were analyzed. For both summer and winter transplants, there was no significant "site × density" interaction. However, both site and density were significant factors. In the summer transplant experiment, survival of small clams at Low Point Wells was significantly lower than at Mid Point Wells or Lews Bay (Student-Newman Kews test, $P < 0.05$). Survival was not significantly different between Mid Point Wells and Lews Bay ($P > 0.05$). For the winter transplant experiment, survival of small clams was significantly different among all three sites ($P < 0.05$), with lowest values at Low Point Wells (Fig. 4).

The majority of small clams not retrieved in the final sample (either as whole or damaged valves) could not be accounted for (Fig. 5). At Lews Bay, the majority of large clams not retrieved were also missing (Fig. 5a and b) presumably due to the storm events. However, at the Point Wells sites, the majority of large clams not retrieved in the final sample were accounted for by either whole valves or damaged valves, retrieved during visual searches carried out during the year. Most retrieved valves were still intact, with no evidence of damage that might indicate predation (Fig. 5). Damaged shells were mainly crushed half valves or had chipped ventral margins.

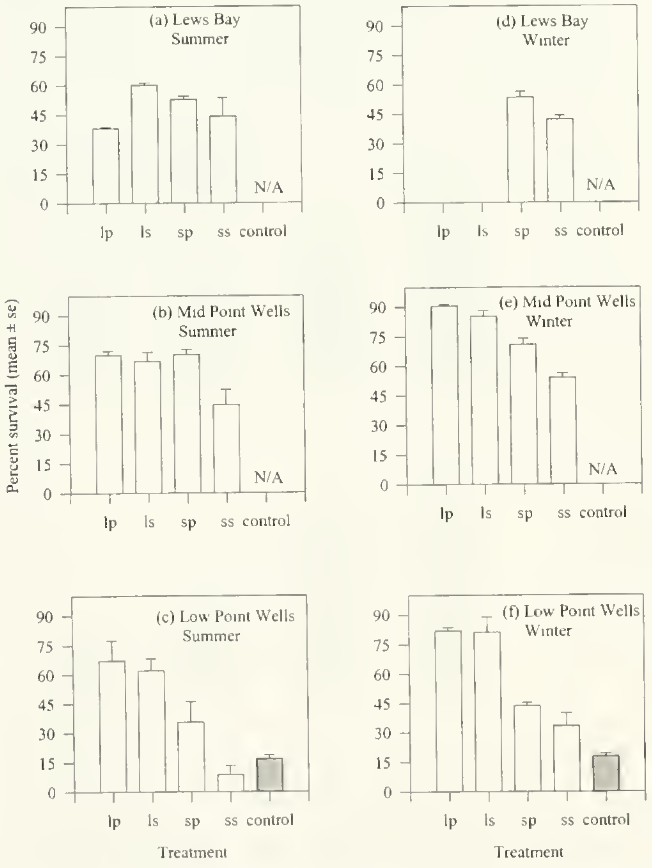

Figure 4. Mean percent survival taken from the number of live tagged cockles retrieved at the end of the experiment (11 mo for the summer transplant and 5 mo for the winter transplant). $n = 3$ replicate plots. Size and density treatments as in Figure 3. Disturbance controls (Low Point Wells site only) are shown in grey.

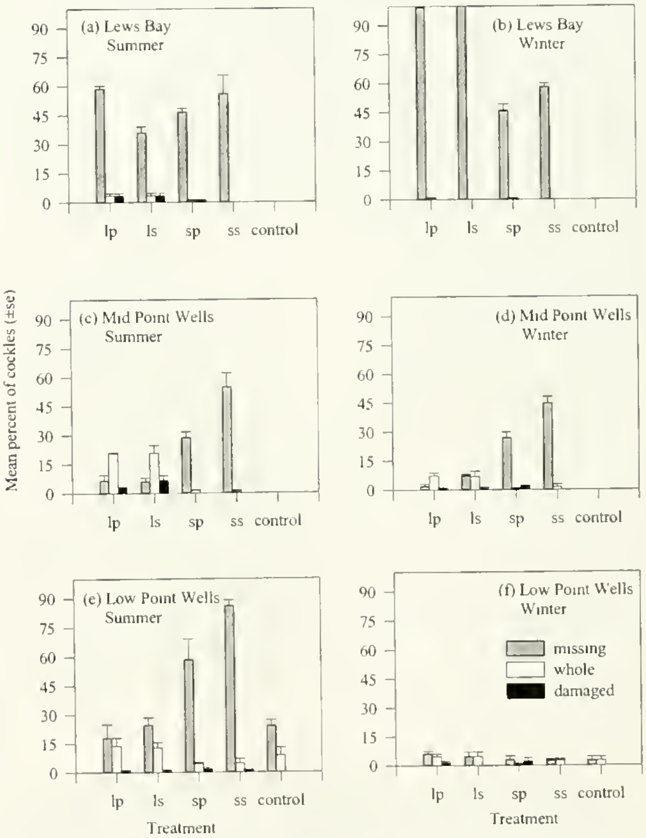

Figure 5. Mean percent of cockles missing, and mean percent of damaged and whole valves retrieved by visual searches at the end of the experiments (11 mo for summer experiment and 5 mo for winter experiment). $n = 3$ replicate plots at each site.

Growth

Mean shell length was initially taken from random samples of 20 individuals taken from each plot every 2 mo. These individuals were considered representative of the experimental population. Inter-replicate variation was found to be virtually zero, so replicate treatments were pooled for graphical representation. Because there was little change in mean size until toward the end of the experiment (when water temperature increased), the difference in growth between treatments was analyzed in terms of the change between the initial mean shell length of transplants and the mean shell length at the end of the experiment.

Growth was analyzed separately for each experiment using a three-way ANOVA with the factors "site," "size class," and "density." For both seasons, there was a significant ($P < 0.05$) three-way interaction ("site × size × density"), but some patterns can be seen in the data. Large clams grew only 1–2 mm during the experiment (Fig. 6). Small clams, however, showed pronounced growth in some treatments, particularly at the Low Point Wells site (Fig. 6). Interestingly, growth in the small clams was readily identifiable by direct observation of the shell. A clear disturbance mark could be seen in the shell, indicating when they were first placed out in the field. No other clams in the area showed such as pronounced growth check. Clams taken from the high shore level at Lews Bay and transplanted to Point Wells grew more than those returned to the site of origin at Lews Bay (Fig. 6).

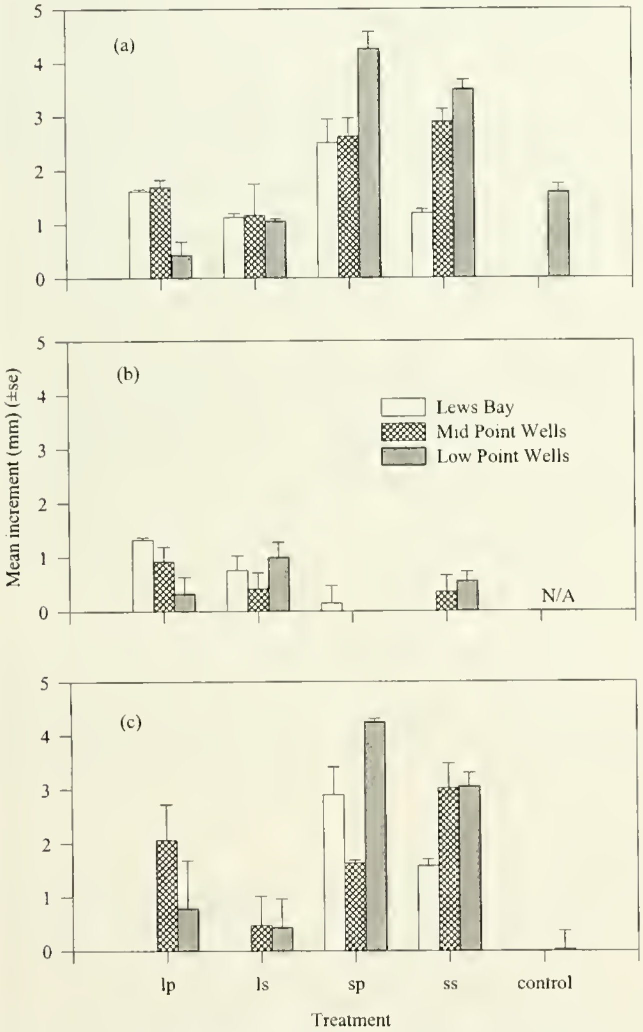

Figure. 6. Mean change in shell length (mm) after 11 months (March–January) for the summer transplant experiment (a) and after 5 months (March–July and September–January) for the summer (b) and winter (c) transplant experiments, respectively. $n = 3$ replicate plots at each site. Control plots (Low Point Wells only) for disturbance are shown in the gray bars.

DISCUSSION

Although the initial collection and tagging of clams did appear to cause a clear growth check mark, the disturbance of clams for ongoing sampling during the experiment appeared to have no effect on growth or survival. The absence of a digging effect is consistent with results from previous manipulations of these clams (Martin, unpubl. data).

Apparent mortality of large clams was lowest when they were returned to their site of origin at Lews Bay. At the end of the experiment, however, no large clams transplanted in winter and few large clams transplanted in summer were retrieved from Lews Bay, due to storm events. Until this point, mortality, estimated both by the numbers of cluckers retrieved and by observations made during ongoing sampling, was lower than for the Point Wells sites. We are confident that this trend of lower mortality at the site of origin would have continued in the absence of storm interference. Lower mortality for clams returned to the site of origin indicates that survival of transplanted clams was affected by environmental characteristics of particular sites, because all clams underwent the same transplant process.

The majority of small clams not retrieved at the end of the experiment could not be accounted for. These clams may have either died and their shells were lost, or they may have migrated out of the area. Small clams were observed to move away from the initial experimental plots more than large clams. If emigration is the main source of "loss," and these clams remain within the general vicinity of the transplant plot, then enhancement may still be considered successful, because these clams will still contribute to the population. If they have migrated away from the area entirely, then the effect on enhancement success is the same as mortality. Tethering of clams would allow better assessment of whether these clams are dying (with subsequent loss of their shells) or if they are migrating. However, we suggest that a minimum survival rate for transplanted clams of 30% (as achieved in our experiment), although not great, may be a realistic management option and preferable to doing nothing and accepting the present situation of declining stocks and poor recruitment.

The majority of enhancement projects are conducted using juvenile shellfish, simply because large numbers can be produced in a hatchery and they do not require costly on-growing to a larger size before planting out. However, planting out of larger shellfish is often more successful (Peterson et al. 1995; Marelli & Arnold 1996), a result attributed to high mortality of juvenile shellfish, possibly from predation. This inverse relationship between size and mortality rate is often explained in terms of prey size refuge (Whetstone & Eversole 1977; MacKenzie 1979; Arnold 1984; Kraeuter & Castanga 1989; Peterson et al. 1995; Marelli & Arnold 1996). For this reason, Marelli and Arnold (1996) consider that broadcasting (i.e., the seeding out of very large numbers of unprotected juveniles) is not an effective stock enhancement technique.

Our experiments enabled a comparison between the survival of small and large transplanted clams. As predicted by the published studies cited above, survival of large clams was much higher than for small clams (generally >60% and up to 90%).

The survival rates obtained in this study compare favorably with transplants of the hard clam *Merceneria merceneria* (mainly juveniles) in the United States. Peterson et al. (1995) achieved 35% survivorship over 14 mo. Marelli and Arnold (1996) were less successful with 95% mortality after 80 d. In earlier trials by Flagg and Malouf (1983), greater than 10% survival was only achieved for those clams larger than 20 mm, and even clams of this size suffered 100% mortality in areas with large whelk populations. A minimum survival for small clams of 30% could well be acceptable for enhancement, but a greater initial number of transplants would be required to compensate for subsequent losses. To achieve the desired level of enhancement, it may also be necessary to follow the initial transplant with a number of smaller secondary transplants. It would be laborious to collect such large numbers of *A. stutchburyi* from the field, and enhancement may only be feasible if viable hatchery production, using local spawning stock, could be established.

Damaged shells recovered in this study had either crushed valves, likely to be attributable to birds or crabs, or chipped ventral margins, likely due to whelks. No large crabs were observed in the area and the only birds observed were lone pairs of oystercatchers, *Haemstropus ostralegus*. Other potential predators may have been stingrays or fish, because a number of feeding pits were observed in the area. However, predation by stingrays would have left a noticeable disturbance in the experimental grid, which was never observed.

Peterson et al. (1995) found that survivorship was greater for *Mercenaria mercenaria* clams planted in late fall/winter, but average growth was greater for clams planted in late summer. However, clams transplanted in summer were in the field for at least 2 mo longer than winter-transplanted clams. This temporal difference is mentioned, but no adjustments were made for comparison of the two seasons. Thus, the period of exposure was compared, and not season.

In our study, growth of transplanted *A. stutchburyi* was more dependent on the time of year than on the treatment. Large clams showed minimal growth, probably due to having already attained near maximum size of 35 mm (Larcombe 1971; Dobbinson et al. 1989). Small clams showed pronounced growth at some sites. This growth did not begin until late winter (September) for clams planted out in March, and was most noticeable in the spring (November). For those clams planted out in September, an increase in size was already noticeable 2 mo later, in November. Small clams showed the greatest increase in shell length when transplanted, from high on the shore at Lews Bay (the original site) to low on the shore at Point Wells. This same shore level effect was found by Dobbinson *et al.* (1989) in Otago Harbour, southern New Zealand. *A. stutchburyi* low on the shore are generally larger than those high on the shore (Wood cited in Morton & Miller 1973; Larcombe 1971; Dobbinson et al. 1989). Larcombe (1971) hypothesizes that this is due to poor growth conditions, in particular food availability (due to increased exposure time), restricting growth at higher shore levels. Clams transplanted to low on the shore at Point Wells grew considerably more than those returned to the site of origin at Lews Bay, indicating that clams high on the shore at Lews Bay are unlikely to realize their full growth potential. If this is the case, then translocating these clams to areas lower on the shore at enhancement sites may be a way of increasing the biomass yield (Dobbinson et al. 1989).

Dobbinson et al. (1989) found virtually no effect of density on growth of *A. stutchburyi*. Density manipulations by Stephenson (1981) and Blackwell (1984), however, resulted in apparent intraspecific competition, limiting growth and increasing mortality. Martin (1984) found higher growth rates in treatments where density was experimentally reduced. The effect of density on growth and survival in our study is difficult to interpret because of an interaction with the effect of size. Higher retrieval of clams in densely packed treatments may have been because clam movement was restricted at the higher density, and therefore more clams were retained. If so, retention of transplanted clams may be improved if they are planted out in dense clumps rather than spaced over a beach. This would also make post-transplant monitoring easier.

In conclusion, high survival for large clams and reasonable survival for small clams indicates that the transplant of New Zealand clams is a feasible technique for enhancement. It is recommended that transplant of clams for enhancement should be undertaken with adult clams, because these show the highest survival, are easier to collect, and are more likely to remain within an area. Recent studies have demonstrated that infaunal bivalve recruitment can be dependent on the adult density (Peterson & Summerson 1992; Peterson et al. 1996; Arnold et al. 1998). Large clams may therefore also have the added benefit of adding to the population sooner (through spawning and subsequent recruitment of new clams), thereby speeding the recovery of an area.

ACKNOWLEDGMENTS

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MORTALITIES OF *ENSIS ARCUATUS* (JEFFREYS) (SOLENACEA) IN WESTERN IRELAND

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ABSTRACT Mortalities of the razor clam *Ensis arcuatus* were widespread in western Ireland in the spring months of 2001. Loss of biomass from one razor clam bed was estimated at 74%. Larger razor clams were more susceptible. Histological and bacteriological examinations and TEM were carried out on moribund and live individuals but no pathological cause was identified. Gonadal staging revealed that large *Ensis arcuatus* were partially or completely spent. Mortality is explained as a post-spawning phenomenon, which was unusually severe in 2001.

KEY WORDS: *Ensis arcuatus*, razor clams, shellfish mortalities, western Ireland

INTRODUCTION

In 1997, a hydraulic dredge fishery for razor clams commenced in the Irish Sea where extensive beds of *Ensis siliqua* had been discovered. A market developed, largely in Spain, and, when the Irish Sea fisheries became exhausted, additional beds were sought on the western Irish seaboard. The inshore waters of Cos Galway and Mayo contain thinly dispersed *E. arcuatus*, which form sufficiently dense concentrations to reward commercial exploitation only in special and limited circumstances. These patches are invariably in the lee of reefs and islands that provide shelter from the Atlantic swell.

Investigations were undertaken on these razor clam beds to provide biological data on the growth and reproduction of *Ensis arcuatus* (Fahy et al. 2001b); in the course of these studies we encountered the phenomenon described here, which is evaluated in the context of information emerging from the wider biological enquiry.

Investigations on the local ecology of *E. arcuatus* had been carried out in Cill Chiaráin Bay, Co Galway, in August 2000. In March of the following year, a mass mortality of the species was reported there by a local fisherman, prompting a second assessment. Cill Chiaráin Bay (9°45′W: 53°20′N) is occupied by a major Irish shellfish cooperative, Comharchuman Sliogéisc Chonamara Teó, managing oyster and scallop, and concern for the possible involvement of a disease in the mortalities prompted further investigations. Other shellfish species were not, however, similarly affected, and the fisherman who first reported the event also reported mortalities of *Ensis arcuatus* over a wide area south of Slyne Head (10°10′W:53°7′N), extending to Inishmann in the Aran Islands (9°36′W:53°24′N). Survey work by one of the authors (E. F.) in the vicinity since has confirmed that the phenomenon was widely distributed and that it has affected large numbers of the species.

MATERIALS AND METHODS

The distribution of *E. arcuatus* in a part of Cill Chiaráin Bay was first investigated in August 2000. Divers sampled by pouring 1 L of granular salt over a quadrat 0.33 m^2 and collecting all razor clams which emerged from the substratum within it. Initially, the area was investigated along north-south and east-west transect lines but, when heavier densities of the animals were located (the razor clam "bed"), sampling was concentrated on those areas. The length of every individual was measured on a fish measuring board and the biomass in each quadrat was estimated from a weight at length curve, the parameters of which were calculated in August 2000. Repeat sampling of the bed was conducted on April 23, May 29, and August 23, 2001.

Samples of razor clams, *Ensis arcuatus*, taken from Cill Chiaráin Bay in April (24 individuals) and May 2001 (29 individuals) were processed for histology. Animals were dissected and immediately fixed in Carson's fixative for 48 h. Cross sections were cut so as to include as many organs as possible. Tissue samples were processed in an automatic processor for 10 h. In the processor, the tissues were passed through different grades of alcohol and xylene and then impregnated and embedded in paraffin wax. Tissue sections were cut at 3 μm and stained in an automatic stainer with hematoxylin-eosin; they were then mounted on silinized slides and air dried.

Some of the razor clams collected in April displayed postmortem tissue changes, so the following month a distinction was made between 13 moribund individuals that were lying on the surface of the substratum and 29 live razor clams that emerged from the substratum in response to granular salt having been spread within a quadrat.

The stage of gonadal development was assigned to the samples collected in May following microscopic examination using the terminology of Gaspar and Monteiro (1998): the six stages of gametogenic development progress from stage 0 (inactive) through stages I (early active gametogenesis), II (late active gametogenesis), III (ripe), IV (partially spent), to V (spent).

Tissues from two moribund individuals were fixed in Carson's fixative for 48 h for electron microscopy. They were washed several times in 24 h in a bath of cold cacodylate buffer (at 4°C) and then fixed in 3% glutaraldehyde. Samples were washed in cacodylate buffer then postfixed in 1% OsO4 (for 1 h at 4°C) and washed again in cacodylate buffer, dehydrated in ascending grades of ethanol, and subsequently embedded in epoxy resin. Thin sections were cut with a diamond knife using an ultramicrotome, stained with uranyl acetate and lead citrate, and examined under an electron microscope.

Bacteriological culture plates were used for primary isolation of pathogens. The media employed included blood and seawater agars and thiosulfate citrate bile salt sucrose agar (TCBS) for detection of *Vibrio*. A BioNor (Norway) monoclonal antibody agglutination kit was used to detect the possible presence of *V. anguillarum*.

TABLE 1.

Single-factor ANOVA comparing razor clam biomasses in Cill Chiaráin on four occasions: August 2000, and April, May, and August 2001.

Summary

Groups	Count	Sum	Average	Variance
36982	21	2502.274	119.1559	8031.401
37012	8	606.9071	75.86338	2704.084
37104	24	2657.337	110.7224	10344.37

ANOVA

Source of Variation	SS	DF	MS	F	*P*-value	*F* crit
Between Groups	11019.4	2	5509.698	0.65988	0.521356	3.182606
Within Groups	41477.1	50	8349.542			
Total	428496.5	52				

RESULTS

When the razor clam bed was examined in April 2001, dead and dying razor clams were much in evidence on the substratum or standing half-clear of it, together with freshly empty but undamaged shells. Similar conditions obtained when the site was visited 1 mo later. Razor clams on the bed in Cill Chiaráin Bay are not so densely concentrated when compared with parts of the *Ensis siliqua* bed on the east coast, for example (Fahy et al., 2001a) and there was wide variance in estimates of biomass on each occasion it was sampled. A single-factor analysis of variance (ANOVA) suggests that mean biomass altered highly significantly (Table 1) and more detailed comparisons revealed the differences in biomass to be highly significant between August 2000 and May 2001 ($P < 0.001$); biomass also differed highly significantly between August 2000 and August 2001. The reduction in biomass between August 2000 and May 2001 was 74% (Fig. 1).

Length frequency distributions of live razor clams recovered by divers in August 2000, May 2001, and August 2001 are shown in Figure 2, along with the length frequency of moribund animals in April 2001. In May, there were signs that some of the larger animals recorded the previous August (corresponding with the moribunds recorded in April 2001), were absent from the population, although smaller size categories were not recorded either in May 2001. A notable difference between the years is the large incidence of juveniles, corresponding to recently spatted 0-group animals in August 2001. To compare the year-to-year changes in length distributions, therefore, only animals greater than 7 cm were considered. The two length frequencies differ significantly ($P < 0.05$) (Table 2); contrary to expectation, however (the moribunds had apparently been the largest size group), the greatest discrepancy is in the abundance of small- to medium-sized animals in the second year.

Gonadal staging of live and moribund razor clams in May (Table 3) revealed that the majority were female, but all were either stage IV or V (partially or completely spent).

Histological examination of razor clams sampled in April 2001 revealed that ciliates were occasional on the gills, and there was a low incidence of *Nematopsis* oocytes (Gregarines) in the connective tissue of the mantle and gills of some animals. Low numbers of *Chlamydia*-like organisms were found in some epithelial cells of the digestive gland of some razor clams. The levels of infestation of any pathogen were so low that they could not be associated with the mortalities. Some animals presented postmortem tissue changes. Abnormal nuclei were found in the connective tissue cells of some razor clams. Transmission electron microscopy (TEM) analysis of these samples confirmed that the abnormalities were related to necrosis and not to a pathogen.

Some razor clams collected in May 2001 had *Chlamydia*-like organisms in the epithelial cells of the digestive gland and some had *Nematopsis* oocytes in the connective tissue. One individual in May had a heavy infestation of metazoan sporocysts. But again, mortalities could not be attributed to any of these. The presence of an unexplained "deposit" in some digestive diverticular cell cytoplasm was noted.

Bacteriological analysis indicated the presence of *Vibrio* sp. (possibly *V. fluvialis*), the natural flora of the environment. Results of serological agglutination tests for *V. anguillarum* were negative.

DISCUSSION

Occasional mass mortalities of razor clams, *Ensis* sp., are attributed to several causes, among them storms (Tebble 1966) and

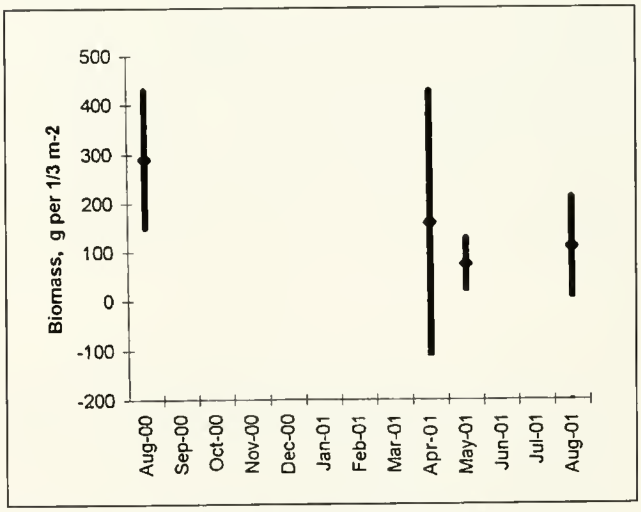

Figure 1. Mean values (±1 SD) of razor clam biomass in Cill Chiaráin Bay at times between August 2000 and August 2001.

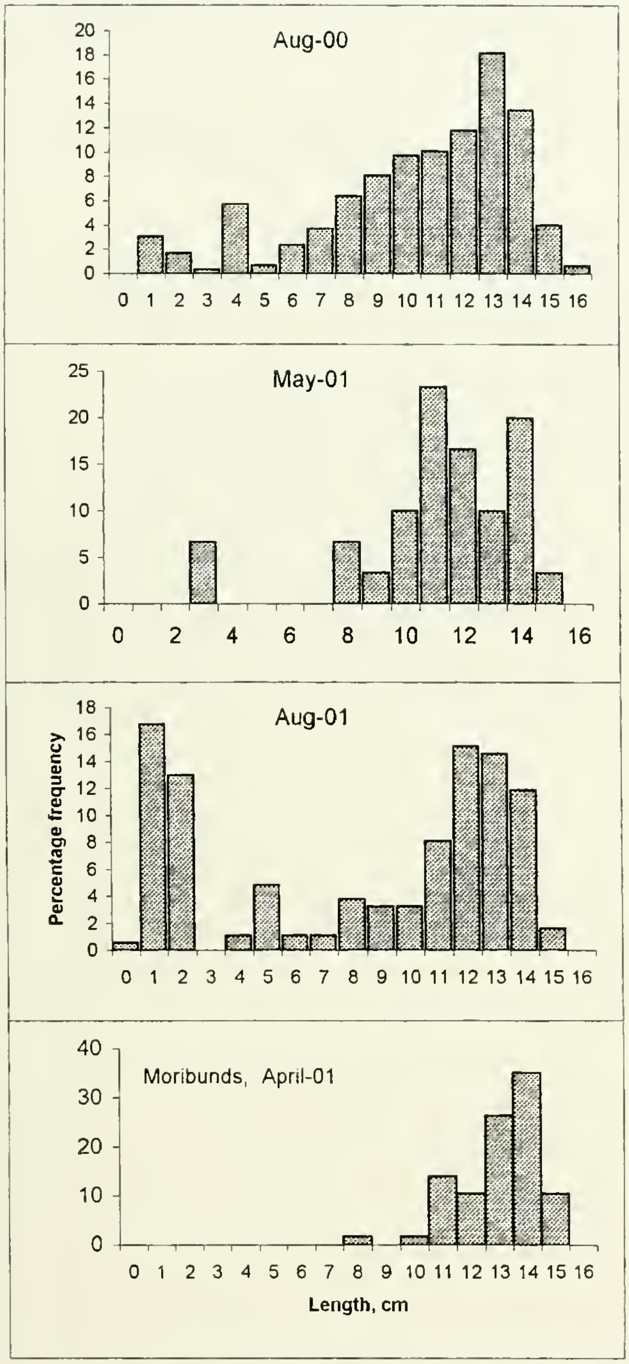

Figure 2. Length frequency distributions of *Ensis arcuatus* in August 2000 and in May and August 2001, and distributions of moribund animals in April 2001.

"adverse environmental conditions" (Howard 1998). Mortalities of the Pacific razor clam, *Siliqua patula*, explained by infection with *Rickettsia*-like organisms, referred to as nuclear inclusion X, have been reported in several locations in the United States (Elston 1986).

Razor clam mortalities in western Ireland in the spring of 2001 were too widespread to be attributed to a local environmental explanation. They occurred within (and outside) a large shellfish cooperative and there was concern lest the oysters and scallops that are managed within the cooperative might be infected with a pathogen, but simultaneous mortalities were not observed among these.

TABLE 2.

Length frequencies (above 7 cm) of razor clams in August 2000 and August 2001, compared by chi-square.

Length (cm)	August 00	August 01	August 00	August 01	Chi-square
7	4	2			
8	7	6	12	8	2.0
9	9	5	9	5	3.4
10	11	5	11	5	7.3
11	12	13	12	13	0.1
12	14	24	14	24	4.5
13	21	23	21	23	0.2
14	16	19	21	22	0.0
15	5	3			
16	1	0			
	100	100	100	100	17.6

$N - 1 = 6$; at 6 degs of freedom, chi-square $= 12.6$, therefore, $P < 0.05$.

The razor clams that made up the mortalities were apparently predominantly large *E. arcuatus*, the dominant bivalve species in this vicinity (Fig. 2). Although it is accepted that larger individuals would be more visible to a diver collecting specimens, the size groups which appeared to have been removed from the population are of small to medium size (Table 2), when August 2000 is compared with August 2001, although sampling in April and May 2001 suggested the largest clams were most affected. The question is why the small- to medium-sized razor clams were not better represented among the moribunds. Razor clams are highly mobile, probably the most mobile of the bivalves after scallops, and they are known, for example, to rapidly recolonize favorable ground after dredging operations, so some immigration to the depleted clam bed from the surrounding lower densities is likely to have taken place after the event. *E. arcuatus* is widely distributed in the vicinity and its length frequency distribution in the areas of greatest clam concentration (the "bed") was similar to that among the more thinly dispersed population in the waters surrounding it in August 2000 (Fahy et al., 2001b). The razor clam bed in this part of Cill Chiaráin Bay is understood to occupy more sheltered and hence more favorable conditions for the animals.

Recorded mortalities were confined to animals of mature size (the smallest mature *E. arcuatus* recorded in the course of biological investigations was 8.5 cm, corresponding with an age of 2–3 y).

TABLE 3.

Gonadal staging for apparently healthy (live) and moribund razor clams from Cill Chiárain Bay in May 2001.

Status	Sex	Gonadal Developmental Stages	Number of Individuals	Percentage
Moribund	Female	V	5	56
		IV	1	11
	Male	V	1	11
		IV	2	22
Live	Female	V	13	65
		IV	4	20
	Male	V	1	5
		IV	2	10

Although it is possible that the smallest dead razor clams were undersampled, the mortalities appeared to be heavily skewed toward the largest, oldest individuals. There are signs that some spawning by *E. arcuatus* takes place in every month of the year, but it is more prevalent in the population from October to April; there were signs of only one spatfall, on the other hand, in June or July. The latter would correspond with spawning in March–April, which must therefore be the most significant spawning time in this species.

If postspawning mortality is a normal occurrence in *E. arcuatus*, the incident in the spring of 2001 must have been exceptional. A regular loss of 74% biomass, although it was followed in this case by a considerably larger spatfall than in the previous year, would have serious consequences for populations of a species with a longevity of up to 18 y, of which 27% of the population on the bed and 38.4% of the more widely dispersed population in the Bay in 2000 were more than 10 y old. These are the age groups that contribute most to the biomass.

A natural postspawning mortality is the proffered explanation for the razor clam deaths in Co Galway in 2001.

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ECOLOGICAL AND MORPHOLOGICAL FEATURES OF THE BIVALVE *ASTARTE BOREALIS* (SCHUMACHER, 1817) IN THE BALTIC SEA NEAR ITS GEOGRAPHICAL RANGE

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ABSTRACT During 1999 and 2000 macrozoobenthos surveys were made in the Mecklenburg Bight (western Baltic Sea). In total 116 stations were investigated between March and September. *Astarte borealis* showed a wide distribution at depths between 12 and 26.5 m. Mean abundance at these depths was 47 ind./m^2 with a biomass (AFDW) of 0.5 g/m^2. Maximum densities observed at these depths were 541 ind./m^2 and 16 g/m^2, respectively. In comparison to a data set of the 1960s, a decreased *A. borealis* population was found. *A. borealis* ranged from 1.2 to 28.7 mm in shell length. Most (78%) individuals in the population measured <10-mm shell length, indicating strong recruitment in the Bight during recent years. Larger size classes (>20 mm) were observed only sporadically and in low numbers. Shell length to wet weight and ash free dry weight correlations are given. Mean wet meat yield was 14.5%. The individual ash free dry weight decreased with increasing shell length from 9.1% (<5 mm) to 5.6% (>25 mm) with a mean value of 7.4%. All results were compared with data from populations in Russian Arctic and adjacent waters.

KEY WORDS: *Astarte borealis*, distribution, abundance, size, meat yield, Baltic Sea, Mecklenburg Bight

INTRODUCTION

The bivalve, *Astarte borealis*, is an arctic-boreal species that occurs in Arctic, North Atlantic and adjacent waters (Zettler 2001). Some data are known from the North Pacific waters such as Japan, Sea of Ochotsk, Behring Street, British Columbia and at the Aleutian Islands (e.g., Coan et al. 2000; Higo et al. 1999; Skarlato 1981). *A. borealis* find its most extensive distribution in Arctic waters of Russia (e.g., Antipova 1978; Filatova 1957; Gagaev 1989; Matveeva 1977). It extends from the Barents Sea, via Kara and Laptev Sea to Chukchi Sea and to areas of northern Alaska, Greenland (Ockelmann 1958) and Spitzbergen (Hägg 1904). At the Grand Banks off Newfoundland, it reaches high abundance in sandy bottoms around 130 m depth (Prena et al. 1999). In Europe this bivalve extends from Iceland (Thorarinsdottir 1997) and off Faeroes and Norway (Brattegart & Holthe 1997) to the northern North Sea (Johansen 1916) via Kattegat (Rasmussen 1973) into the western Baltic Sea and reaches there, its eastern limit of distribution in the Bornholm basin (see Fig. 1) (Demel & Mulicki 1954; von Oertzen & Schulz 1973). Thus, the Baltic population represents the most southern occurrence, an outpost of the mainly arctic area of distribution. The largest populations in the Balic are found in the Kiel and Mecklenburg Bights in depths below 15 m (Kühlmorgen-Hille 1963; Schulz 1969; Zettler et al. 2000). *A. borealis* is among the longest living species in the Baltic and is an important indicator of environmental conditions. Beside salinity and sediment structure, oxygen concentration has a strong influence on the composition of Baltic Sea fauna and flora. Although *A. borealis* is highly resistant to oxygen depletion (von Oertzen 1973; Oeschger 1990) frequent and long lasting periods of anoxic conditions finally diminish or kill the species. This has resulted in a severe decrease of the Baltic Sea population of *A. borealis* during recent decades in the deeper parts of the Mecklenburg Bight (Gosselck et al. 1987; Schulz 1968).

Information on the population biology and morphological features (growth, age, size) of this species is limited. Some investigations on production, growth, population size, and morphological features were carried out in Russian Arctic Waters (Antipova 1978; Gagaev 1989; Matveeva 1977). Within the framework of an autecological analysis of glacial relict species in the Baltic Sea, investigations on the reproduction of *A. borealis* were carried out in addition to experiments concerning its resistance and metabolic adaptations (von Oertzen 1972, 1973; von Oertzen & Schulz 1973). Schaefer et al. (1985) studied biometric features of *A. borealis* in Kiel Bight, the westernmost part of the Baltic Sea. They investigated several relationships between shell length and weight and their applicability for taxonomical distinguishing of species of the genus *Astarte*.

The purpose of this study was to investigate the distribution, frequency and biomass of *A. borealis* in Mecklenburg Bight as the first extensive study on the population characteristics of this important indicator species near its geographical range. A further aim was to compare these results with existing data of the 1960s compiled by Schulz (1969). From one monitoring station (stn. 018), we have a long time data set to show the development of *A. borealis* within the last decades.

Area of Investigation

The Mecklenburg Bight is part of the Belt Sea and belongs to the transition area between North Sea and Baltic Sea (Fig. 1 and Fig. 2). 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.

During 1999 and 2000 macrozoobenthos surveys were made in the Mecklenburg Bight. In total 116 stations were sampled between March and September (Fig. 2). Station depth ranged from 5 to 29.6 m. The sediment varied from fine sand at the shallowest stations to sand mixed with silt and clay at the deepest stations. Sediment characteristics and current data for the area have been 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. 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 oxygen levels determined by Winkler titration. Benthic samples were taken with a 0.1 m^2 Van Veen grab. Due to sediment conditions, grabs of different weights were used. Three replicates of

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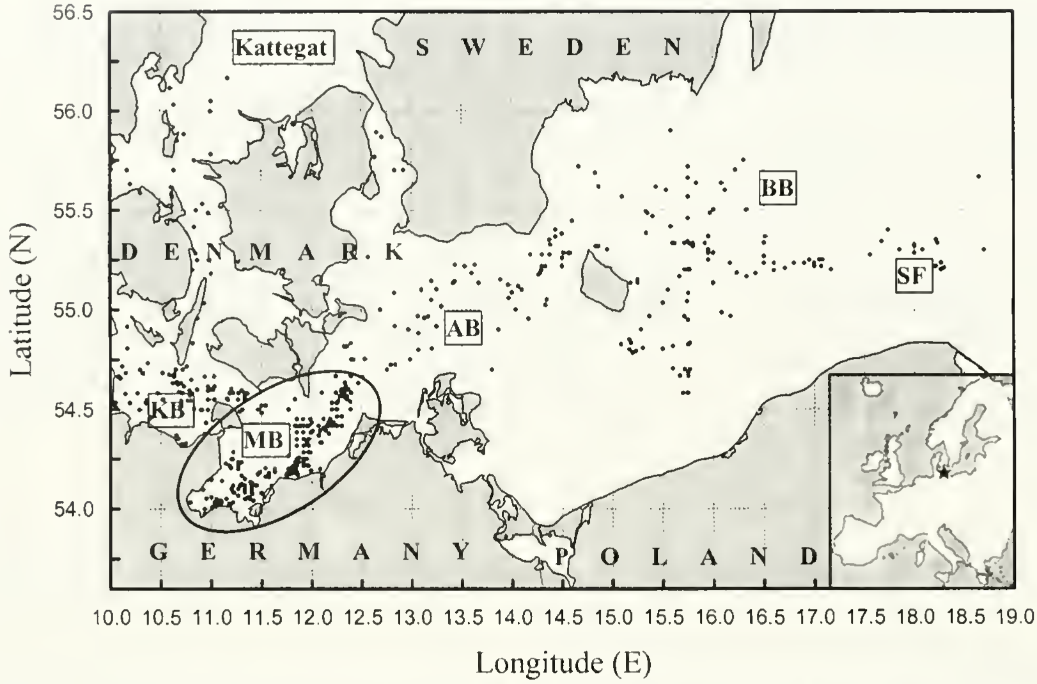
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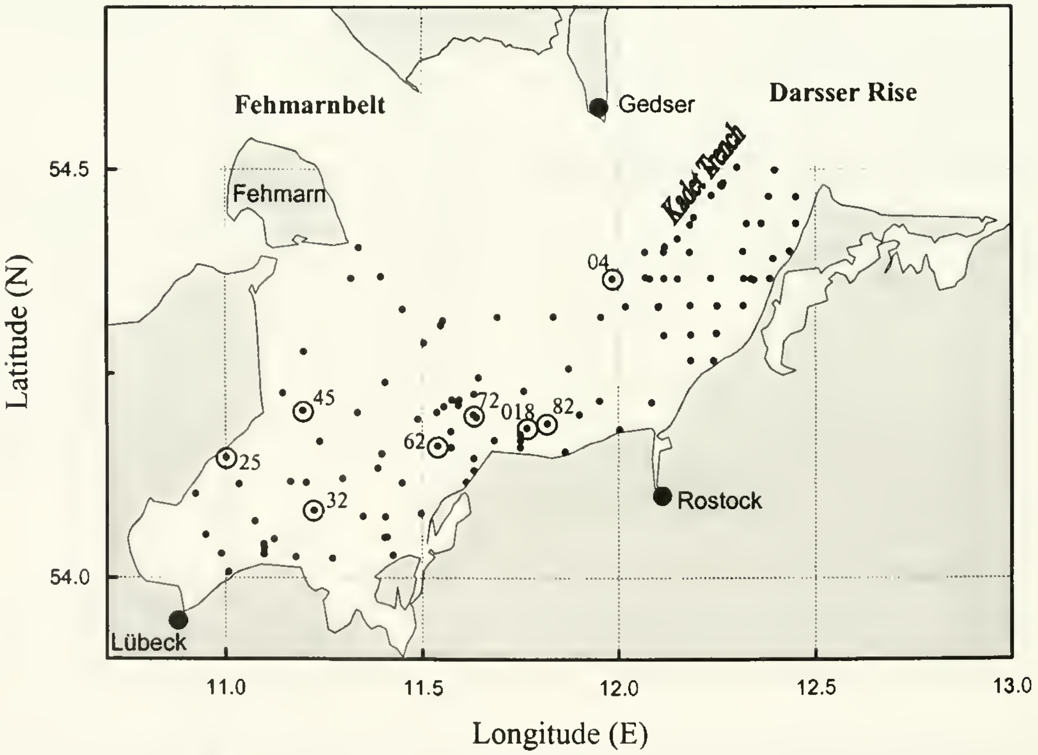
Longitude (E)

Figure 1. Distribution of *Astarte borealis* within the Baltic Sea. The sources of the data are Demel and Mulicki (1954), Kühlmorgen-Hille (1963), Löwe (1963), von Oertzen and Schulz (1973), Petersen (1918) and own observations. Due to a strong decrease and wide disappearance of this species mainly in the Bornholm and Arkona Basins not all dots represent recent locations. (KB-Kiel Bight, MB-Mecklenburg Bight, AB-Arkona Basin, BB-Bornholm Basin, SF-Slupsk Furrow, circled area refer to the present study and Fig. 2).

grab samples were carried out at each station. The samples were sieved through a 1-mm screen and animals preserved with 4% formaldehyde in the field. For sorting in the laboratory a stereomicroscope with 10–40× magnification was used.

The shell length of all collected individuals was 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 414 specimen were measured. The valves and the wet meat of the


Fehmarnbelt
Gedser
Darsser Rise
Kadet Trench
Fehmarn
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82
62
25
32
Rostock
Lübeck
54.5
54.0
Latitude (N)
11.0
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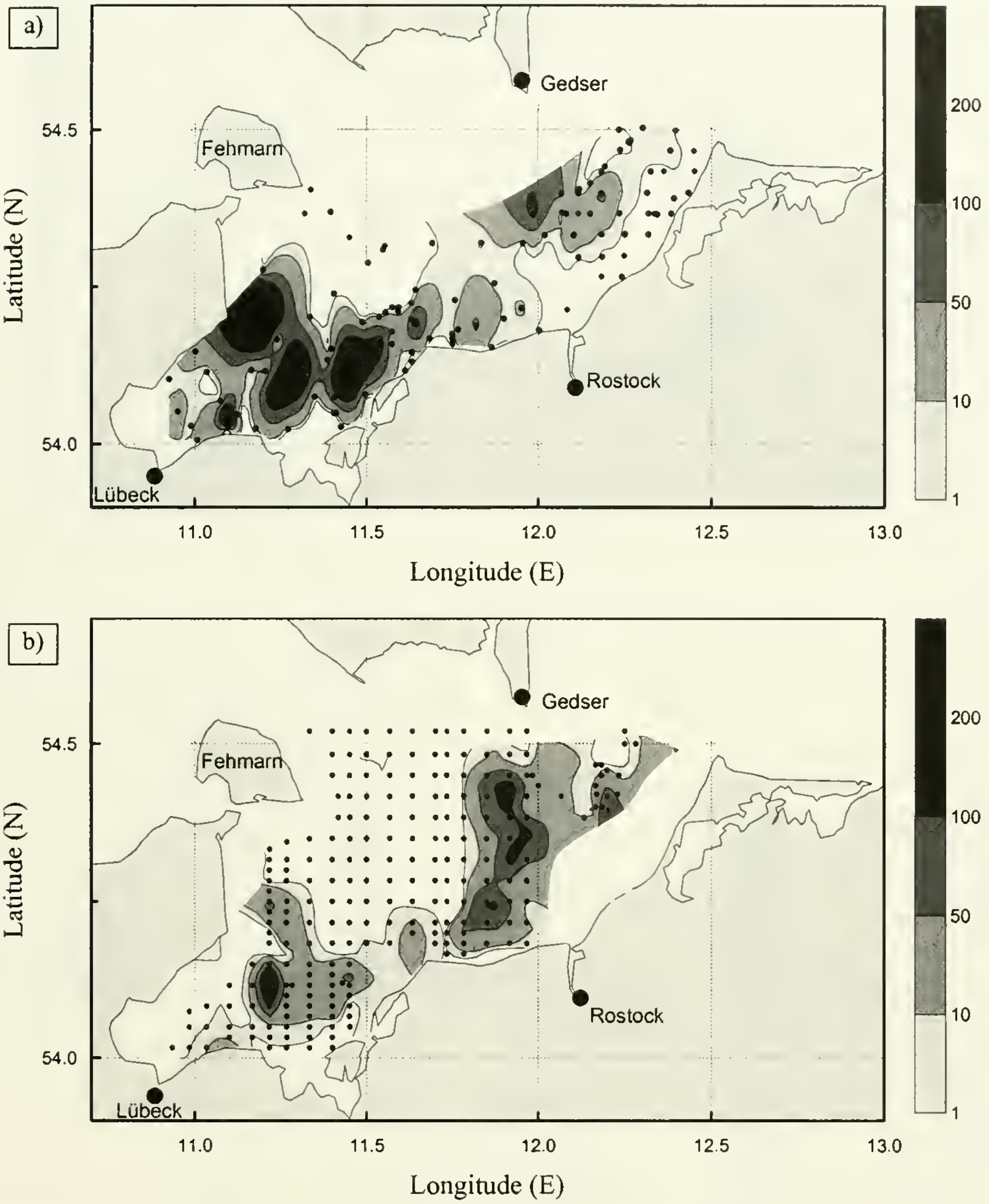
13.0
Longitude (E)

Figure 2. A map showing the investigation area with 116 stations in the Mecklenburg Bight (circled stations refer to text and Figs. 5 and 8).


a)
Gedser
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Rostock
Lübeck
54.5
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Latitude (N)
11.0
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Longitude (E)
200
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50
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b)
Gedser
Fehmarn
Rostock
Lübeck
54.5
54.0
Latitude (N)
11.0
11.5
12.0
12.5
13.0
Longitude (E)
200
100
50
10
1

Figure 3. Distribution of *Astarte borealis* (ind./m^2): (a) in 1999/2000 and (b) during the investigation period 1962–1965 (Schulz 1969).

specimen were weighted separately. Dry weight (DW) and ash free dry weight (AFDW) were determined to the nearest 0.01 mg. Length-frequency distribution for each station was calculated for 5 mm size classes. For the shell length to height relationship, 221 individuals from the 1999/2000 survey and 49 valves of *A. borealis* from the Zoological Collection of the University of Rostock (sampled in the 1980s from the Mecklenburg Bight) were measured.

The distribution map of *A. borealis* in the Mecklenburg Bight was made using Surfer 7.0 programme of Golden Software Inc. The recent distribution was compared with the results from Schulz (1969), whose data were transformed into the Surfer program to obtain a comparable map. For the long time series (1985 to 2000) of the monitoring station 018, the data of the Baltic Sea Research Institute Warnemünde and data from literature were used (Al-Hissni 1989; Voigt 1991).

RESULTS

Bottom Water Variables

Salinity throughout the water column ranged between 7.5 and 27.8 psu, while bottom water salinity of areas inhabited by *A. borealis* varied between 11.0 and 26.3 psu in 1999/2000. No oxygen depression was observed during the surveys. Up to a depth of 18 m, more than 5.0 mg/l oxygen was measured. In deeper parts of the Bight the oxygen content decreased to a minimum of 1.0 mg/l. *A. borealis* were found in an oxygen range of 1.5 to 8.1 mg/l. The

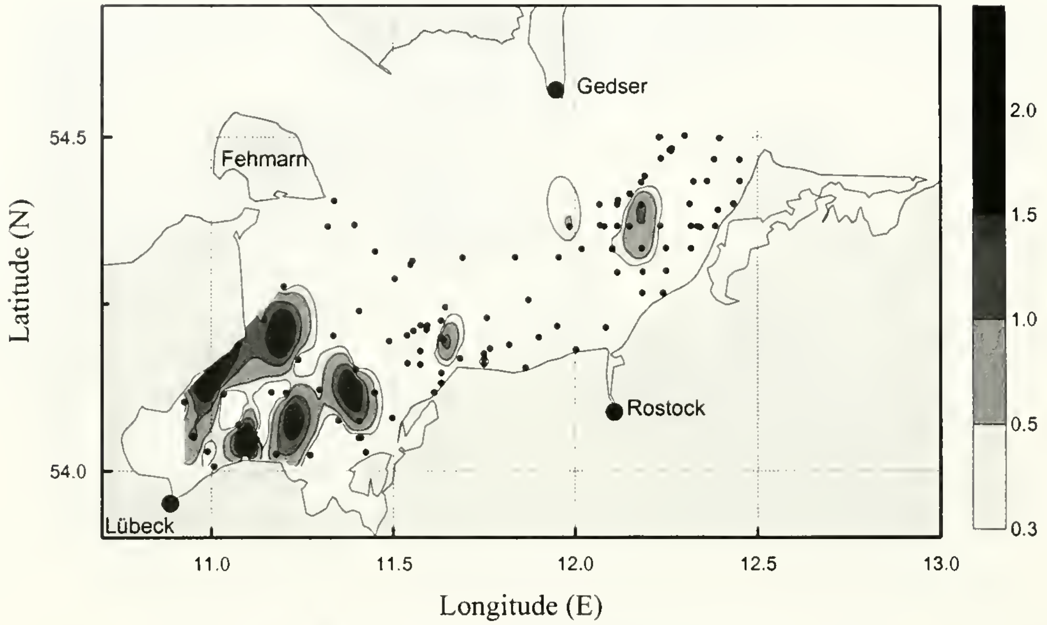

Figure 4. Biomass distribution of *Astarte borealis* (AFDW g/m^2) in 1999/2000.

mean oxygen content in areas inhabited by the bivalve was 6.27 ± 1.98 mg/l.

Distribution, Abundance and Biomass

In 1999/2000, *A. borealis* was distributed between 12 and 26.5 m depth in the Mecklenburg Bight. The species showed a very patchy density distribution (Fig. 3a). In the shallow areas 10 m depth with fine sand sediments, no *A. borealis* were found. Furthermore, the muddy zones of the outer Mecklenburg Bight and the outer Kadet Trench with strong currents and stony substrates were not inhabited. The highest abundance was found in the southeastern part of the Bight with a maximum of 400 to 500 ind./m^2 and an AFDW of 4 g/m^2 (water depth around 16.5 m). Thirty-five years ago (in the mid 1960s), *A. borealis* reached medium densities between 10 and 50 ind./m^2 with a maximum of 100 ind./m^2 in the eastern central part of the Bight (Schulz, 1969) (Fig. 3b). At depths below 20 m of the innermost area (Lübeck Bay) and western outer Bight no *A. borealis* were found. The comparison of the main distribution areas of the 1960s with the results of the present study showed a clear change.

The highest biomass was observed at the coast off Holstein and Mecklenburg (water depth about 16.0 m) with an ash free dry weight (AFDW) of 5–16 g/m^2 (Fig. 4). Within water depths of 12 and 27 m, *A. borealis* reached a mean abundance of 47 ind./m^2 and a biomass (AFDW) of 0.5 g/m^2.

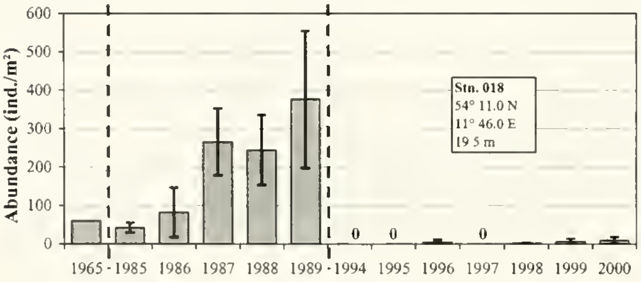

Figure 5. Changes in mean abundance (±S.D.) of *Astarte borealis* at stn. 018 over the last 15 years in comparison with data from the mid 1960s (Schulz 1969).

The development of the abundance of *A. borealis* at stn. 018 (see Fig. 2 for location) during the last 15 years is shown in Figure 5. From 1985 to 1989 abundance increased from around 50 ind./m^2 to about 400 ind./m^2. By the mid 1990s and through to 2000 abundance at the same station declined dramatically to 10–20 ind./m^2.

In the present study the highest abundance was observed in depths between 12 and 22 m (Fig. 6). In this depth range, *A. borealis* settled in an abundance of between 20 and 100 Ind./m^2.

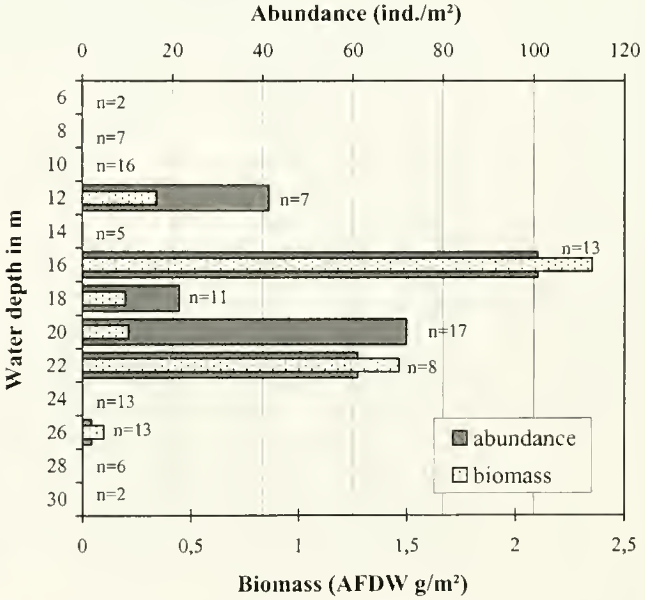

Figure 6. The vertical distribution of abundance (ind./m^2) and biomass (AFDW g/m^2) of *Astarte borealis* in the Mecklenburg Bight in 1999/2000 (n = number of included stations of each depth intervall).

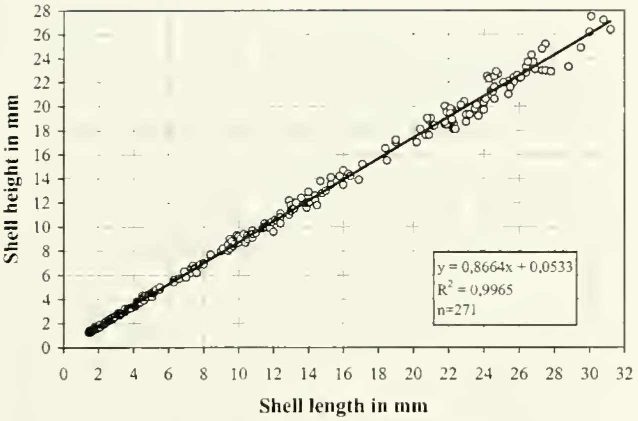

Figure 7. Shell height-length relationship of *Astarte boeralis* from the Mecklenburg Bight.

The biomass (AFDW) reached average amounts of 1.8 g/m^2 at 15–22 m with a maximum of 2.4 g in 15–16 m interval. In shallower and deeper areas no or only single specimens were found.

Population Structure, Meat Yield and Growth

Measured shell lengths of *A. borealis* ranged from 1.2 to 31.2 mm (Note, this range included both the survey and the collection material, see earlier). The shell length to shell height relationship is linear as indicated in Figure 7. The mean ratio length to height was 1.15 ± 0.04 and varied from 1.05 in minimum up to 1.25 in maximum, independent of shell length.

The population structures of selected stations are shown in Figure 8. The size structure varied between the stations. The 0–10 mm size class composed about 78% of the population. Larger size classes (>20 mm) were observed only sporadically and in low numbers. The dominant 0–5 mm size class at most of the investigated stations represents the survivors of the settlement during the last two years. Only at stn. 25 was the size structure dominated by older specimens.

Relationships between shell length and individual weights (whole wet weight, wet meat weight and ash free dry weight) are shown in Figure 9. These graphs include all measured specimen (1.2 mm to 28.7 mm) from the 1999/2000 survey. The smallest meat wet weight was 0.09 mg at a shell length of 1.2 mm and the largest one was 1.17 g at 28.3-mm shell length. The estimated mean regression line indicates the average meat yield per unit shell length of *A. borealis*. Differences between the station means were not statistically significant. The ash free dry weight varied between 0.08 mg (1.9 mm in length) and 0.29 mg (28.5 mm in length). The estimated regression lines of weights for different stations did not differ significantly. The results indicate that throughout the Mecklenburg Bight *A. borealis* contained approximately the same meat per unit shell length for the range of length considered. The mean wet meat yield (percentage of total wet weight) of different size

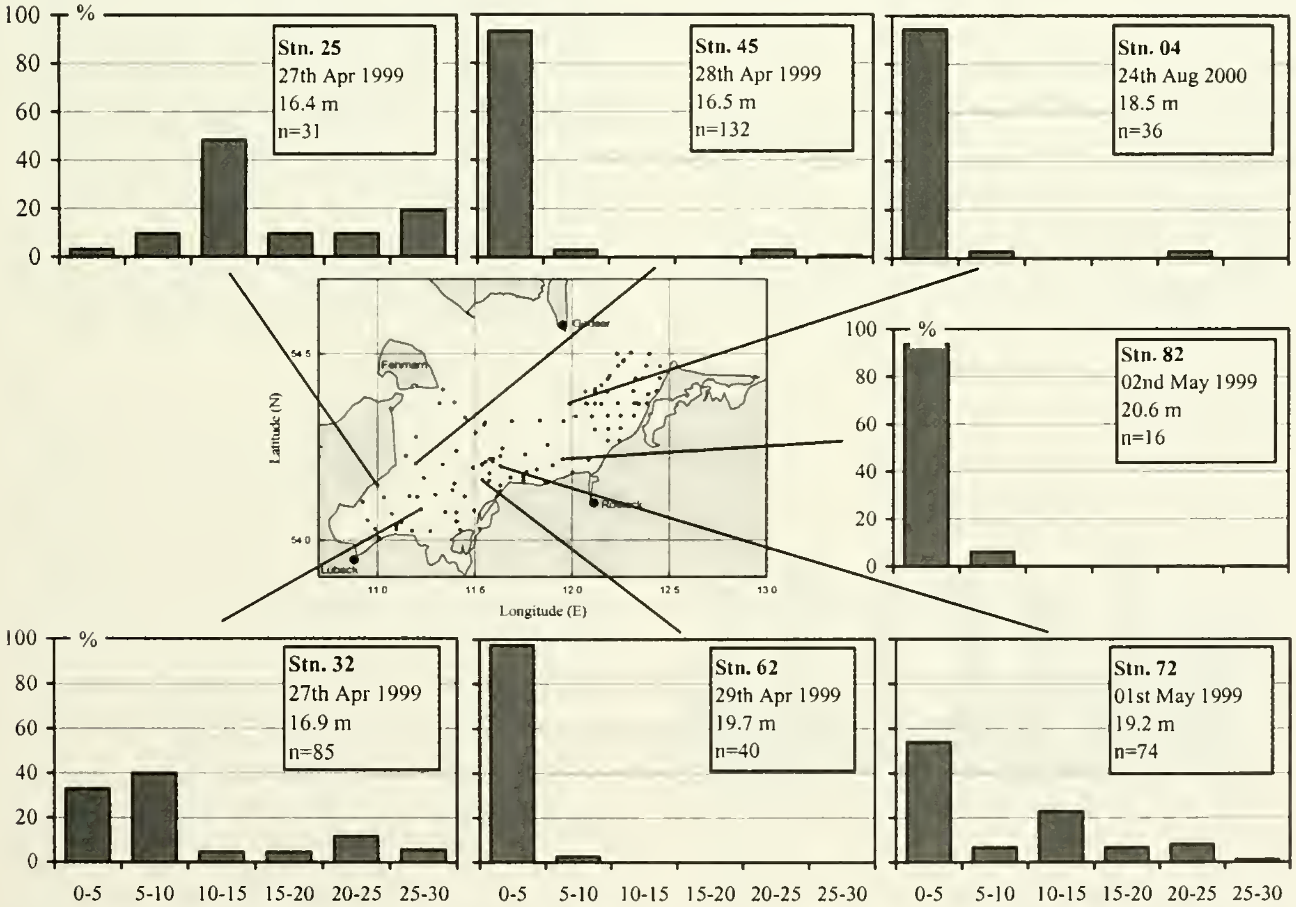

Figure 8. Shell length-frequency distribution for 5 mm size classes of *Astarte borealis* at several stations in depths between 16.4 m and 20.6 m in Mecklenburg Bight in 1999/2000.

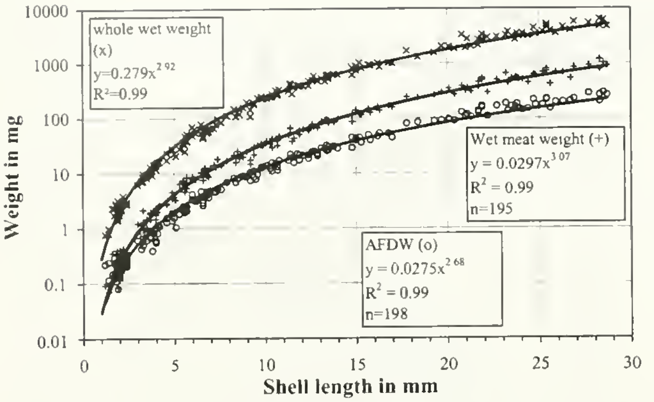

Figure 9. Estimated relationships between shell length and whole wet weight (+), wet meat weight (×) and ash free dry weight (o) for *Astarte borealis*. The corresponding equations for the calculated best fit regression lines are given in the figure.

classes varied in a range between 12.21% and around 16.4% with a minimum at 5% and a maximum at 23% (Fig. 10). The mean meat was 14.5%. With 9.1%, the highest amount of organic content (ash free dry weight) appeared in the 0–5 mm shell size class. The lowest organic content was 5.6% in the 25–30 mm size class. The difference was significant (*t*-test, $p < 0.001$). The mean organic content of dry weight ranged from 4.05% to 31.2% with a mean value of 7.4%.

DISCUSSION

Distribution, Abundance and Biomass

The occurrence of *A. borealis* in the Mecklenburg Bight has been known since the 19th century when the first investigations on the benthic fauna of the Baltic Sea took place (von Martens 1871; Wiechmann 1869/70). While the main Baltic populations live in the Kiel Bight and in parts of Sound and Belt, the distribution in the Mecklenburg Bight and the Arkona Basin represent the most eastern recent occurrence of this species in the Baltic Sea (see Fig. 1 and von Oertzen & Schulz 1973). Due to the decreasing salinity, *A. borealis* has its natural limit of distribution in areas east of Arkona Basin. Formerly, the distribution reached the deep parts of the Bornholm Basin as far as the Slupsk Furrow (Demel & Mulicki 1954; Jaeckel 1952; von Oertzen & Schulz 1973). Due to long lasting oxygen depletion in the last decades the bivalve had nearly disappeared in this area (Andersin et al. 1978). Today, *A. borealis* occurs in this region only in the Slupsk Furrow and only in depths between 60 and 70 m (Warzocha 1995).

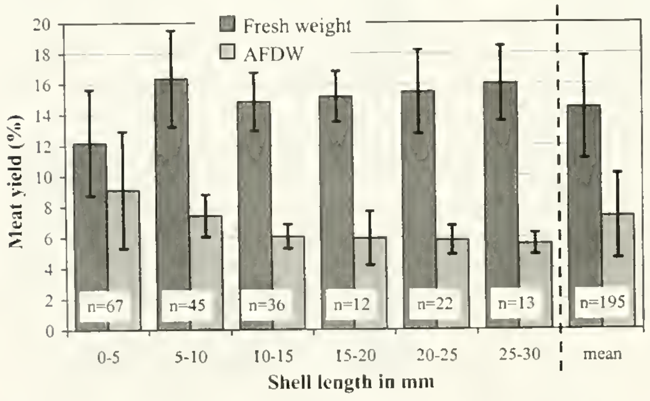

Figure 10. Mean individual percentage of wet meat yield and ash free dry weight in 5-mm size classes of *Astarte borealis* from the Mecklenburg Bight in 1999/2000 (±S.D.).

The investigation of Schulz (1969) from 1962 to 1965, giving the distribution pattern of *A. borealis* in the Mecklenburg Bight, was used for comparison with the present study. In the 1960s, densities were between 10 and 50 ind./m^2 in mean (maximum 320 ind./m^2). During the 1980s, the abundance of the bivalve increased in waters near 20 m depth (e.g., at the monitoring station 018) (Köhn 1989; Voigt 1991). Due to a long period of oxygen depletion in the deeper part of the Lübeck Bay (the inner part of the Mecklenburg Bight), no *A. borealis* were found in the 1980s (Gosselck et al. 1987; Prena et al. 1997). In the 1990s, the density of *A. borealis* decreased in shallower regions of the Mecklenburg Bight, too. Recently, a mean abundance of approximate 50 ind./m^2 in depths between 11 and 26 m could be observed. The highest abundance and biomass was observed in the region between 16 and 22 m. The mean biomass reached 1 g/m^2 AFDW (17 g/m^2 wet weight). In comparison, at the beginning of the 1950s during the investigations of Kühlmorgen-Hille (1963) in the Kiel Bight *A. borealis* occurred in a mean abundance of between 4 and 52 ind./m^2. At the beginning of the 1970s Arntz et al. (1976) observed in the Kiel Bight the highest frequency and abundance in depths between 10 and 20 m with 74–83 ind./m^2 (max. 570 ind./m^2, 1240 g/m^2 wet weight). In adjacent waters of the Arkona Basin Löwe (1963) found *A. borealis* in quite high amounts of 13 g/m^2 wet weight. The largest biomass with about 70 g/m^2 he observed off the island of Falster and at the entrance of the Sound. Outside the Baltic in Russian Arctic waters, the main distribution area of the species, *A. borealis* colonises the littoral zone and reaches an abundance of about 200 ind./m^2 and a biomass of about 620 g/m^2 wet weight (Antipova 1978; Gagaev 1989; Matveeva 1977). *A. borealis* belongs to the most productive bivalves in this region (Gagaev 1989).

According to von Oertzen (1972), *A. borealis* has an extremely prolonged period of ripe eggs and sperms. The main spawning season is presumably in spring with the possibility of "portion spawning" the whole year (Köhn 1989; Matveeva 1977; von Oertzen 1972). *A. borealis* prefers sandy substrates and mixed sediments avoiding muddy sediments (e.g., Arntz et al. 1976; Schulz 1969). In the deeper parts (>22 m) of the Mecklenburg Bight, oxygen depletion in late summer prevents successful recruitment and growth of the juveniles. However, *A. borealis* is one of the most tolerant species to oxygen deficiency and to hydrogen sulphide (von Oertzen 1973; Oeschger 1990). Probably, the possibility of the high tolerance to oxygen deficiency is restricted to the adults, whereas the juveniles are more susceptible, causing settlement only in favourable years and depths. The lack of recruitment events is described for populations of Kiel and Mecklenburg Bight by Werner et al. (1974) and Köhn (1989). In 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 suboptimal, probably due to the lower nutrition supply, lower salinity and higher temperature (Arntz et al., 1976; von Oertzen, 1973). In depths shallower than 11 m the mean salinity, 8–12 psu, is probably too low. The range of potency of *A. borealis* is 8–35 psu with an optimum between 14 and 30 psu (Jaeckel 1952; von Oertzen 1973).

Population Structure

The largest living *A. borealis* found in this survey was 28.3 mm long and 24.7 mm high. Valve material of the Zoological Collection of the University of Rostock (sampled in the 1980s) had a maximum length of 31.2 mm (26.4-mm height). Lenz (1882) found specimen of 36 mm in length (31-mm height) in the Lübeck Bay. The shell is quadrate to subtrigonal and compressed with a total shell length of 38 mm at boreal outposts (e.g., Baltic Sea, Jaeckel, 1952) and 55 mm in the Arctic Sea (Coan et al. 2000, Filatova 1957) with a mean of 25–45 mm (Dance 1977). In general, the shell length exceeds the height and the height/length indices vary from 0.8 to 0.9 (e.g., Ockelmann 1958). However, *A. borealis* is a variable species with several forms and varieties (see Zettler 2001). In the past, the great variability in morphological features resulted in a number of new species and subspecies descriptions (see Zettler 2001). Recent investigations of Hopner Petersen (2001) show a large variety of shell morphology within the genus *Astarte*. The material discussed in the present study had a high constancy in respect to the relation between length and height (Fig. 7).

Statements on population dynamics or size structure of *A. borealis* are very sparse in the literature. Some Russian studies (Gagaev 1989; Matveeva 1977) and few investigations on Baltic populations (Köhn 1989; Voigt 1991; Werner et al. 1974) have been carried out. During the studies of Köhn (1989) and Voigt (1991) in the Mecklenburg Bight the populations were dominated (89%) by individuals <6 mm in length. Larger sizes were observed only sporadically. In the present study, juvenile individuals were dominant at most of the stations investigated. Adult specimens (>20 mm) were only observed in high abundance in the inner part of the Mecklenburg Bight (Fig. 8). Köhn (1989) pointed out that only individuals >20 mm are reproductive. In the White Sea *A. borealis* reaches sexual maturity in sizes >17 mm (Matveeva 1977). The maintenance of the stock in the Mecklenburg Bight depends on few adults and/or probably on drifting of lecitothrophic (non-pelagic) larvae from regions nearby. In areas with strong currents, longer immigration distances are imaginable (Rasmussen 1973). In the Kiel Bight and in Russian Arctic waters however, the population structures are more balanced (Werner et al. 1974; Gagaev 1989).

Meat Yield

The mean wet meat yield of *A. borealis* of 14.5% in the present study is similar to reported values of Köhn (1989). He found organic contents (wet) between 9% and 30% for a population in the Mecklenburg Bight. Ansell (1975) observed a soft tissue content for *A. elliptica* of 14.3–23.5% and dry tissue weights of 3–5% in British waters depending on the reproductive cycle. In the Mecklenburg Bight, in the present study, the mean individual ash free dry weight of *A. borealis* decreased from 9.1% to 5.6% with increasing size classes. The increasing mean wet yield and the decreasing ash free dry weight with increasing shell length indicates relatively higher water content of bigger bivalves. The length specific wet weight (shell length – wet weight relationship) in this study was similar to that reported for *A. borealis* of the Kiel Bight and Russian Arctic waters. In the Mecklenburg Bight, the calculated wet weight for an individual of 25 mm shell length was 3.42 g. The wet weight of *A. borealis* in the East Siberian Sea reported by Gagaev (1989) was 3.49 g and in Kiel Bight 4.22 g (Schaefer et al. 1985). According to Köhn (1989) the meat wet weight of a 25-mm specimen of *A. borealis* is 534 mg compared with 572 mg in the present study.

In conclusion, the present study shows ecological and morphological features of *Astarte borealis* in the Mecklenburg Bight near its eastern distributional boundary within the Baltic Sea. Further investigations must deal with growth rates and morphology of *A. borealis* within the Baltic and adjacent waters. The causes of the decline of this cold-adapted arctic-boreal species in much of the Baltic remain unclear. The comparative population dynamics at different water depths and/or within different sediment structures and the dispersion and settlement patterns of the larvae in this "border" area are of special interest.

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IS SURVIVAL GENOTYPE-DEPENDENT IN NORTH AMERICAN POPULATIONS OF FARMED BLUE MUSSELS, *MYTILUS* SPP?

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ABSTRACT We monitored survival and allele frequencies at five enzyme loci in single year-class cohorts of cultured *Mytilus* spp. during a 14 month period from sleeving of seed to harvest at three commercial mussel farms in Notre Dame Bay, Newfoundland, Canada. Among-site genetic heterogeneity at all five individual loci and over all loci combined was evident among the three seed sources. Significant temporal genetic heterogeneity occurred at all three sites. The observed intra-site temporal heterogeneity was not related to genotypic state since neither the relative proportions of homozygotes and heterozygotes at each of the five loci individually, nor the mean heterozygosity over five loci, changed significantly within sites over time. Significant change in genotypic frequencies at the *Gpi* locus was the primary contributing factor to the overall temporal genetic heterogeneity within each of the three sites. Significant temporal changes in genotypic frequencies at each of the *Mpi*, *Lap*, *Pgm*, and *Odh* enzyme loci inconsistently appeared among the three sites. A significant directional shift in genotypic frequencies, consistent across all three sites, gave evidence of genotype-dependent survival selection differentially favoring survival of *Gpi* genotypes with electrophoretically slower alleles in comparison to genotypes with electrophoretically faster *Gpi* alleles during sub-tidal rope culture of *Mytilus* spp. populations in Newfoundland. We also conclude that temporal genetic heterogeneity is a common occurrence in suspended rope culture of Newfoundland blue mussel populations and is likely a significant contributory factor in the extensive geographic genetic population structuring previously reported among mixed *M. edulis* - *M. trossulus* populations.

KEY WORDS: *Mytilus*, genotype-dependent, survival, genetic heterogeneity

INTRODUCTION

In northeastern North America, two separate mytilid species, *Mytilus edulis* and *M. trossulus* are recognized (Koehn et al. 1984; Varvio et al. 1988; McDonald et al. 1991). In Atlantic Canada, the distribution of both species is widespread (McDonald et al. 1991; Mallet & Carver 1992) and the two form hybridized mixed-species assemblages (Saavedra et al. 1996). Newfoundland is, apparently, a zone of natural distributional overlap between these two mytilid species since most populations consist of mixtures of both species varying widely in relative frequency (Bates & Innes 1995; Penney & Hart 1999).

Within the overlap zone in Newfoundland, sites in close proximity to each other display inter-site genetic heterogeneity on a magnitude scale comparable to sites much larger distances apart (Penney & Hart 1999). Such scales of genetic heterogeneity suggest the potential for significant stock- or site-related variability in growth, survival, or other characteristics among adjacent sites throughout the mixed-species zone. For commercial industry, factors that significantly affect productivity within and among mussel farms is of great importance. Genotype-dependent temporal survival selection processes have previously been noted in inter-tidal, mixed-species communities involving *M. galloprovincialis* and *M. edulis* in England (Gardner & Skibinski 1991; Skibinski & Roderick 1991; Gardner 1994) and in a mixed *M. edulis* and *M. trossulus* intertidal assemblage in Nova Scotia (Pedersen et al. 2000).

In cultured stocks, inter-site variation in mortality rates related to genetic (stock) differences are known to exist (Mallet et al. 1987, 1990) and may significantly affect production in suspended culture (Mallet & Carver 1989). There is also evidence that between-site mortality differences are maintained when the stocks are transferred to new sites (Mallet et al. 1990; Myrand & Gaudreault 1995) suggesting that among-site survival variation is related to stock genetics rather than environmental factors. Mortality and growth variation related to genotype (*M. edulis* vs. *M. trossulus*) was reported to significantly influence productivity at one farm site in Nova Scotia (Mallet & Carver 1995). Since the combination of stock mortality and growth rate primarily define production in suspended culture, among-site stock and/or genotype-dependent variation in either parameter due to selective processes may be an important determinant of inter-site variability in farm production indices.

In this study, we investigate survival patterns in three hybridized *M. edulis* - *M. trossulus* assemblages of cultured mussels in Newfoundland, Canada, over the latter phase of the production cycle, namely from sleeving of seed mussels to harvest. We use empirical observations of temporal change in allele frequencies at five enzyme loci to test the hypothesis that genotype-dependent selection processes occur within rope-cultured mixed *M. edulis*-*M. trossulus* populations and, by inference, may be a significant contributory factor influencing genetic heterogeneity, survival, and production variability among mussel farms in Newfoundland.

METHODS

Seed mussels from collector ropes were sleeved in standard commercial plastic mesh on each of three farms in Notre Dame Bay, Newfoundland in August, 1998 (Fig. 1). In each case, the seed mussels were derived from collector ropes set out on each farm in 1997. Seed mussels were mechanically de-clumped prior to sleeving to ensure random distribution across all sleeves. Each sleeve was 1 meter in length and was vertically suspended at 0.5-meter intervals from horizontal mainline ropes with flotation sufficient to ensure stable suspension at depths of approximately 10–12 meters. One week after gear placement, three replicate sleeves were retrieved from each farm site. From measured sections of each sleeve, all the mussels were removed and counted and 100 individuals were randomly selected for allozyme analysis (total of 300 from each site). The one-week delay in taking the first

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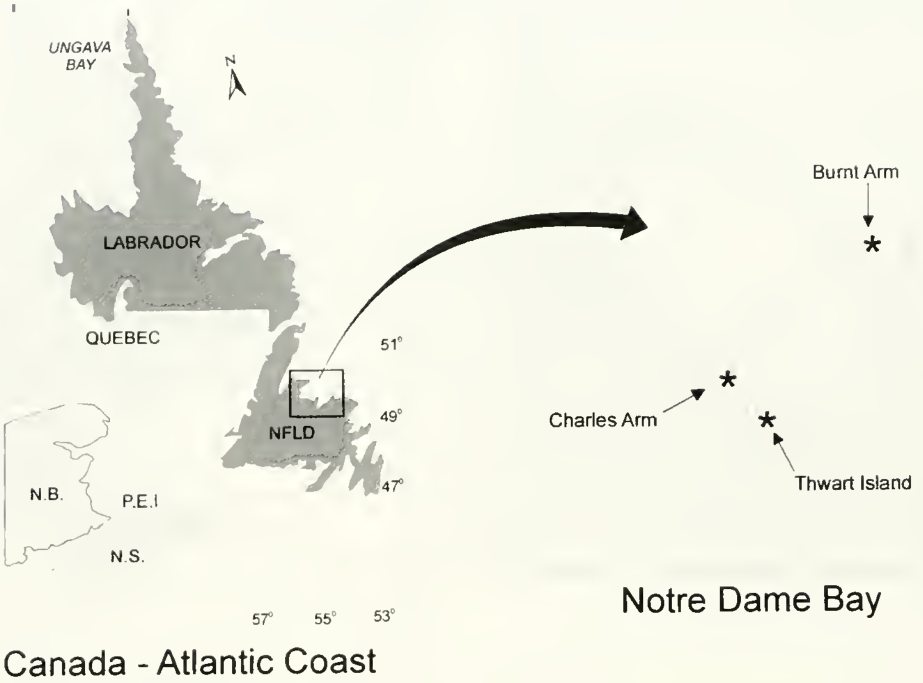

Figure 1. Geographic location of the three farm sites in Notre Dame Bay, Newfoundland.

sample was necessary to allow for mussel acclimation and attachment to the sleeving material and thus eliminate drop-off of any mussels that failed to properly attach themselves via their byssal threads.

Each farm site was similarly sampled in May, 1999 and again in October, 1999 at which time the majority of mussels had reached or exceeded the minimum commercial harvest size of 55 mm. Survival over time was calculated as the difference in mean number of mussels sleeve^{-1} in May, 1999 and in October, 1999, compared to the initial sample. In this context, changes in survival do not distinguish between changes due to mortality versus drop-off from the culture gear.

Hepatopancreas tissue was excised from each selected mussel, lyophilized, and stored at 5°C for later allozyme analysis. Five polymorphic loci were investigated: mannose phosphate isomerase (*Mpi*, EC 5.3.1.8), aminopeptidase-I (*Lap*, EC 3.4.11.-), phosphoglucomutase (*Pgm*, EC 2.7.5.1), glucose-6-phosphate isomerase (*Gpi*, EC 5.3.1.9), and octopine dehydrogenase (*Odh*, EC 1.5.1.11). A small amount of freeze-dried material was ground to a fine powder with 0.5M Tris HCL pH 8.0 buffer containing 20% glycerol and 0.2% NAD. Subsequent electrophoresis and staining on cellulose acetate plates followed the general procedures of Hebert and Beaton (1989), although a constant current of 2 mA per plate was used during the electrophoretic runs. The procedure for *Lap* was modified to run with Tris glycine pH 8.6 buffer. For *Odh*, we modified the stain for *Gpi* suggested by Hebert and Beaton (1989) to use octopine as the substrate instead of fructose-6-phosphate. Allele nomenclature is similar to that employed by previous authors (Koehn et al. 1984; McDonald & Koehn 1988) with the exception that alleles at the *Odh* locus were numbered consecutively from 0–9 in order of increasing electrophoretic mobility from the origin. The latter exception was necessary since we were unable to match *Odh* electromorphs on our plates with previously published allele frequencies.

Analysis of allele frequencies for population differentiation and probability estimation of log-likelihood G tests of genetic heterogeneity within and among genotype classes and within and among sites utilized the software package F-Stat for Windows™, version 2.9.1 (Goudet et al. 1996). All other statistical analyses used the SAS software system (SAS Institute Inc. 1988). Significance levels of all test statistics involving multiple comparisons were Bonferroni-adjusted. Non-parametric analysis of variance of ranks and Kruskal-Wallis rank test statistics (SAS Institute Inc. 1988; Sokal & Rohlf 1995) were calculated to test for directional, intra-site, temporal trends in genotypic frequency distributions at the *Gpi* locus. For these analyses, the twelve observed Gpi alleles were assigned an ordinal rank score based on electrophoretic mobility, with faster alleles scoring lower than slower alleles (e.g., Gpi^{110} = rank 1; Gpi^{107} = rank 2, etc.). Thus for diploid loci, the genotypic rank assigned each individual is the mean rank score of its two constituent alleles (e.g., for an individual of genotype $Gpi^{110,107}$, rank = 1.5, etc.). For Kruskal-Wallis tests with $n > 2$ sample dates, SAS calculates a χ^2 approximation of the Kruskal-Wallis test statistic for significance testing (SAS Institute, Inc. 1988).

RESULTS

Seed mussels were collected from three farms located in Burnt Arm, Charles Arm, and at Thwart Island in Notre Dame Bay on the northeast coast of Newfoundland (Fig. 1) and transferred to commercial plastic mesh sleeves in August, 1998, for grow-out. Electrophoretic assays of the three seed mussel populations detected four alleles at the *Mpi* locus, six at the *Lap* locus, eight at the *Pgm* locus, eleven at the *Odh* locus, and twelve at the *Gpi* locus. Gene diversity per locus at the three sites was very low (<0.20) at the *Odh* locus, moderate (0.40–0.50) at the *Mpi* locus, relatively high (0.60–0.75) at *Lap* and *Pgm*, and highest (>0.80) at *Gpi*. Log-likelihood G test scores from inter-site comparison of the three seed mussel populations in August 1998, revealed significant ($p < 0.001$) among-site population genetic heterogeneity at all loci individually as well as over all loci combined (Table 1). At the *Mpi* locus, Mpi^{100} predominated at Burnt Arm and Charles Arm while Mpi^{94} was the allele at highest frequency at Thwart Island (Fig. 2). Pgm^{100} was the most common allele at the *Pgm* locus at both Burnt Arm and Charles Arm while the frequency of Pgm^{111} was highest at Thwart Island. At the *Lap* locus, Lap^{98} was the most common allele at Charles Arm but at Burnt Arm and Thwart Island the frequency of Lap^{96} was greatest. Gpi^{98} predominated at Charles Arm while, at both Burnt Arm and Thwart Island, the frequency of Gpi^{96} was highest. At *Odh*, the three sites differed only in frequency of relatively rare alleles. The frequency of Odh^{3} was greatest for all three sites.

In August, 1998, mean mussel density on the sleeves varied among sites from 450 mussels m^{-1} at Charles Arm to 790 mussels m^{-1} at Burnt Arm and 890 mussels m^{-1} at Thwart Island. Survival to May, 1999 (calculated as within-site change in mean mussel density since August, 1998) was not significantly different among sites (ANOVA, $P > 0.05$). Mean survival during the first 9 months of grow-out (to May, 1999) was 81.2% at Charles Arm, 76.9% at Thwart Island, and 78.0% at Burnt Arm (Fig. 3). After 14 months on the sleeves (October, 1999), mean survival at Charles Arm was significantly less than the other two sites (ANOVA, $P < 0.05$). Mean survival at Charles Arm was only 37.4% compared to 51.6% at Thwart Island and 51.7% at Burnt Arm. Log-likelihood G test scores for among-site comparisons of population genetic structure after 9 and 14 months of grow-out (May and October, 1999 respectively) were also significant (Table 1) indicating the initial among-site genetic heterogeneity was maintained during the course of the grow-out period.

During the same 14 month grow-out period, significant intra-site temporal changes in allele frequencies at one or more loci were found at all three sites (Table 2). After the first 9 months of grow-out (to May, 1999), log-likelihood G test scores were significant over all loci combined at all three sites indicating significant temporal change in genetic structure had occurred over this period. Significant intra-site temporal population differentiation at the *Gpi* locus was evident at all three sites. Intra-site population genetic differentiation was also evident at the *Mpi* locus at Burnt Arm, the *Pgm* and *Odh* loci at Charles Arm, and at the *Odh* locus at Thwart Island. This pattern continued through to October, 1999 after 14 months of grow-out by which time further genetic differentiation at the *Odh* locus had become evident at Burnt Arm and at the *Lap* locus at Thwart Island.

To determine whether the observed intra-site temporal genetic differentiation revealed by the log-likelihood G tests gave evidence of selective survival patterns, we further examined the dataset for (a) non-random mortality related to genotypic state (e.g., differential survival of homozygotes vs. heterozygotes) at either of the five loci individually or over all loci combined; and (b) non-random mortality favoring specific single locus genotypes. With respect to (a), for all five loci individually, the relative proportions of homozygotes vs. heterozygotes within all three sites did not significantly change over the 14 month grow-out period (Fisher's exact test; $P > 0.05$). Within-site variation in mean multi-locus heterozygosity was also not significantly related to time (sample date) and the interaction effect of time × site was not significant (ANOVA; $P > 0.05$) at all three sites (Table 3). Thus, we conclude the observed intra-site temporal variation in population genetic structure revealed by the log-likelihood G tests is not explained by selective survival related to genotypic state at either of the five loci individually or on the basis of multi-locus heterozygosity (multi-locus homozygotes vs. heterozygotes). Multi-locus heterozygosity did significantly vary however among sites (ANOVA, $P < 0.01$; Table 3). Mean multi-locus heterozygosity was lowest at Charles Arm, highest at Burnt Arm and intermediate between these two at Thwart Island (Fig. 4). However, only the May and October means at Burnt Arm and Charles Arm were significantly different (Tukey, $P < 0.05$).

TABLE 1.

Log-likelihood G test (Goudet et al. 1996) probability estimates for inter-site population genetic differentiation at the time of socking (August, 1998), in May, 1999, and at harvest in October, 1999.

	Locus					
Date	Mpi	Lap	Pgm	Gpi	Odh	All
Aug 98	***	***	***	***	***	***
May 99	***	***	***	***	***	***
Oct 99	***	***	***	***	***	***

*** $p < 0.001$

With respect to (b), we then tested the hypothesis that the observed intra-site temporal genetic differentiation could be explained by non-random mortality favoring specific single locus genotypes. To accomplish this, we tested for temporal patterns of change in genotypic frequencies (χ^2) with specific individual alleles (homozygotes and heterozygotes combined) versus those without at each site × locus combination wherein the log-likelihood G test was significant. Homozygotes and heterozygotes were combined for this analysis due to the previously noted non-significant temporal change in frequencies of homozygotes and heterozygotes at all three sites. With respect to the *Lap*, *Pgm*, and *Odh* loci, the observed temporal changes in frequency of genotypes with or without all individual alleles were not significant after Bonferroni-adjustment of significance levels for multiple test comparisons. For the *Mpi* locus, a significant G test score was observed only at Burnt Arm (Table 2). At this site, the relative frequencies of genotypes which included the Mpi^{100} allele were significantly reduced while the relative frequencies of genotypes which incorporated the Mpi^{94} allele were significantly increased during the August, 1998, to October, 1999 period. These frequency changes remained significant after Bonferroni correction of probabilities for multiple comparisons. Frequency changes at Burnt Arm for genotypes with all other *Mpi* alleles were not significant after Bonferroni correction of probabilities.

At the *Gpi* locus, χ^2 tests of temporal patterns of change in genotypic frequencies involving specific alleles varied across the three sites (Fig. 5). At Burnt Arm, significant (χ^2, $P < 0.05$) frequency reductions occurred within genotypes which included the Gpi^{107}, Gpi^{102}, and Gpi^{96} alleles, while genotypic frequencies with the Gpi^{93}, Gpi^{89}, Gpi^{86}, and Gpi^{83} alleles all increased. At Charles Arm, a significant reduction in individuals with genotypes that included Gpi^{107} also occurred while genotypic frequencies with Gpi^{100}, Gpi^{96}, and Gpi^{89} all increased. At Thwart Island, a trend towards increasing frequency of individuals with Gpi^{93} and Gpi^{86} occurred with reductions in the frequency of individuals with the more electrophoretically mobile Gpi^{98} and Gpi^{96}. Frequency changes over time for all other *Gpi* alleles at each of the three sites

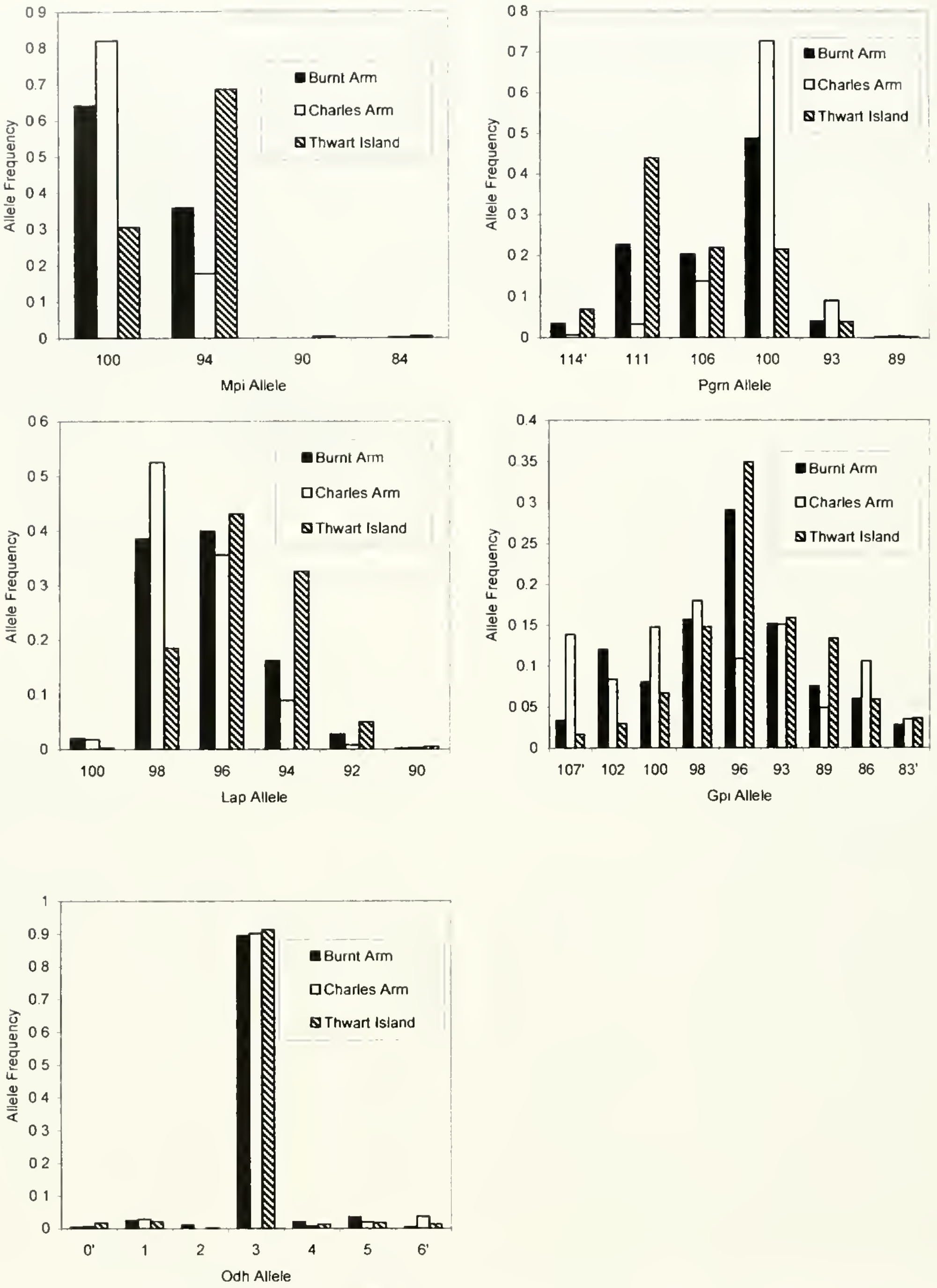

Figure 2. Inter-site comparison of allele frequencies at the *Mpi*, *Pgm*, *Lap*, *Gpi*, and *Odh* enzyme loci in seed mussel populations at Burnt Arm, Charles Arm, and Thwart Island at the start of the rearing trials in August, 1998. Allele frequency numbers with a ' (e.g., *Pgm* allele114') include frequencies of adjacent rare alleles.

were not significant (χ^2, $P > 0.05$) when significance levels were Bonferroni-adjusted for multiple comparison tests.

However, while the frequencies of the same individual *Gpi* alleles were not consistently significantly differentiated among sites over time, the overall *Gpi* genotypic frequency distribution significantly shifted in favor of electrophoretically slower alleles at all three sites (Table 4). At the *Gpi* locus, mean genotypic rank scores within all three sites significantly increased over time. Also, Kruskal-Wallis H tests revealed the median genotypic rank score also significantly increased within all three sites over time. These results indicate a consistent directional change in the overall genotypic frequency distribution had occurred at all three sites. *Gpi* genotypes carrying alleles of higher electrophoretic mobility were significantly reduced in favor of those with alleles of relatively lower electrophoretic mobility at all three sites during the 14-month grow-out period. We infer this directional shift in *Gpi* genotypic frequencies which is consistent across all three sites is evidence of genotype-dependent selective survival processes dif-

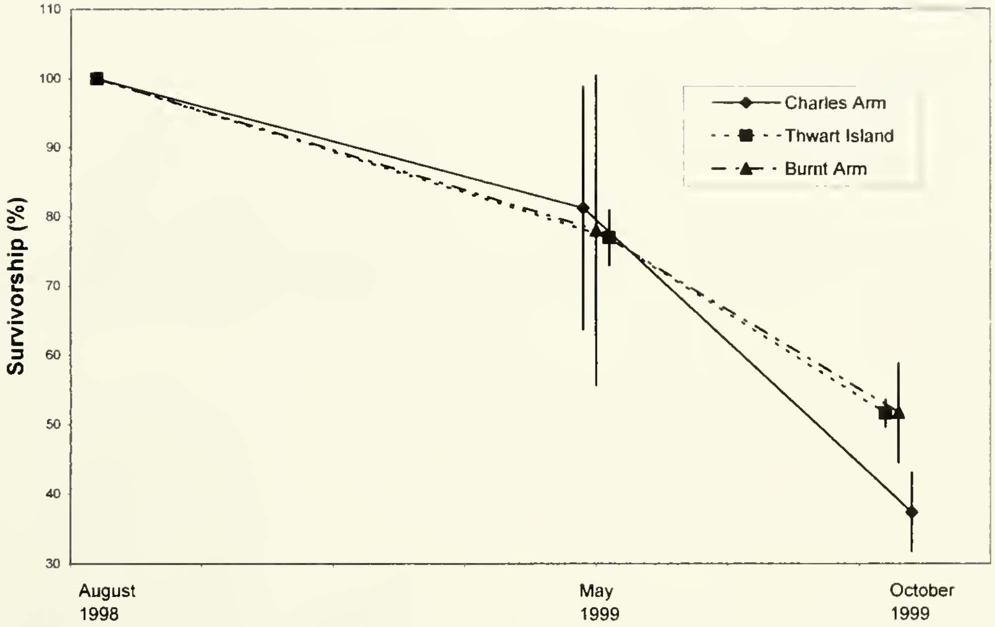

Figure. 3. Comparative survival from sleeving in August, 1998, to harvest in October, 1999 at the three farm sites. Plotted points are means of replicate socks with ±2 se.

ferentially favoring alleles of relatively lower electrophoretic mobility at this locus during sub-tidal, rope culture of *Mytilus* spp. in Newfoundland.

DISCUSSION

Two recent studies (Gilg & Hilbish 2000; Pedersen et al. 2000) have provided evidence that both cohort mixing as well as selective survival processes work to produce significant temporal genetic heterogeneity in mixed-species, intertidal mytilid communities. However, ours is the first to examine the potential for similar selection processes within suspended rope-cultured populations in commercial culture. In the present work, we have found significant within-site genetic heterogeneity among samples taken over a 14 month grow-out period for three mixed-species populations. The probability that our results were affected by cohort mixing seems remote. This would have required an undetected re-settlement on the culture sleeves with a new larval cohort some time after the original gear setup date in August, 1998. Such an occurrence would have been easily detected due to the disparity in length frequencies between the older mussels and the new cohort on the sleeves. Our length frequency data show no evidence of such cohort mixing. A more parsimonious explanation for the observed intra-site differences among sample dates is that temporal genetic heterogeneity occurred within the original populations from time of sleeving in August, 1998, through to harvest size in October, 1999.

Both Gilg and Hilbish (2000) and Pedersen et al. (2000) reported genotype-dependent mortality patterns in *Mytilus* spp. populations that resulted in population structuring along species lines. In the case of the former, an intertidal mixed population of newly-settled *M. edulis, M. galloprovincialis*, and their hybrids in SW England became heterogeneous on the basis of tidal height. In the latter, a directional selective mortality pattern favoring *M. trossulus* at the expense of *M. edulis* was demonstrated in a mixed intertidal *M. edulis* and *M. trossulus* assemblage in Nova Scotia. In our study, the mussel populations at all three sites could be classified as hybridized, mixed-species (*M. edulis, M. trossulus*, and their hybrids) stocks based on their *Mpi* genotypes (Varvio et al.

TABLE 2.

Log-likelihood G test (Goudet et al. 1996) probability estimates for intra-site temporal genetic differentiation during the 14 month growout period at all loci individually and over all loci combined.

Site	Interval (months)	Locus					
		Mpi	Lap	Pgm	Gpi	Odh	All
Burnt Arm							
	9	**	n.s.	n.s.	*	n.s.	*
	14	*	n.s.	n.s.	***	*	***
Charles Arm	9	n.s.	n.s.	*	***	***	***
	14	n.s.	n.s.	*	***	***	***
Thwart Island	9	n.s.	n.s.	n.s.	***	*	**
	14	n.s.	*	n.s.	***	**	***

* $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$

TABLE 3.

Analysis of variance (ANOVA) test results of variation in the number of heterozygous loci (out of five) per individual among the three sites and sample dates. F ratios without superscripts are not significant ($P > 0.05$).

Variable	d.f.	MS	F
Date	2	0.62	0.56
Site	2	6.31	5.72**
Date × Site	4	0.07	0.06
Residual	2652	1.05	

** $P < 0.01$

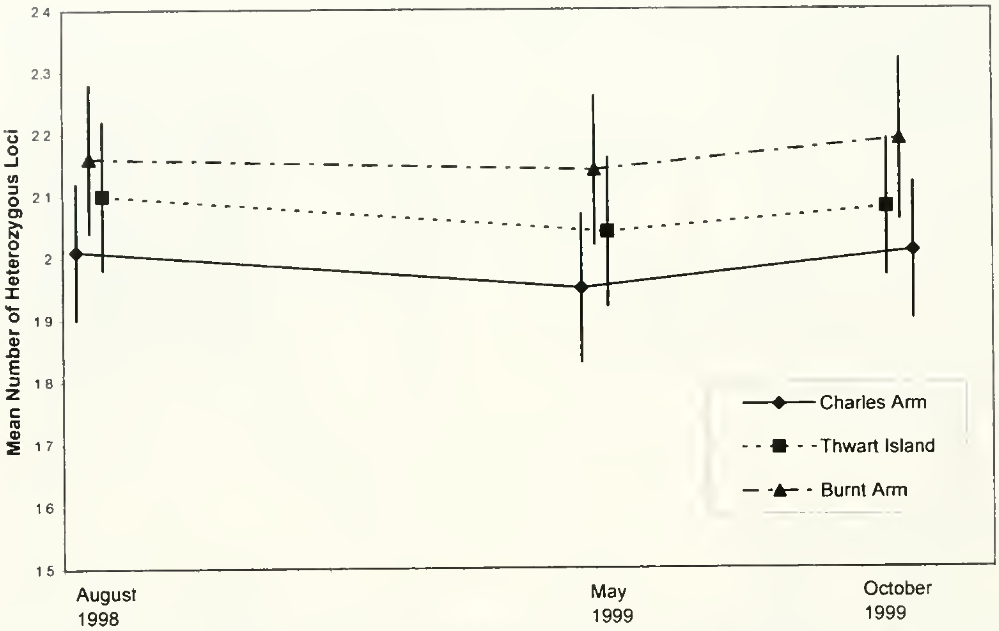

Figure 4. Mean multi-locus heterozygosity (mean number of heterozygous loci out of five) ±2 se in August 1998, May 1999, and October 1999 at each of the three farm sites.

1988; McDonald & Koehn 1991). All three populations showed evidence of significant temporal genetic heterogeneity over the 14 months from sleeving to harvest. However, the relative proportions of *Mpi* genotypes changed only at one site, Burnt Arm. This seems to go against the general applicability of the hypothesis that genotype-dependent mortality among *Mpi* genotypes (e.g., *M. edulis*, *M. trossulus*, or their hybrids) might be the primary cause of the temporal genetic heterogeneity we observed among cultured Newfoundland stocks as Pedersen et al. (2000) found within one rocky intertidal community in Nova Scotia.

Several factors may be contributory to the different conclusions we have reached compared to Pedersen et al. (2000). Presumably, selective processes favoring one genotype over another will only manifest themselves under conditions that induce differential degrees of stress. Since both *M. edulis* and *M. trossulus* are endemic throughout Atlantic Canada, we may infer that both species are generally adapted to the range of environmental conditions and habitat types encountered throughout that range. However, it is entirely likely that naturally occurring pelagic larval dispersal and settlement will sometimes result in mussels being placed in habitats where differential selection of genotypes can occur. The intentional transfer of seed mussels to new areas, as in the case of commercial mussel farming, could likewise produce an environment where differential selection could occur. Thus the appearance of differential selection processes would be site-specific, perhaps even on micro-geographic scales, and may change with time on any particular site as stressful conditions appear and disappear due to natural environmental variability. The range of environmental conditions experienced by rope-cultured, sub-tidal mussels is less extreme and likely less stressful than those experienced by natural inter-tidal assemblages and this may explain the different conclusions we have reached compared to Pedersen (2000). Alternatively, selection processes may also change with age (Gilg & Hilbish 2000). Our study was not directly comparable to that of Pedersen et al. (2000) on the basis of age since we started our work with seed mussels considerably older than the newly recruited mussels they studied.

In our study, the temporal genetic patterns we observed may have been produced not by mortality per se but rather by drop-off from the culture gear. On vertically suspended sleeving substrates used in commercial mussel culture, selective processes affecting strength of attachment by byssal threads may result in drop-off. While not sufficient to cause mortality per se, such episodes nevertheless cause loss to the culture system and result in apparent if not real mortality. Such selective processes may not be apparent in intertidal habitat where mussels with similarly weakened byssal attachment are physically better supported and thus better able to retain their placement. We know that byssal thread formation varies in mytilids in response to environmental variation (Young 1985). Strength of attachment also varies among species due to interactions between hydrodynamic forces and interspecific variation in shell shape (Willis & Skibinski 1992; Bell & Gosline 1997). Co-occurring cultured *M. edulis*, *M. trossulus* and their hybrids differ in shell shape within the populations we studied (Penney & Hart 1999). Whether or not interactions among strength of attachment, genotype-dependent physiological variability, and/or shell shape contributes to the temporal genetic heterogeneity we observed is the subject of continuing research efforts.

Previously, heterozygosity at individual loci or mean heterozygosity over several loci have been implicated in selective survival mechanisms among mytilid populations; (Koehn & Gaffney 1984, Tremblay et al. 1998). A higher degree of mean multi-locus heterozygosity over several enzyme loci has been associated with lower energy requirements for maintenance metabolism (Diehl et al. 1986; Hawkins et al. 1986, 1989) thus imparting a differential survival advantage to heterozygotes. In the present work, variable survival in relation to heterozygosity, either at individual loci or as a mean over all loci, was not a significant factor.

In our study, all enzyme loci did not contribute equally to the observed temporal genetic heterogeneity. Only the *Gpi* locus pro-

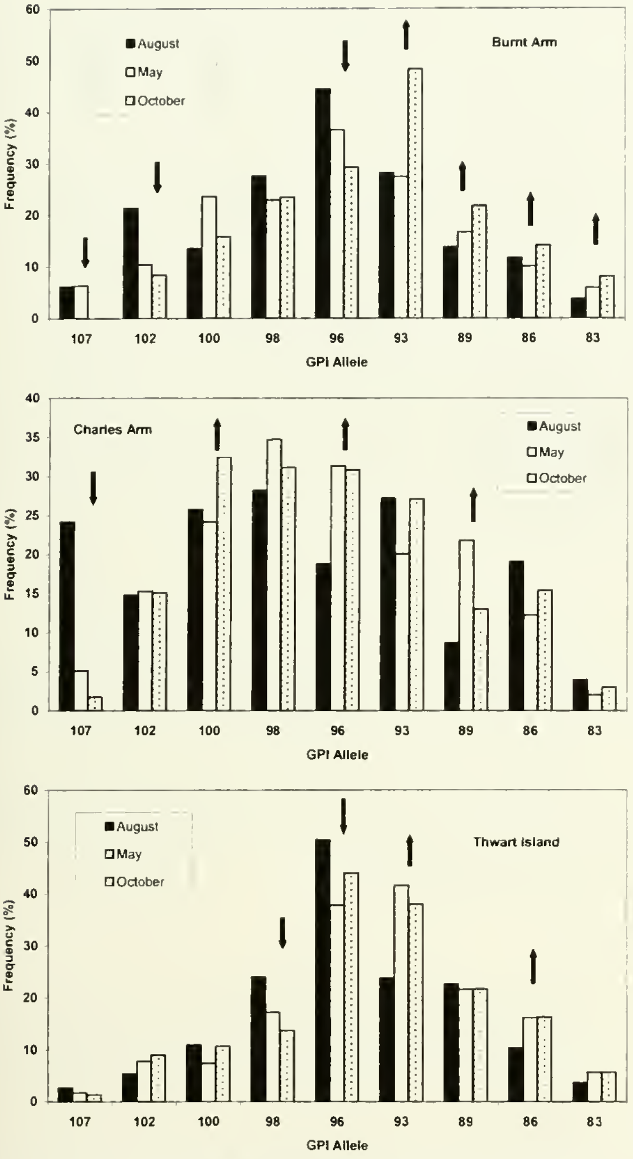

Figure 5. Intra-site change in genotypic frequencies with various *Gpi* alleles (homozygotes and heterozygotes combined) from August, 1998 to October, 1999. Arrows indicate specific alleles for which the χ^2 test for change in frequency over time of those with the allele (homozygotes and heterozygotes combined) vs. those without was significant after Bonferroni adjustment of significance levels for multiple tests. Arrow direction indicates an increase (upwards) or decrease (downwards) in frequency over time. Alleles without arrows did not significantly change in frequency over time. Allele frequency numbers with a ' (eg. *Gpi* allele 83') include frequencies of adjacent rare alleles.

TABLE 4.

Mean genotypic rank scores, non-parametric analysis of variance of ranked genotypic frequencies, and χ^2 approximation of the Kruskal-Wallis test for differences among median genotypic rank scores within sites over time at the *Gpi* locus.

Site	Sample Date	Mean Rank	Non-Parametric F Score	Kruskal-Wallis H (χ^2 approx.)
Burnt Arm	August, 1998	5.78	20.28***	37.04***
	May, 1999	5.96		
	October, 1999	6.49		
Charles Arm	August, 1998	5.43	3.30*	6.43*
	May, 1999	5.72		
	October, 1999	5.73		
Thwart Island	August, 1998	6.33	4.78**	12.09**
	May, 1999	6.64		
	October, 1999	6.60		

*** $P < 0.0001$; ** $P < 0.01$; * $P < 0.05$

duced significant temporal genetic heterogeneity with any pattern suggesting the presence of consistent selection processes. Temporal variation among electrophoretic variants at any locus implies the existence of selective processes differentially imparting a survival advantage over time. This does not necessarily imply a direct relationship between polymorphisms at a particular locus and survival. Variation at individual enzyme loci may simply serve as an indirect marker of another linked genetic condition that is subject to selective pressure (Beaumont et al. 1990). During our commercial rearing trials at three sites in Newfoundland, the population genotypic frequency distribution at the *Gpi* locus was shifted over time in favor of electrophoretically slower alleles. We infer from this frequency shift that selective survival processes favoring survival of *Gpi* genotypes incorporating relatively slower alleles exist during sub-tidal rope culture of blue mussels in Newfoundland. Although, this pattern of temporal change (high mobility alleles reduced, low mobility alleles increased) was consistent among all three sites, the same *Gpi* alleles were not always significantly implicated across all three populations. We found no evidence that any individual *Gpi* allele was consistently selected for or against at all three sites during the grow-out period. With the current dataset, we are unable to identify a causal mechanism that would adequately explain these observed patterns. However, our failure to detect a significant relationship between survival and the same specific individual *Gpi* alleles which was consistent over all three sites may be related to the widely different genetic population structure at these three sites. All three sites had allele frequencies significantly different from one another at all loci on all three sample dates during the rearing trials. A different result may well have occurred if the three populations had been genetically homogeneous initially. Until such time that a causal physiological mechanism is found, our inference of a genotype-dependent selective relationship between *Gpi* polymorphisms and survival in subtidal mussel culture based on directional shifts in genotypic frequencies will remain contentious.

Several previously published studies offer some insights which may help identify a causal physiological mechanism for the observed relationship between *Gpi* genotype and survival in subtidal, rope culture. The *Gpi* locus exhibits a considerable degree of polymorphism among most species of bivalve molluscs (Koehn et al. 1976) including North American mussel populations (McDonald et al. 1991; Penney & Hart 1999). Latitudinal clines that parallel thermal gradients have been observed at the *Gpi* locus in a variety of marine finfish and invertebrate species (see Hall 1985) suggesting a possible relationship between allelic variation at the *Gpi* locus and temperature. Available evidence in support of this possibility is inconclusive. In a series of laboratory experiments with larval and juvenile mussels, Beaumont et al. (1988, 1990) recorded significant genotype-dependent mortality at the *Gpi* locus. However, in neither case was this related to either temperature or salinity.

In contrast, the work of Hall (1985) does provide some evidence to support a potential relationship between *Gpi* and temperature. Glucosephosphate isomerase (*Gpi*) acts in the glycolytic pathway catalyzing the interconversion of fructose-6-phosphate and glucose-6-phosphate (Hall 1985). In experiments involving purified extracts of glucosephosphate isomerase derived from *Gpi*^{100} and *Gpi*^{96} genotypic individuals, Hall (1985) demonstrated the two variants had similar catalytic activity at 5–10°C but the electrophoretically faster *Gpi*^{100} enzyme became more efficient at higher temperatures between 15–25°C. In a similar experiment involving the sea anemone, *Metridium senile*, Hoffmann (1984) noted superior activity at higher temperatures for genotypes with the electrophoretically faster of two *Gpi* alleles and that the population variation in frequency of these two alleles exhibited a latitudinal cline along the eastern North American coast with the faster allele dominant in populations south of Cape Cod.

Both these studies suggest a possible selective advantage favoring relatively faster *Gpi* alleles in high temperature environments and further suggest a possible explanation for the observed decline in electrophoretically faster alleles in favor of slower ones in our study. The seed mussels in our study are at least in part derived from spawning of intertidal mussels which must regularly encounter periods of high air temperatures in the 20–25°C range during periods of emersion in summer. We may speculate that this produces selective temporal adaptation among intertidal populations in favor of electrophoretically faster *Gpi* genotypes while electrophoretically slower *Gpi* genotypes would be favored in suspended subtidal culture where temperature maxima are much lower (<20°C). Alternatively, the observed temporal variation at the *Gpi* locus may be linked to other factors unrelated to temperature such as selection for continuous immersion versus periodic emersion, a factor which might provide a different genotype-survival relationship in natural, intertidal populations versus those in sub-tidal rope culture.

In summation, our work has established that temporal genetic differentiation is apparently a common feature among rope-cultured blue mussel populations in Newfoundland and a *Gpi* genotype-dependent selective survival relationship significantly influences survival of *Mytilus* spp. during sub-tidal rope culture. This temporal genetic heterogeneity is likely a significant contributory factor in the extensive geographic genetic population structuring noted earlier among mixed *M. edulis* - *M. trossulus* populations in Newfoundland (Penney & Hart 1999). Identification of causal mechanisms controlling these temporal shifts in genotypic frequencies may permit commercially motivated selection of seed stocks with enhanced survival probabilities for culture operations. Commercial utilization of such seedstocks may help boost farm production and profitability.

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EFFECTS OF FOOD QUALITY AND QUANTITY ON FEEDING AND ABSORPTION IN BLACK-RIBBED MUSSELS, *SEPTIFER VIRGATUS* (WIEGMANN) (BIVALVIA: MYTILIDAE) DOMINATING WAVE-EXPOSED HABITATS IN HONG KONG

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ABSTRACT The black-ribbed mussel *Septifer virgatus* is an ecologically important species dominating exposed rocky shores in tropical and subtropical areas of Asia. The present study investigates its feeding and digestive responses to variations in food quantity and composition under laboratory conditions. Individuals were exposed to either one of five rations composed of different percentages of silt and the green microalga *Dunaliella tertiolecta*. Clearance rate (CR: $l\ h^{-1}$) was correlated weakly with both food quantity and quality whereas rejection rate (RR: $l\ h^{-1}$) showed a strong positive correlation with total particulate matter (TPM: $mg\ l^{-1}$), with the lowest threshold of TPM for pseudofaeces production estimated at $10\ mg\ l^{-1}$. Organic fraction in pseudofaeces was also significantly lower than organic content in water (f), evidence of pre-ingestive selection. Absorption efficiency (AE) was a negative exponential function of TPM and a negative quadratic function of organic ingestion rate (OIR). Gut passage time (GPT) showed a negative relationship with food quantity and ingestion rate (IR: $mg\ h^{-1}$). As food concentration increased (TPM), GPT of *S. virgatus* remained high when OIR was low ($<0.4\ mg\ h^{-1}$); further increases in OIR resulted in reductions of both GPT and AE. A prolonged GPT at low OIR resulted in a rapid increase in AR from 0.1 to $0.3\ mg\ h^{-1}$; further increases in OIR, however, did not cause a significant increase in AR. The existence of the regulative mechanism facilitates maximum absorption in wave-exposed habitats characterized by low seston concentrations.

KEY WORDS: *Septifer virgatus*, feeding, seston composition

INTRODUCTION

Both the concentration and composition of suspended particles in intertidal seawater undergo large fluctuations as consequences of the effects of tides, waves, currents, discharges of freshwater from rivers, and temporal variations in the abundance of phytoplankton and zooplankton. The feeding behavior of suspension-feeding organisms, therefore, is affected strongly by fluctuations in the food environment. To compensate for fluctuations in food availability, various mechanisms have been developed in bivalves which include regulation of feeding rate, production of pseudofaeces, selection of nutritious particles, and alteration of digestive efficiency (Hawkins et al. 1990; Bayne et al. 1993; Bacon et al. 1998). The relationships between suspension feeding and food concentration and quality have been studied in temperate, but not, generally, tropical bivalves. Such studies have provided insights into the trophic roles of bivalves in temperate aquatic ecosystems (Navarro et al. 1992; Hawkins et al. 1996) and identified optimum food conditions for aquaculture purposes (Beiras et al. 1993; Navarro et al. 1996).

In contrast to the Green mussel *Perna viridis* which is extensively cultured in southeast Asia and dominates sheltered and polluted harbours in Hong Kong characterized by turbid waters, the Black-ribbed mussel *Septifer virgatus* (Wiegmann) dominates wave-exposed rocky shores in Hong Kong characterized by clean water and low seston concentrations (Morton & Morton 1983; Seed & Richardson 1999). It forms a conspicuous band approximately 1.0 m wide in the eulittoral and the structural complexity of the mussel bed provides refuge and suitable habitat for a wide diversity of associated organisms (Ong Che & Morton 1992; Seed & Brotohadikusumo 1994). The species matures at a shell length of approximately 15 mm, about 1 year after recruitment and, like other local mytilids, *S. virgatus* is dioecious with a bimodal pattern of spawning and recruitment in any one year (Morton 1995). Although it is an ecologically important species on wave-exposed shores, far less is known about its ecology (Seed & Richardson 1999) and feeding behaviour as compared with that of temperate species such as *Mytilus edulis* and the tropical and subtropical mussel *Perna viridis* (Hawkins et al. 1998; Wong & Cheung 1999, 2001a,b). The present study is the first to investigate the feeding and digestive responses of *S. virgatus* to variable food quantity and quality under laboratory conditions.

MATERIALS AND METHODS

Collection and Maintenance of Experimental Animals

In December 1999, individuals with shell lengths of between 38 and 42 mm were collected from the eulittoral population of *Septifer virgatus* at Cape D'Aguilar on the southeastern extremity of Hong Kong Island, Hong Kong, and transported to the laboratory. Individuals of this size range were used because this was the dominant size group in the habitat. Epibionts on the shell of each individual were removed, and the mussels acclimated to laboratory conditions for 25 days before experimentation. During acclimation, individuals were fed daily with the green microalga, *Dunaliella tertiolecta*.

Preparation of Diet

Five diets with different quantities of sediments and the microalga *Dunaliella tertiolecta* were prepared. The sediments were collected adjacent to the population of *Septifer virgatus* at the study site and dried and ashed at 600°C to burn off organic matter in the sediments. The residual matter was grounded up in a pestle, sieved through a 37 μm sieve and mixed with *D. tertiolecta*, for

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which the organic matter content had been pre-determined. Diet characteristics, including total particulate matter (TPM: mg l^{-1}), particulate organic matter (POM: mg l^{-1}), particulate inorganic matter (PIM: mg l^{-1}) and organic content (f = POM/TPM), are identified in Table 1. The ranges of TPM and f used in the present study were comparable to those reported for the study site where TPM ranged from 4.5 to 36.0 mg l^{-1} with an annual mean of 9.5 mg l^{-1} and f ranged from 0.15 to 0.57 with an annual mean of 0.33 (unpublished data).

Experimental Procedures

Each individual of *Septifer virgatus* was kept in a separate beaker (250 ml) and supplied with one of the diets via continuously-flowing seawater pumped from a reservoir by an 8-channel peristaltic pump. A beaker without animals was considered as the control. The experiment was replicated so that results of fourteen mussels were obtained for each diet. The particles in the reservoir were kept in suspension by aeration and stirring. A preliminary experiment was done to determine the appropriate flow rate to be used in this study. We have tested four flow rates (20, 40, 60, and 80 ml min^{-1}) with seven individuals each. Results showed that clearance rate was significantly lower at 20 ml min^{-1} whereas no significantly difference was obtained among the other three flow rates. A flow rate of 40 ml min^{-1}, therefore, was used in this study. The reduction in particle concentration at this flow rate was found to be less than 40%. This helped to ensure significant particle reduction between inflow and outflow for accurate determination of CR at the same time avoiding recirculation of water in the beakers which would otherwise have resulted in underestimation of CR (Hildreth & Crisp 1976; Hawkins et al. 2001). Seawater samples were collected from a beaker without an experimental mussel (i.e., the control) at fixed time intervals of about 30 min. The collected samples were filtered through ashed and pre-weighed 25 mm GF/C filters and rinsed with isotonic ammonium acetate solution. The filter papers were dried in an oven at 90°C, weighed, ashed in a muffle furnace at 450°C and reweighed to determine TPM (mg l^{-1}), POM (mg l^{-1}), PIM (mg l^{-1}) and f values for the filtered particles.

Before the start of the experiment, individuals of *Septifer virgatus* were kept in filtered seawater to empty their guts for about 50 min which was the maximum evacuating time being obtained in a preliminary experiment for a 40 mm individual. The maximum evacuating time was determined by feeding the mussels with the microalga *Dunaliella tertiolecta* for 24 h. They were then starved in beakers containing flowing filtered seawater and pseudofaeces and faeces produced were collected continuously. The time when no more faeces and pseudofaeces produced was considered as the maximum evacuating time which was estimated at 50 min. After emptying the gut, all pseudofaeces and faeces produced in the first hour were removed. Each individual was then exposed to an experimental diet for 150 to 180 min and faeces and pseudofaeces were collected with caution during and at the end of the experiment to prevent resuspension of faeces and pseudofaeces. After food supply was stopped, the mussels were maintained in the beakers for another 40 to 50 min until no more faeces and pseudofaeces were produced. The minimum gut passage time rather than the mean gut passage time (Decho & Luoma 1991) was measured and defined as the difference in time between initial filter-feeding by each individual and the first appearance of faeces disregarding extracellular and intracellular digestion. The total, inorganic and organic weight of pseudofaeces and faeces were determined by the same methods as those described for seawater samples. The following rates were then computed: total matter rejection (RR: mg h^{-1}), organic matter rejection (ORR: mg h^{-1}), inorganic matter rejection (IRR: mg h^{-1}), total egestion (defecation) (ER: mg h^{-1}), organic matter egestion (OER: mg h^{-1}) and inorganic matter egestion (IER: mg h^{-1}) (Hawkins et al. 1996).

TABLE 1.

Characteristics of experimental diets.

Diet	TPM (mg l^{-1})	PIM (mg l^{-1})	POM (mg l^{-1})	f (POM/TPM)
A	6.13 ± 0.21	3.72 ± 0.12	2.41 ± 0.11	0.39 ± 0.007
B	8.10 ± 0.22	5.69 ± 0.18	2.41 ± 0.11	0.30 ± 0.011
C	10.08 ± 0.72	8.01 ± 0.74	2.07 ± 0.12	0.22 ± 0.02
D	10.35 ± 1.05	9.28 ± 1.00	1.07 ± 0.11	0.11 ± 0.01
E	20.33 ± 1.28	14.34 ± 0.06	5.98 ± 0.46	0.30 ± 0.02

TPM: total particulate matter; PIM: particulate inorganic matter; POM: particulate organic matter; f: organic content of suspended matter. Values are mean ± S.D.

Calculation of the Feeding Parameters

Food processing rates (feeding and absorption) were calculated following Iglesias et al. (1992). Assuming that absorption of inorganic matter through the digestive system was negligible (Cranford & Grant 1990), the sum of IRR and IER was considered to represent the rate of inorganic matter filtration (IFR: mg h^{-1}). Clearance rate (CR: l h^{-1}) was then estimated as CR = IFR/PIM. Filtration rate of total particulate (FR: mg h^{-1}) was computed as FR = CRxTPM and filtration rate of particulate organic matter (OFR: mg h^{-1}) as OFR = CRxPOM. Ingestion rates of total particulate matter (IR: mg h^{-1}) and of particulate organic matter (OIR: mg h^{-1}) were estimated as IR = FR-RR and OIR = OFR-ORR and hence, the organic content of absorbed food could be estimated as AR (mg h^{-1}) = OIR-OER, and absorption efficiency (AE) = AR/OIR.

Preingestive selection efficiencies for total filtered organic matter (SEo) were estimated as: SEo = 1 – (p/f) (Navarro et al. 1992), where p is the organic content of the pseudofaeces and f is the organic content of the suspended matter. In view of a very narrow size range of animals we used, no attempt has been made to weight-standardized the measured feeding rates.

Statistical Procedures

To obtain functional relationships between feeding responses and food parameters, a set of regression equations was fitted to experimental data, following standard least-squares procedures. Regression analyses were performed by simple linear and nonlinear procedures, depending on the most appropriate function to be fitted in each case (Zar 1999). Multiple regression analysis was conducted when feeding behavior was correlated with more than one diet parameters, and the collinearity between the independents was tested with collinearity statistics of SPSS (Belsley et al. 1980; SPSS Inc. 1999a). When the independent(s) were highly correlated with others, they would be eliminated from the independent list and the model was reconstructed until all the intercorrelations between the independents were removed from the regressive model. Residuals were also analyzed to check the normality, constant variance of predicted dependents and other necessary assumptions of the regression model. Data were transformed if necessary to meet the regressive requisites, i.e., normality of datum distribution and homogeneity of variances.

Analysis of variance (ANOVA) was used for comparisons among different treatment groups. Prior to analysis, raw data were diagnosed for normality of distribution and homogeneity of variance with Kolmogorov-Smirnov test and Levene test, respectively. The specific function and procedures followed for each feeding response will be presented with the results. All the statistical procedures were performed with software SPSS for windows, release 9.0 (SPSS Inc. 1999a,b).

RESULTS

Clearance Rates and Filtration Rates

The relationship between CR and food availability was weak but significant and can be described by the multiple regression equation as:

$$CR = 0.18 + 0.09/TPM + 0.13\ f(r^2 = 0.19,\ F_{2,67} = 6.56,\ P < 0.001)$$

There was no significant relationship between FR and f. FR, however, was positively related to POM and the equation that describes the relationship is:

$$FR = 0.79 + 0.57\ POM\ (r^2 = 0.72,\ F_{1,68} = 185.57,\ P < 0.001)$$

Pseudofaeces Rejection, Selection Efficiency and Ingestion of Filtered Food

The organic content of particulate matter (f) was significantly higher than that of the pseudofaeces for Diet C, D and E of which pseudofaeces were produced (Table 2). As TPM increased, pseudofaeces production increased with the increase in FR (Fig. 1) and can be described by the equation:

$$RR = -0.439 + 1.014\ \ln(FR)\ (r^2 = 0.84,\ F_{1,68} = 365.04,\ P < 0.001).$$

There was a significant positive relationship between TPM and RR ($RR = 0.54 + 0.08\ TPM\ (r^2 = 0.786,\ P < 0.001)$). No significant relationship, however, could be established between RR and f, showing that the rejection rate was related to the quantity (TPM) but not the quality (f) of suspended matter. As pseudofaeces were only produced for Diets C, D, and E, the lowest threshold of TPM for pseudofaeces production was about 10 mg l^{-1}.

Selection efficiency (SEo) is a measure of the efficiency in selecting the organic fraction of food when pseudofaces are being produced. There was an inverse relationship between SEo and TPM. No significant relationship between SEo and f, however, could be established. The corresponding equation is:

TABLE 2.

Comparisons between the organic content of the diet (f) and that of the pseudofaeces (e) for diet C, D and E where pseudofaeces were produced.

Diet	f (mean ± SD)	e (mean ± SD)	t value	Degree of Freedom	p
C	0.22 ± 0.02	0.12 ± 0.058	5.10	8	<0.001
D	0.11 ± 0.01	0.071 ± 0.016	6.91	7	<0.001
E	0.30 ± 0.02	0.199 ± 0.068	5.56	13	<0.001

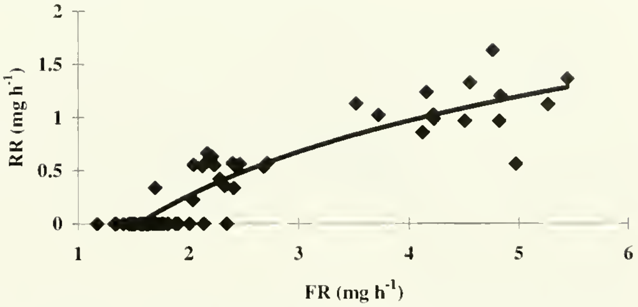

Figure 1. The relationship between filtration rates (FR: mg h^{-1}) and rejection rates (RR: mg h^{-1}) in *S. virgatus*.

$$SEo = 0.77 + 1.51/TPM\ (r^2 = 0.49,\ F_{1,68} = 66.34,\ P < 0.001)$$

Ingestion rate (IR) is estimated as the difference between filtered food and rejected pseudofaeces. Both IR and OIR were not significantly correlated with f but IR was a positive power function of TPM and OIR a linear function of POM which can be described by the following equations:

$$IR = 1.02\ TPM^{0.06}\ (r^2 = 0.82,\ F_{1,68} = 328.11,\ P < 0.001)$$

$$OIR = 0.048 + 0.186\ POM\ (r^2 = 0.91,\ F_{1,68} = 672.81,\ P < 0.001)$$

The more the particles were filtered, the more they were ingested, resulting in a positive linear relationship between IR and FR:

$$IR = 0.50 + 0.64\ FR\ (r^2 = 0.94,\ F_{1,68} = 1013.45,\ P < 0.001)$$

Absorption Rate and Absorption Efficiency

AR was a positive linear function of f (Fig. 2). As OIR increased, AR increased rapidly until OIR reached about 0.4 mg h^{-1} (Fig. 3). The rate of increase of AR, however, was slower when OIR increased further. The corresponding equations are:

$$AR = 0.09 + 0.85\ f(r^2 = 0.63,\ F_{1,68} = 126.27,\ P < 0.001)$$

$$AR = 0.45 - 0.05/OIR\ (r^2 = 0.74,\ F_{1,68} = 192.70,\ P < 0.001)$$

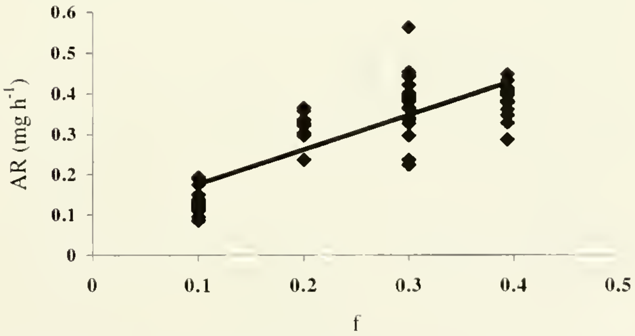

Figure 2. Absorption rates (AR: mg h^{-1}) as a function of organic content (f) of seston in *S. virgatus*.

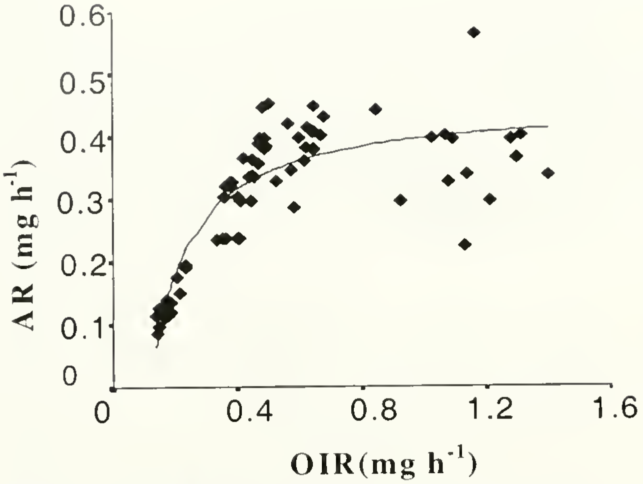

Figure 3. The relationship between absorption rates (AR: mg h^{-1}) and organic ingestion rates (OIR: mg h^{-1}) in *S. virgatus*.

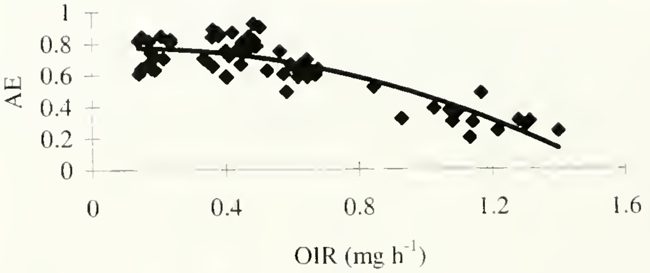

Figure 5. The relationship between absorption efficiency (AE) and organic ingestion rates (OIR: mg h^{-1}) in *S. virgatus*.

AE decreased exponentially with an increase in TPM in the water and was a negative quadratic function of both POM (Fig. 4) and OIR (Fig. 5). The corresponding equations are:

$$AE = 0.962e^{-0.041TPM} \ (r^2 = 0.42, F_{1,68} = 61.28, P < 0.01)$$

$$AE = 0.726 + 0.037 \ POM - 0.017 \ POM^2 \ (r^2 = 0.73, F_{2,67} = 88.03, P < 0.001)$$

$$AE = 0.77 + 0.07 \ OIR - 0.38 \ OIR^2 \ (r^2 = 0.74, F_{2,67} = 96.36, P < 0.001)$$

Gut-Passage Time

Gut-passage time (GPT) for particles was a negative exponential function of TPM (Fig. 6) and a negative quadratic function of OIR (Fig. 7), showing that the time the food particles stayed in the gut was prolonged when OIR was low. GPT, however, was much reduced when OIR increased. The respective equations are:

$$GPT = 59.32 \ e^{-0.07TPM} \ (r^2 = 0.76, F_{1,68} = 213.54, P < 0.001)$$

$$GPT = 21.28 + 49.07 \ OIR - 43.04 \ OIR^2 \ (r^2 = 0.42, F_{2,67} = 24.16, P < 0.001)$$

DISCUSSION

Rates of Clearance, Filtration and Pseudofaeces Production

Food availability is regarded as one of the most important factors affecting the feeding behaviour of bivalves. To compensate for fluctuations in food availability, various mechanisms have been developed which include regulation of feeding rate, pseudofaeces production, selection of nutritious particles and alterations to digestive efficiency (Hawkins et al. 1990; Bayne et al. 1993; Bacon et al. 1998). As particle concentration increases, ingestion is commonly regulated by a reduction in clearance rate and the production of pseudofaeces in mussels (Widdows et al. 1979), scallops and clams (Navarro et al. 1992; Bacon et al. 1998). A similar reduction in clearance rate with food availability was also recorded for another locally dominant mussel, i.e., *Perna viridis*, under both laboratory (Wong & Cheung 1999) and field conditions (Hawkins et al. 1998; Wong & Cheung 2001a,b). In contrast, the correlation between clearance rate and food availability (TPM and f) in *Septifer virgatus* was weak, showing that ingestion of particulate organic matter was not regulated through clearance rate. In *Mytilus trossulus*, CR was independent of increasing seston quality at relatively high seston loads (20 and 50 mg l^{-1}) (Arifin & Bendell-Young 1997). Positive correlations between clearance rates and the TPM of natural seston have been reported upon for *Mytilus edulis* (Newell & Shumway 1993; Hawkins et al. 1996) and *Cerastoderma edule* (Iglesias et al. 1992). CR of *Crassostrea gigas* was maintained high and constant when TPM was 50 mg l^{-1} but decreased with further increases (Barillè & Prou 1994). Nevertheless, all species should have an upper limit to the rate of particle processing (Bayne & Newell 1983). It was expected, therefore, that the CR of *S. virgatus* would ultimately decrease when TPM increased to a level higher than that experienced in the present study.

With clearance rate relatively independent of food quantity, filtration rate increases with POM, as shown here for *Septifer*

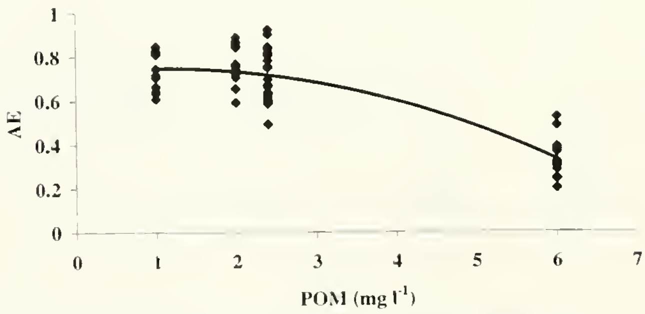

Figure 4. Absorption efficiency (AE) of *S. virgatus* as a function of particulate organic matter (POM: mg l^{-1}) in the water.

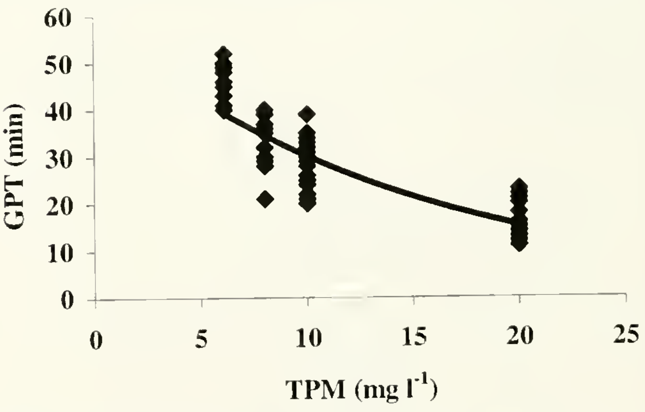

Figure 6. Gut passage time (GPT: min) of *S. virgatus* as a function of total particulate matter (TPM: mg l^{-1}) in the water.

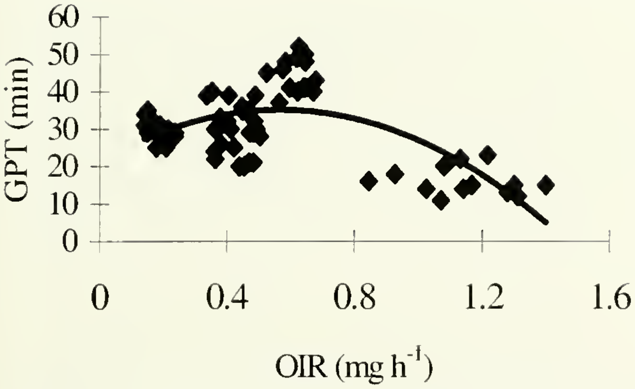

Figure 7. The relationship between gut passage time (GPT: min) and organic ingestion rates (OIR: mg h^{-1}) in *S. virgatus*.

virgatus. Reports on the relationship between filtration rate and food concentration, however, were variable. Winter (1973) showed that the filtration rates of *M. edulis* decreased with increasing food concentration such that the bivalves could keep the number of filtered algae in unit time relatively constant within the range of 10×10^6 to 40×10^6 cells l^{-1}. Contrary results, however, were reported by Winter (1978) and Riisgård and Randløv (1981), that is, that filtration rates increased quickly with increasing particle concentration. Griffiths and Griffiths (1987) reviewed the relationship between food quality and quantity and filtration rate of various species of suspension-feeding bivalves and concluded that conflicting experimental results were largely attributed to differences in particle concentration. At low concentrations, a positive relationship between filtration rate and food quantity was established. When similar experiments were conducted at low to intermediate particle concentrations, and the concentration ranges were relatively narrow, no obvious relationship between filtration rate and particle concentration was observed. In the present study, four out of the five treatment groups had particle concentrations either equal to or lower than 10 mg l^{-1}. A reduction in filtration rate, therefore, is expected at higher particle concentrations when CR decreases.

Pseudofaeces production is regarded as one of the most important regulative processes in bivalves to organically enrich ingested matter by the selective rejection of inorganic matter versus organic matter and thus, as a consequence, to optimize energy uptake. The production of pseudofaeces is strongly related to both the density and organic content of suspended seston in the water (e.g., Foster-Smith 1975; Bacon et al. 1998; Baker et al. 1998; Beninger et al. 1999). *Septifer virgatus* started to produce pseudofaeces when TPM was ~10 mg l^{-1}; its production rate was also highly correlated with TPM and FR. Ingestion rate, therefore, increased across the full range of food availability. This agrees with the observations of Kiørboe at al. (1980) and Hawkins et al. (1996) on *Mytilus edulis* but is not consistent with those of Widdows et al. (1979) who demonstrated that maximum IR coincided with the threshold TPM above which pseudofaeces were produced. Hawkins et al. (1996) anticipated that IR would reach maximal values in association with reduced CR and/or an increase in the ratio RR/FR, as observed for *M. edulis* feeding upon pure strains of cultured algae (Foster-Smith 1975).

The SEo index represents the efficiency with which bivalves can organically enrich ingested matter by selectively rejecting inorganic matter via the palps. For both *C. edule* (Iglesias et al. 1992, 1996) and *P. viridis* (Wong & Cheung 1999), SEo was a curvilinear function of f whereas in another study on *C. edule*, SEo was positively related to f and negatively related to seston concentration (Navarro & Widdows 1997). In *S. virgatus*, SEo varied from 0.74 to 1.0 and was negatively correlated with TPM but not significantly correlated with f. Such high values of SEo in *S. virgatus* can probably be attributed to low seston concentrations and it is expected that SEo would decrease when seston concentration increased beyond that experienced by the individuals used in this study. A maximum selection efficiency of 0.60 was obtained for *C. edule* when fed at low seston concentrations, and this decreased to the lowest value of 0.10 when seston concentration increased to 600 mg l^{-1} (Navarro & Widdows 1997). The SEo of *Perna viridis* was comparable to *S. virgatus*, with a maximum efficiency of 0.75 (Wong & Cheung 1999).

The present study demonstrates inter-specific differences in pre-ingestive feeding behaviour between two mussels in Hong Kong. *S. virgatus* is a dominant species on exposed rocky shores in the eastern waters of Hong Kong characterized by low TPM values (Morton & Morton 1983). Seasonal variations in TPM at Cape d'Aguilar, Hong Kong, where the animals used in the present study were collected from, have been studied for one year (unpublished data). TPM varied from 4.5 to 36 mg l^{-1} with an annual mean of 9.5 mg l^{-1}. With the relative independence of clearance rate from particle concentration, and positive relationships between RR and FR and between IR and FR, the ingestion rate of organic particulate matter, therefore, could be enhanced under the low food concentrations which prevail in this environment. In contrast to *S. virgatus*, *P. viridis* occurs predominantly in sheltered environments in which the water is often turbid and sometimes heavily polluted (Lee 1985; Cheung 1993). This species possesses large labial palps with strong ciliary rejection tracts (Morton 1987; Seed & Richardson 1999) to cope with the high sediment loadings often associated with sheltered, low energy habitats. Ingestion of particulate organic matter by *P. viridis* was regulated through pseudofaeces production, as herein demonstrated for *S. virgatus*, although the rate was much higher at ~9 mg h^{-1} (Wong & Cheung 1999) as compared with *S. virgatus* of a similar size (~1.6 mg h^{-1}). Regulation was further enhanced by controlling clearance rate which has not been observed in *S. virgatus*.

Absorption and Gut Passage Time

The absorption efficiency of suspension-feeding bivalves has been shown to change over time scales according to variability in seston characteristics (Bayne & Newell 1983) and is mainly controlled by organic food quantity and/or quality when feeding on either natural seston or on suspensions resembling natural conditions (Bricelj & Malouf 1984). In the present study, the AE of *S. virgatus* was negative quadratic functions of both POM and OIR. Digestion in bivalves is biphasic involving the stomach and digestive diverticula as compartments for extra- and intra-cellular digestion, respectively (Purchon 1968). Food may undergo extracellular digestion in the stomach followed by intestinal absorption and is voided as "intestinal faeces". Some of the food may then also undergo intra-cellular digestion and absorption within the digestive diverticula and eliminated as "glandular faeces" (Bricelj & Malouf 1984). With higher rates of food processing, the proportion of particles by-passing the digestive diverticula would increase and result in reductions in both GPT and AE (Navarro & Iglesias 1993). In models combining gut passage time and feeding behaviour. Willows (1992) also showed that GPT generally decreased with an increase in the quality of food at high levels of food availability. These observations agree with the present study, i.e., as food concentration increased (TPM), the GPT of *S. virgatus* remained at high levels and was relatively constant when OIR was low (<0.4 mg h^{-1}). Further increases in OIR resulted in a reduction in GPT. The high and constant values of AE under relatively low OIR (up to 0.4 mg h^{-1}), therefore, might be attributed to the prolonged GPT and resulted in a rapid increase in AR from 0.1 to 0.3. Further increases in OIR, however, did not cause significant increases in AR. Similar observations were also reported upon for *Placopecten magellanicus* (Brillant & MacDonald 2000) and *Aulacomya ater* (Griffiths & King 1979) that AE of the latter species decreased rapidly with increasing algal concentration and AE approached zero when algal concentrations were $>32 \times 10^6$ cells l^{-1}. Using algal concentrations ranging from 50 to 100×10^6 cells l^{-1} (equivalent to 1.25 – 2.5 mg l^{-1} POM), Gerdes (1983), however, found that AE of the Pacific Oyster, *Crassostrea gigas*, was independent of ration with AE of ~75% being obtained for all three kinds of diet with different TPM and POM. As the range of POM used in our study on *S. virgatus* varied from 1 to 6 mg l^{-1} as compared to a narrower range (1.25 – 2.5 mg l^{-1}) used by Gerdes (1983), AE of *C. gigas* is expected to decrease, as what has been observed in *S. virgatus* and other bivalves, when POM increases further.

The ranges of seston quantity (TPM) and quality (f) used in this study were 20 mg l^{-1} and 0.4, respectively, and within the ranges recorded for the habitat where *S. virgatus* was collected for this study. AR reached asymptote when OIR was only one third that of the highest value. This suggests that *S. virgatus* is particularly well-adapted to a low seston concentration environment. Food quality was also an important factor controlling AR. For example, AR is a positive quadratic function of POM in *P. viridis* (Wong & Cheung 1999) and is positively linearly related to the different measures of diet quality in *Placopecten magellanicus*, even when the organic content of seston (f) was as high as 80% (Cranford 1995). This agrees with the results obtained for *S. virgatus* in this study.

The present study demonstrated regulative mechanisms in the subtropical mussel *S. virgatus* which is dominant on locally clean and wave-exposed habitats characterized by low seston concentrations. As food concentration increases, particulate organic matter ingestion is enhanced through pseudofaeces production with preferential ingestion of particulate organic matter, although clearance rate is relatively independent of food quantity. GPT was a negative function of TPM and OIR such that AE is relatively constant and absorption rate reaches asymptote at low seston concentrations.

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COMPARATIVE SETTLEMENT DEPTHS OF *MYTILUS EDULIS* C. LINNAEUS, 1758 AND *M. TROSSULUS* GOULD, 1850: I. A MESOCOSM STUDY

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ABSTRACT Production on many commercial mussel farms in Eastern Canada is hampered by the presence of *Mytilus trossulus*, a comparatively thin-shelled but close relative of *M. edulis*, the principal species collected and grown. Exploitation of a species-based difference in depth preference could potentially increase the collection of *M. edulis* at mixed species sites. As a first step in the investigation, larval cultures of each species were reared to settlement in replicated mesocosms in a study conducted at Dalhousie University's Aquatron facility in Halifax, Nova Scotia. Depth preference for settlement was examined with and without a thermocline. Both *M. edulis* and *M. trossulus* had the largest percentage of larvae settling at the surface. Settlement occurred below the thermocline at 6 m in both species, although in significantly lower proportions. In mixed water, a significant species–depth interaction was detected. This was driven by a significantly large number of *M. edulis* spat settling at 8 m, a pattern not found in *M. trossulus*.

KEY WORDS: *Mytilus edulis*, *M. trossulus*, settlement, depth, mesocosms

INTRODUCTION

Farm recruitment [1]of mussels in Eastern Canada depends on the timing and inter-relationship of a number of natural events combined with judiciously applied husbandry practices. The deployment of a suitable settlement medium, at an appropriate time, is key to maintaining an uninterrupted cycle of spat collection, growout, and harvest. Traditionally, spat collecting is performed on the farm site, usually by deploying plastic mesh (Vexar™) on headropes near the surface several days before the anticipated onset of settlement. This technique has proven successful over the relatively short history of Eastern Canadian mussel farming. Nevertheless, spat collections at some farms comprise two species (*Mytilus trossulus* Gould, 1850 and *M. edulis* C. Linneaus 1758) and the presence of the former is cause for concern to many growers (Freeman 1996).

Although it was realized early in the industry that certain farms often produced mussels of varying quality, it was not initially appreciated, at least in certain cases, that the problem was species-based. After this had been demonstrated (Koehn et al. 1984), investigations into distributions of the two species showed that Eastern Canadian *Mytilus* populations vary from purely one or the other species to varying proportions of each (Penney & Hart 1999; Mallet & Carver 1999). Although the two are often superficially similar, *M. trossulus* is characterized by comparatively fragile shells and generally lower meat weight per shell length. For example, it has been found that the initial number of *M. trossulus* farmed would have to be multiplied 1.7 times to achieve the same economic return as with *M. edulis* alone (Mallet & Carver 1995). Furthermore, anecdotal reports suggest that on some farms the proportion of *M. trossulus* to *M. edulis* is gradually increasing (Freeman 1996). Importation of pure *M. edulis* seed has been the sole means producers have to overcome production shortcomings associated with farm-collected *M. trossulus*, however, researchers have been looking for other options. It had been believed, for example, that comparison of early life events of both species might suggest alternate husbandry strategies at sites with both species, where growers might wish to harvest only the more commercially desirable *M. edulis* (Freeman 1996)

Examination of spawning times and duration of larval phases revealed close synchrony between *M. edulis* and *M. trossulus* (Freeman et al. 1994), indicating that any separation technique employed as part of routine husbandry would have to be based on some other criterion. Anecdotal references to different settlement depths for each suggested that looking for larval depth variations between the two would be an appropriate first step. It is presumed that initial settlement would "preferentially" occur at a depth chosen by the larvae upon their attainment of competence, assuming the larvae had immediate accessibility to a suitable substrate at that depth. Following recent success of mesocosm studies of *Placopecten magellanicus* larvae at the Dalhousie University Aquatron in Halifax, Nova Scotia (e.g., Manuel et al. 1996; Gallagher et al. 1996), additional experiments were performed at the same facility using laboratory-reared larvae of *M. edulis* and *M. trossulus* (MacQuarrie 1995; Freeman & MacQuarrie 1999).

Here we present data on depth settlement preferences of both *M. edulis* and *M. trossulus* as observed in the laboratory. Settlement behavior was examined in columns of mixed water, and when a thermocline was present at 6 m.

METHODS

Broodstock Selection

Naturally conditioned broodstock animals were obtained from a commercial mussel farm near Lunenburg, Nova Scotia. The collection was made in early June 1995, a time corresponding to peak natural spawning at this site. Animals were selected on the basis of apparent shell weight (a completely subjective judgment) combined with shell length to shell height ratio derived from direct measurements. Those animals with a shell length to shell height ratio >2.2 and deemed 'light weight' for their length, were considered *M. trossulus*, whereas those animals with a ratio of <2.0 and deemed 'heavy' for their length were regarded as *M. edulis*. Subsequent isoenzyme analysis in earlier work confirmed the accuracy of this visual identification method at this site (Freeman et al. 1994). Furthermore, a more recent shell analysis of mature, DNA-identified mussels has indicated that the length-to-height

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TABLE 1.

Chronology of mesocosm experiment, 1995. *M. edulis* larvae produced on June 13, and *M. trossulus* larvae produced on June 20.

Species	Mesocosm Replicate	Date Larvae Placed	Larval Age at Placement	Larvae per Mesocosm ($\times 10^6$)	Date & Age (days) of Mussels at Interval Collector	
					Deployment	Removal
M. edulis	E1	June 18	5 days	1.43	July 7 (24)	July 31 (48)
M. edulis	E2	June 18	5 days	1.43	July 7 (24)	July 31 (48)
M. edulis	E3	June 18	5 days	1.43	July 7 (24)	July 31 (48)
M. edulis	Nt E4	June 18	5 days	1.43	July 7 (24)	July 31 (48)
M. trossulus	T1	June 22	2 days	1.60	July 11 (21)	July 31 (41)
M. trossulus	T2	June 22	2 days	1.60	July 11 (21)	July 31 (41)
M. trossulus	T3	June 22	2 days	1.70	July 11 (21)	July 31 (41)
M. trossulus	Nt T4	June 22	2 days	1.70	July 11 (21)	July 31 (41)

Nt = no thermocline mesocosm.

proportions, as a guide to species identification at Corkums Island, remain valid. Greater assurance of species identification was attained by selecting animals toward the extreme ends of the shell length/shell height scale, which at this site, ranged from approximately 1.75 to 2.50. One hundred putative *M. edulis* and 92 *M. trossulus* were chosen from several dozens of specimens examined. These animals were taken to the former Halifax Fisheries Research Laboratory, Halifax, Nova Scotia.

Spawning and Fertilization

Animals were held in ultraviolet-irradiated, 1-μm filtered ambient temperature (<10°C) seawater to encourage gut content release prior to spawning attempts. On spawning occasions, mussels were brushed clean of epiphytic growth, wiped with dilute bleach solution, rinsed in 1-μm filtered seawater, and then placed in 500-mL containers, one animal per container. Seawater (20°C) was added to each container and changed after the first 30 min if spawning had not commenced; any mussel not spawning after this first rewarming was discarded. Sperm–seawater mixtures from several *M. edulis* males were combined in one labeled container, and egg–seawater mixtures from several females of the same species were combined in a second labeled container. To remove fecal matter and other detritus, all the eggs were passed through a 100-μm filter, and the sperm mixtures were passed through a 40-μm filter. Gametes from *M. trossulus* were treated similarly. *M. edulis* broodstock was spawned on June 13, and *M. trossulus* on June 20, 1995.

Within species, pooled sperm was well mixed and 25 mL of the mixture were added to the pooled eggs in a 20-L bucket and gently mixed again. Egg–sperm mixtures were examined microscopically to ensure that the sperm concentration was not excessive; further sperm additions were made if concentrations appeared low. Two hours following fertilization, a well-stirred sample of each batch of eggs was examined microscopically and the percentage of fertilization was estimated by noting the proportion of intact eggs to embryos having attained at least the first division. Within 3 h of fertilization, each batch of fertilized eggs was separated into seven roughly equal lots, placed into seven 135-L plastic containers, filled with treated (20°C, 1 μm) seawater, and left standing. One day later, the upper 80% of each container was carefully siphoned off and retained. The final 20% containing detritus, unfertilized eggs, and many substandard trochophores, was discarded. The 135-L containers were sanitized, the retained larvae replaced in them, and refilled with treated seawater. At 48 h, the entire contents of the 135-L tubs, now containing straight-hinge (veliger) larvae, were siphoned through 40-μm filters, and rinsed with, then resuspended in treated seawater. At this stage, the *M. trossulus* larvae were enumerated using a Coulter Counter, then equally divided among four sanitized, covered, 20-L buckets and transported to the 10-m-deep tower tank at Dalhousie University (for a complete description of the facility, see Gallagher et al. 1996). For logistic reasons, *M. edulis* larvae were held for 5 days at the Department of Fisheries Laboratory before final counting, separation into four lots, and being transported to the tower tank. While being held at the Fisheries laboratory, *M. edulis* larvae were fed Tahitian *Isochrysis* daily, and the water was changed at 2 and 4 days.

Tower Tank Procedure

Each mesocosm was a 8.5-m-deep by 0.6-m-diameter polyethylene cylinder sealed at the bottom, supported at the surface by a

TABLE 2.

Nested ANOVA of the transformed percentage of mussel spat settled at depth by species.

Source	DF	Sum of Squares	*F* Ratio	Probability > *F*	Power
Sp. code	1	0.009075	0.2934	0.5903	0.083
Depth	8	12.437850	50.2592	<0.0001	1.000
Sp. code × depth	8	0.364035	1.4710	0.1896	0.600
Mesocosm [Sp. code]	4	0.273567	2.2109	0.0799	0.610
Depth × mesocosm [Sp. code]	32	2.099770	2.1212	0.0072	0.990

This analysis was based on data from mesocosms with no thermocline.

TABLE 3.

M. edulis: **Comparisons for all pairs of depths (m) using Tukey-Kramer honestly significant difference (HSD) test.**

Depth	0	2	3	1	4	5	6	8	7
0	−0.35	−0.02	**0.03**	**0.04**	**0.08**	**0.16**	**0.57**	**0.59**	**0.83**
2	−0.02	−0.35	−0.30	−0.29	−0.24	−0.16	**0.24**	**0.26**	**0.50**
3	**0.30**	−0.30	−0.35	−0.34	−0.29	−0.21	**0.19**	**0.21**	**0.45**
1	**0.04**	−0.29	−0.34	−0.35	−0.31	−0.23	**0.18**	**0.20**	**0.44**
4	**0.08**	−0.24	−0.29	−0.31	−0.35	−0.27	**0.13**	**0.16**	**0.39**
5	**0.16**	−0.16	−0.21	−0.22	−0.27	−0.35	**0.05**	**0.08**	**0.32**
6	**0.57**	**0.24**	**0.19**	**0.18**	**0.13**	**0.05**	−0.35	−0.33	−0.08
8	**0.59**	**0.26**	**0.21**	**0.20**	**0.16**	**0.08**	−0.33	−0.35	−0.11
7	**0.83**	**0.50**	**0.45**	**0.44**	**0.40**	**0.32**	−0.09	−0.11	−0.35

Positive values in bold show pairs of means that are significantly different at $\alpha = 0.05$.
A thermocline was present at 6 m.
Depths are ordered to position significantly different pairs together.

styrofoam floatation collar, and filled with 2.40 m^3 of 1.0-μm filtered water. Hot and cold water circulating in vertically adjustable piping circling the inside walls of the tank maintained a thermocline, in this instance at 6 m. The temperature above the thermocline was 19°C; below it 9°C. Of the *M. edulis* larvae, 1.43×10^6 were placed in each of four mesocosms suspended in the tower tank. For *M. trossulus*, two mesocosms were charged with 1.65×10^6 larvae and two others with 1.70×10^6 larvae. One of the latter *M. trossulus* and one *M. edulis* mesocosm was fitted with an outer polyethylene bag containing water and an airlift system, which kept the water stirred. This system eliminated the thermocline in these two mesocosms, and held their internal temperatures at 12°C, top to bottom. Logistic constraints eliminated the possibility of replicating the nonthermocline mesocosms. A 12-h day/night photoperiod established during the larval stages was continued to the end of the settlement experiment. The feeding regime established by the mid-larval period was also continued. Mixtures of Tahitian *Isochysis* and *Chaetoceros gracilis* were distributed throughout the depths of each mesocosm by use of perforated hoses. Final concentrations of 5.0×10^3 cells/mL of each algal species were maintained to the end of the experiment. Algal counts were assessed and adjusted every 2 days.

Collectors

Duplicate, preconditioned, 13-mm diameter, 25-cm lengths of polypropylene rope were suspended by weighted monofilament lines in all eight mesocosms. The portions of rope were positioned more or less horizontally, at 1-m depth intervals, from the surface to 8.0 m. The use of fine-diameter, smooth-surfaced monofilament line was intended to discourage newly settled spat from migrating vertically. The collectors were installed in the *M. edulis* mesocosms when these larvae were 24 days old, and in the *M. trossulus* mesocosms when these larvae were 21 days old. In each case, settlement began about 5 days after collector installation.

On July 31, the 25-cm segments of polypropylene rope from these collectors with their attached spat were retrieved, placed in a container of 80% ethyl alcohol, and labeled by species, replicate, and depth. *M. edulis* collectors were removed at 48 days of age; those of *M. trossulus* were removed at 41 days. Spat were subsequently separated from the rope segments with a light brushing, then counts of the total number of spat per segment were done microscopically. The chronology of events is shown in Table 1.

Data Treatment

Thermocline at 6 m

Spat counts from the collectors (two replicates for each mesocosm with a thermocline) were converted to percentages settled at each depth per collector, $\log_{10}$ transformed, then tested for normality using the Shapiro-Wilk W test. Normal Q–Q plots of the residuals were visually inspected for deviations. For each factor, homogeneity of variance was assessed with Levene's test. Data were then subjected to analysis of variance (ANOVA). All analyses were computed with JMP® version 3.1 software. The initial analysis was designed to test the hypothesis that there was no significant difference between species in settlement depth distribution in the presence of a thermocline at 6 m. The mesocosms were nested within each species. The model is expressed as

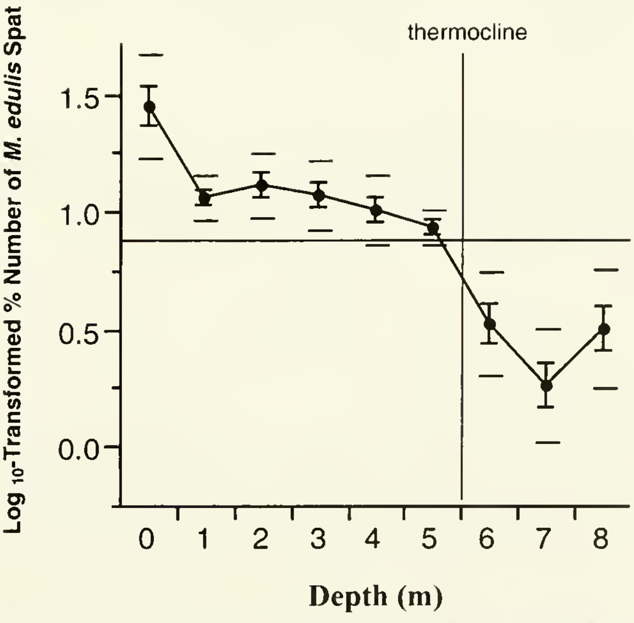

Figure 1. Log_{10}**-transformed percent number of** *M. edulis* **spat. Mean, standard error, and standard deviation are indicated for each depth (0–8 m). The mean across all depths is also indicated (solid line). A thermocline was present at 6 m in this experiment.**

TABLE 4.

M. trossulus: Comparisons for all pairs of depths (m) using Tukey-Kramer honestly significant difference (HSD) test.

Depth	0	4	1	2	5	3	6	7	8
0	−0.46	**0.00**	**0.01**	**0.02**	**0.07**	**0.21**	**0.53**	**0.59**	**0.67**
4	**0.00**	−0.46	−0.45	−0.44	−0.39	−0.25	**0.06**	**0.12**	**0.20**
1	**0.01**	−0.45	−0.46	−0.45	−0.40	−0.26	**0.06**	**0.12**	**0.20**
2	**0.02**	−0.44	−0.45	−0.46	−0.41	−0.27	**0.05**	**0.11**	**0.19**
5	**0.07**	−0.39	−0.40	−0.41	−0.46	−0.32	−0.01	**0.05**	**0.13**
3	**0.21**	−0.25	−0.26	−0.27	−0.32	−0.46	−0.15	−0.09	−0.01
6	**0.53**	**0.06**	**0.06**	**0.05**	−0.01	−0.15	−0.46	−0.40	−0.32
7	**0.59**	**0.12**	**0.12**	**0.11**	**0.05**	−0.09	−0.40	−0.46	−0.38
8	**0.67**	**0.20**	**0.20**	**0.19**	**0.13**	−0.01	−0.32	−0.38	−0.46

Positive values show pairs of means that are significantly different at $\alpha = 0.05$.
A thermocline was present at 6 m.
Depths are ordered to position significantly different pairs together.

$$X_{ijkl} = \mu + A_i + B_j + AB_{ij} + C(B)_{k(j)} + AC(B)_{jk(i)} + \varepsilon_{l(ijk)}$$

where A is depth with nine levels (0, 1, 2, 3, 4, 5, 6, 7, and 8 m) treated as an ordinal value, B is species with two levels (*M. edulis* and *M. trossulus*), and C is mesocosm with three levels nested within species. Depth was orthogonal to both factors.

Tukey-Kramer honestly significant difference (HSD) tests were performed in post-hoc analyses of all ANOVA results. Multiple comparisons of all pairs of means were performed. The significance level (alpha) in the one-way ANOVA for all comparisons was set at 0.05. The mean values of the $\log_{10}$-transformed percentages were plotted for each depth and species along with descriptive statistics.

No Thermocline

A second model was used to test the hypothesis of no difference in the percent of spat of each species settling at depth, in the absence of a thermocline. Because there was only one mesocosm per species, a two-way ANOVA model was used:

$$X_{ijk} = \mu + A_i + B_j + AB_{ij} + \varepsilon_{ijk}$$

where A is depth with nine levels (0, 1, 2, 3, 4, 5, 6, 7, and 8 m), B is species code with two levels (*M. edulis* and *M. trossulus*), and AB is the interaction term. The data were converted to percentage settled at each depth per collector rope and $\log_{10}$ transformed. Normality, homogeneity of variance, and post-hoc tests were performed as above.

RESULTS

Spawning and Fertilization

Of the putative *M. edulis* subjected to spawning inducement, 28 females and 27 males (55%) spawned. Of the putative *M. trossulus* subjected to spawning inducement, 18 females plus 22 males (43%) spawned. Two hours after batch fertilization, 84% of the *M. edulis* eggs had progressed to at least first divisions and for *M. trossulus*, 86%. From past experience with broodstock from the same farm, the percentage spawning and the subsequent progress of fertilization and embryo development fell well within expectations. Approximately 5.72×10^6 *M. edulis* larvae and 6.60×10^6 *M. trossulus* 2-day-old veliger larvae were taken to the Dalhousie University tower tank.

Analyses of Spat Settlement, Thermocline Present at 6 m

The transformed data produced a distribution that was not significantly different from normal at $P = 0.01$ (Shapiro-Wilk W test). Untransformed percentages did not meet the assumptions of the tests. The variances were homogeneous within depth and species according to Levene's test ($P = 0.45$ and 0.74, respectively). Normal Q–Q plots of the residuals indicated that the residuals were normally distributed.

The ANOVA model explained approximately 80% of the variance in the data set (adjusted $R^2 = 0.81$). Table 2 provides the test results. These indicate that both species had significantly different numbers of spat settling at different depths and that those distributions differed according to mesocosm. The spat were not uniformly distributed by depth, however, no significant differences between the species were observed.

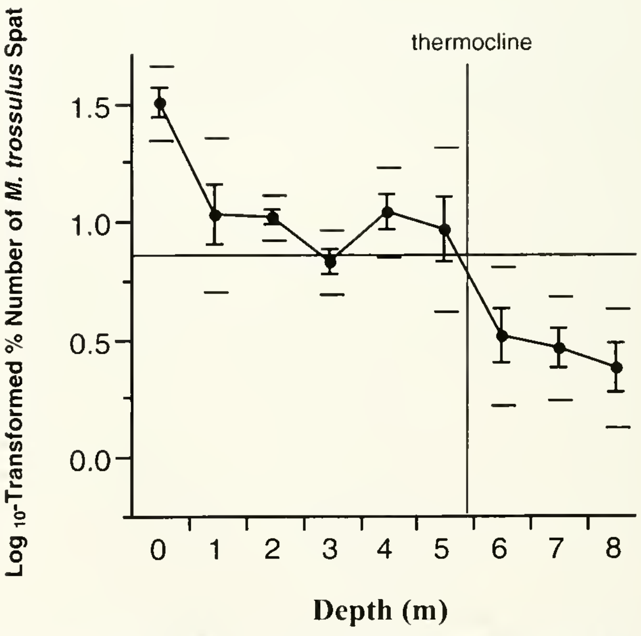

Figure 2. $\log_{10}$-transformed percent number of *M. trassulus* spat. Mean, standard error, and standard deviation are indicated for each depth (0–8 m). The mean across all depths is also indicated (solid line). A thermocline was present at 6 m in this experiment.

TABLE 5.

Two-way ANOVA of the transformed percentage of spat settled at depth by species.

Source	DF	Sum of Squares	*F* Ratio	Probability > *F*	Power
Sp. Code	1	0.007876	0.3184	0.5795	0.083
Depth	8	0.947826	4.7893	0.0028	0.877
Sp. Code * Depth	8	0.609815	3.0814	0.0225	0.559

No thermocline was present.

Post-hoc analyses of the depth distributions of spat of each species in the presence of a thermocline at 6 m depth were performed. For *M. edulis*, the number of spat settling at the surface (depth 0 m) was significantly greater than those settling at any other depth except 2 m. All collections taken above 6 m were significantly greater than those settling below 6 m, where the thermocline was positioned (Table 3). The mean values of the transformed data are illustrated in Figure 1 for each depth. The general pattern is seen quite clearly. These results are mirrored in the depth settlement distribution of *M. trossulus*, however, the significance of the pattern is not as strong (Table 4, Fig. 2). In this species the surface samples (0 m) had significantly more spat than any other depth, whereas the shallower depths generally had significantly more spat than those below the 6 m thermocline.

Analyses of Spat Settlement, No Thermocline

The transformed data produced a distribution that was not significantly different from normal (Shapiro-Wilk *W* test, P = 0.873). The variances were homogeneous within depth and species according to Levene's test (P = 0.06 and 0.098, respectively). Normal *Q–Q* plots of the residuals indicated that the residuals were normally distributed. The results of this analysis are summarized in Table 5. Once again, there is a significant effect of depth, but no difference between the species. The interaction term is also significant and appears to be influenced by the differences in the means of each species below 6 m (see Figs. 3 and 4).

Post-hoc analyses were performed on the data. *M. edulis* had large percentages of spat setting at the surface and also on the deepest collectors (8 m). This suggests that the thermocline was a barrier to settlement (Table 6, Fig. 3), because there was low settlement below 6 m when the thermocline was present, but high numbers when the water was mixed. However, the only significantly different pair of depths was between 0 and 6 m (Table 6), with a greater percentage of spat at the surface. In contrast, *M. trossulus* did not show an increase in settlement with depth when the thermocline was removed. The only significantly different pairs of depths were between 0 and 8 m (Table 7, Fig. 4). This difference between the species at the 8-m depth accounts for the significant interaction term in the ANOVA (Table 5).

CONCLUSIONS

Larval depth distributions of *M. edulis* and *M. trossulus* have been examined with a similar experimental design to that used in our study (MacQuarrie 1995; Freeman & MacQuarrie 1999). Halfway through the larval stage, vertical distributions over time in larvae of both species were similar to each other, with major concentrations found just above the 6 m thermocline, with slightly lesser amounts at the surface, and lower numbers elsewhere. This

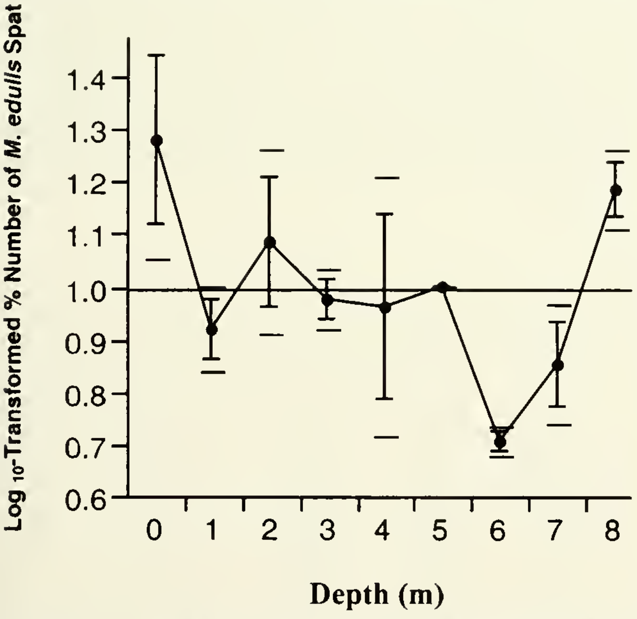

Figure 3. Log_{10}-transformed percent number of *M. edulis* spat. Mean, standard error, and standard deviation are plotted. The sample mean across all samples is illustrated (solid line). No thermocline was present in this experiment.

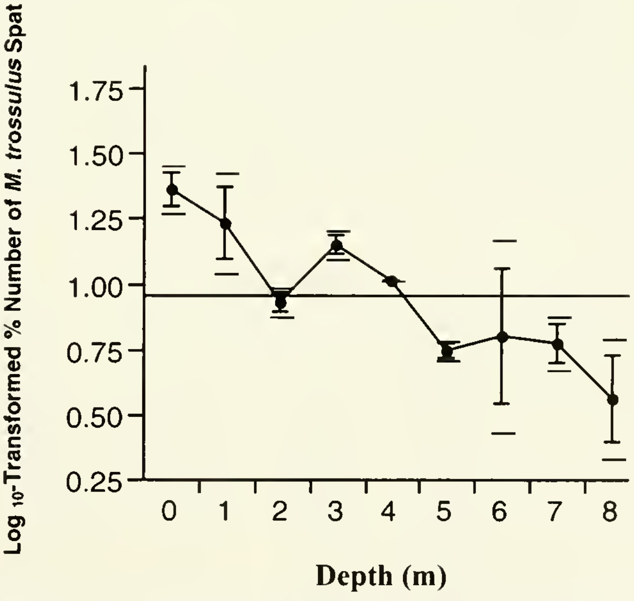

Figure 4. Log_{10}-transformed percent number of *M. trossulus* spat. Mean, standard error, and standard deviation are plotted. The mean across all samples is illustrated (solid line). No thermocline was present in this experiment.

TABLE 6.

M. edulis: Comparisons for all pairs of depths (m) using Tukey-Kramer honestly significant difference (HSD) test.

Depth	0	8	2	5	3	4	1	7	6
0	−0.56	−0.46	−0.36	−0.28	−0.26	−0.24	−0.20	−0.13	**0.02**
8	−0.46	−0.56	−0.46	−0.37	−0.35	−0.34	−0.30	−0.23	−0.08
2	−0.36	−0.46	−0.56	−0.47	−0.45	−0.43	−0.39	−0.32	−0.18
5	−0.28	−0.37	−0.47	−0.56	−0.53	−0.52	−0.48	−0.41	−0.26
3	−0.26	−0.35	−0.45	−0.53	−0.56	−0.54	−0.50	−0.43	−0.29
4	−0.24	−0.34	−0.43	−0.52	−0.54	−0.56	−0.52	−0.45	−0.30
1	−0.20	−0.30	−0.39	−0.48	−0.50	−0.52	−0.56	−0.49	−0.34
7	−0.13	−0.23	−0.32	−0.41	−0.43	−0.45	−0.49	−0.56	−0.41
6	**0.02**	−0.08	−0.18	−0.26	−0.29	−0.30	−0.34	−0.41	−0.56

Positive values in bold show pairs of means that are significantly different at α = 0.05.
No thermocline was present.
Depths are ordered to position significantly different pairs apart.

clustering of larvae at the thermocline was not surprising, because the behavior has been noted in field studies of lamellibranch larvae (e.g., Southward & Barrett 1983; Scrope-Howe & Jones 1986). In addition, *M. trossulus* larvae lack a diel migration (MacQuarrie 1995), although Freeman and MacQuarrie (1999) report a modest (1.6 m) mean diel migration in 11-day-old *M. edulis* larvae with a thermocline present. However, these diel migrations are unlikely to be statistically significant and are in contrast to observations made on scallop larvae (Manuel et al. 1996).

Although thermocline accumulations of larvae decreased as the veligers matured, the settlement patterns, as reported here (Figs. 1–4), are incongruous, because it was expected that a reflection of the earlier attraction to the thermocline would be clearly evident in the distribution of spat which were harvested immediately upon settling. *M. edulis* and *M. trossulus* showed similar patterns of spat distribution with depth in the presence of a thermocline. Spat of both species settled below the thermocline, but at lower numbers than above and the highest numbers were recorded at the surface. In mixed water, *M. edulis* showed a strong preference for settlement at depth (8 m), a feature not seen in *M. trossulus*, although both had high numbers settling at the surface. Reasons for these different patterns are currently speculative, but a recognized weakness in the experiment was that we did not obtain genetic data to support the status of broodstock used. This leaves open to question whether these laboratory observations might have been affected by species contamination. Nevertheless, in subsequent field experimentation (see Kenchington et al. in this issue), DNA markers on field-collected mussels support the observation of behavioral differences by species seen in the mesocosm work.

The industry custom of deploying spat collectors at or near the surface suggests that yields are better there, as was observed in this study. The high degree of similarity in the distribution of the spat of both species augurs little hope for growers wishing to preferentially select *M. edulis* over *M. trossulus* at mixed species sites. However, the significant species × depth interaction in the mixed water offers some basis for further hypothesis testing. Furthermore, the complexity of natural conditions may provoke disparities between the species that are not seen under the controlled conditions of the tower tank.

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TABLE 7.

M. trossulus: Comparisons for all pairs of depths (m) using Tukey-Kramer honestly significant difference (HSD) test.

Depth	0	1	3	4	2	6	7	5	8
0	−0.68	−0.55	−0.47	−0.33	−0.25	−0.11	−0.09	−0.06	**0.12**
1	−0.55	−0.68	−0.60	−0.46	−0.38	−0.24	−0.22	−0.19	−0.01
3	−0.47	−0.60	−0.68	−0.54	−0.46	−0.32	−0.30	−0.27	−0.09
4	−0.33	−0.46	−0.54	−0.68	−0.60	−0.46	−0.44	−0.41	−0.23
2	−0.25	−0.38	−0.46	−0.60	−0.68	−0.55	−0.52	−0.50	−0.31
6	−0.11	−0.24	−0.32	−0.46	−0.55	−0.68	−0.66	−0.63	−0.45
7	−0.09	−0.22	−0.30	−0.44	−0.52	−0.66	−0.68	−0.66	−0.47
5	−0.06	−0.19	−0.27	−0.41	−0.50	−0.63	−0.66	−0.68	−0.50
8	**0.12**	−0.01	−0.09	−0.23	−0.31	−0.45	−0.47	−0.50	−0.68

Positive values in bold show pairs of means that are significantly different at α = 0.05.
No thermocline was present in this experiment.
Depths are ordered to position significantly different pairs apart.

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COMPARATIVE SETTLEMENT DEPTHS OF *MYTILUS EDULIS* C. LINNAEUS, 1758 AND *M. TROSSULUS* GOULD, 1850: II. FIELD OBSERVATIONS

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ABSTRACT Controlled mesocosm experiments using larvae of *Mytilus edulis* and *M. trossulus* have shown that the two species have different settlement patterns with respect to depth, and that the presence of a thermocline can modify the depth distribution. A field program was conducted over 3 y at one site, and at two sites during the final year to determine the settlement patterns of these species in the wild. In all analyses, there was a greater percentage of *M. edulis* postlarvae settling at 5 m than of *M. trossulus*. In addition to opening up certain ecological questions, these results show promise for direct application to husbandry practices at mussel farms having both species, where preferential collection of the *M. edulis* is desirable.

KEY WORDS: *Mytilus edulis, M. trossulus,* settlement, depth, annual variability, spatial variation

INTRODUCTION

The relative depth at which *Mytilus edulis* C. Linnaeus, 1758 and *M. trossulus* Gould, 1850 settle is of direct concern to aquaculturists in Atlantic Canada, and elsewhere, most of whom depend upon the collection of spat from the wild. Although similar in morphology, the lower yield and propensity for shell breakage observed in *M. trossulus* can cause significant production losses on mixed species farms (Mallet & Carver 1995).

The possibility of using ecological information to preferentially collect *M. edulis* postlarvae over *M. trossulus* has been explored (e.g., Freeman et al. 1994). Freeman et al. (2002) have shown that in experiments conducted an indoor tank at Dalhousie University (Halifax, Nova Scotia, Canada), the two species have different depth preferences for settlement. Both species had high percentages of postlarvae settling at the surface, however, in the absence of a thermocline, *M. edulis* showed a strong preference for settling at 8 m depth, which was not seen in *M. trossulus*. Should these results be confirmed in the field, they offer a practical solution to growers who have traditionally deployed surface collectors.

In designing our experiment, we were particularly concerned with identifying patterns that were stable in time and space in order to generate advice to the mussel industry. Although our field experiments were limited in many ways, we were able to examine postlarval settlement patterns in *M. edulis* and *M. trossulus* with depth through the spawning season, across 3 y at one site, and between sites in 1 y.

MATERIALS AND METHODS

Study Sites

Two study sites were chosen to examine temporal and spatial variability in the depth preferences of the mussel spat. One site was located in St. Margarets Bay and the other in Ship Harbour, Nova Scotia (Fig. 1). Both sites were associated with mussel farms that collect, as part of their operation, wild spat for culture, and were known to have both *M. edulis* and *M. trossulus*. Although both sites were located in coastal inlets, the oceanography of the two sites is quite different.

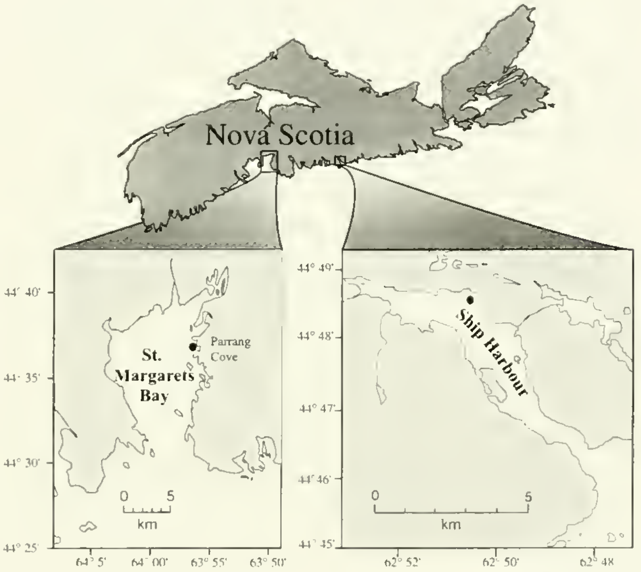

Figure 1. Location of sampling sites at Parrang Cove in St. Margarets Bay and at Ship Harbour, Nova Scotia.

Parrang Cove, St. Margarets Bay

St. Margarets Bay is situated approximately 40 km west of Halifax, Nova Scotia. Our study site was located near the head of the bay at Parrang Cove (44°37′N, 65°56′W) on the eastern shoreline (Fig. 1). St. Margarets Bay is one of the larger bays along the Nova Scotian Atlantic coast and has an axis length of 23.7 km, a surface area of 138.0 km^2, a total volume of 5191 × 10^6 m^3, and an opening of the sea that is ~5 km wide. The bay is subjected to freshwater input from a watershed of 819.1 km^2 (Gregory et al. 1993). In addition to direct runoff, freshwater inputs come from five rivers and the tidal/freshwater volume ratio is 416.32 (Gregory et al. 1993). There is a large, deepwater basin of 80 m maximum depth, and there is a sill at a depth of 35 m (Platt et al. 1972).

As with all coastal inlets in Nova Scotia, the water in winter is well mixed. A thermocline establishes in late spring or early summer between 4 m and 10 m, but in summer the warm surface layer may extend to 30–36 m, depending on direction and strength of winds (Platt et al. 1972). The thermocline can be destroyed through sustained SE or NW winds and can also be pushed offshore by the summer southwesterlies resulting in a cold upwelled nearshore area.

Ship Harbour

Located 90 km east of Halifax, the much smaller Ship Harbour has an axis length of 8.3 km, a surface area of 6.6 km^2, a total volume of 47×10^6 m^3, and an opening to the sea at the mouth of 0.6 km. It is subjected to freshwater input from a watershed of 444.7 km^2 (Gregory et al. 1993), including two rivers and two brooks. The tidal/freshwater volume ratio is 23.87 (Gregory et al. 1993). There is a small, deepwater basin of 27 m and an extensive sill at 7 m.

As with other inlets along the coast, in Ship Harbour the water column turns over in the fall, with a thermocline reestablishing according to wind direction and velocity, the next spring. Thermoclines are strongest in the summer months, establishing around 5 m (Strain 2002). Within Ship Harbour, the study site was located near the head of the harbour at 44°48.65′N, 62°50.53′W, along the northeastern shoreline (Fig. 1).

Sampling Design

Mussel growers at each site reported settlement beginning in mid- to late July and continuing through August and into September. Settlement very late in the year occasionally occurs (December), and may be attributed to either a second fall spawning or to a protracted spawning period. In the first year of sampling (1998), replicate collector ropes were deployed at Parrang Cove on July 24. These were removed on July 31 and replaced with a new pair of collectors. Spat collection continued through the spawning season (Table 1). The schedule for the deployment and retrieval of collectors is given in Table 1. Sampling continued at Parrang Cove at a reduced frequency in 1999 and 2000, according to the schedule in Table 2. In 2000, replicate collectors were also placed at Ship Harbour in order to examine geographic variation in the observed setting pattern. These were deployed and retrieved on the same dates as the Parrang Cove collectors (Table 2).

During 1998, a small experiment was run to determine whether the active postlarval mussels would move along the collector rope after settlement. Two collectors were left in the water from July 24 until October 16 (85 days) at Parrang Cove, in order to compare the depth distribution of older mussels with that of newly settled spat.

Spat Collection

Spat were collected using 5-m lengths of 13-mm-diameter yellow polypropylene rope, weighted at the lower end and suspended at each site from buoyed, surface headropes. Prior to deployment, all collectors were soaked for 2 wk in running seawater to remove any chemicals that might inhibit settlement.

As collectors were retrieved, they were cut into 1-m lengths and placed in polyethylene bags and sealed. Care was taken to avoid cross-contamination of the sample segments. In the laboratory, spat were preserved by adding 85% ethanol to each bag. Subsequently, each 1-m rope portion was removed and the attached spat were separated from the rope by a gentle brushing, and then placed in vials with 80% ethanol. Although mussels from all five 1-m lengths were saved, only those from 0–1, 2–3, and 4–5 m were analyzed (hereafter referred to as 1, 3, and 5 m), with one exception. All five 1-m rope segments were analyzed from the two collectors which were deployed at Parrang Cove on July 24, 1998, and left in the water until the fall. The number of spat at 1, 3, and 5 m were recorded for two time periods at each of two sites in 2000.

TABLE 1.

Deployment and retrieval dates of mussel spat collectors at Parrang Cove, Nova Scotia in 1998.

Time	Deployment Date	Retrieval Date
1	July 24	July 31
2	July 31	August 7
3	August 7	August 21
4	September 4	September 18
5	September 18	October 2
6	October 2	October 16

TABLE 2.

Deployment and retrieval dates of mussel spat collectors at the two experimental sites grouped according to three time periods used in the ANOVAs.

Time	Location	Year	Deployment Date	Retrieval Date
1	Parrang Cove	1998	July 24	July 31
1		1999	July 14	August 11
1		2000	July 18	August 15
1	Ship Harbour	2000	July 19	August 15
2	Parrang Cove	1998	August 7	August 21
2		1999	August 11	September 8
2		2000	August 15	September 12
2	Ship Harbour	2000	August 15	September 12
3	Parrang Cove	1998	September 4	September 18
3		1999	September 8	October 6
3		2000	September 12	October 10
3	Ship Harbour	2000	September 12	October 10

Species Determination

Approximately 30 individual spat from each sample depth replicate/time/site were subsequently decanted and examined under the microscope to ensure that the shell contained tissue. In some cases, there were too few animals to meet the sample size objective, in which case all available animals were sampled. For the two collectors left in the water for 85 days, only animals greater than 9 mm were analyzed in order to ensure sampling of animals from the earliest settlement period.

Individuals were then separately picked, crushed, and rinsed with high TE (1 M Tris 0.5 M ethylenediaminetetraacetic acid [EDTA], pH 8) and ddH$_2$O three subsequent times (Heath et al. 1995). Tissue was resuspended directly in a 20.4-μL extraction solution (19 μL H$_2$O, 1 μL 10 × SFG buffer, 0.4 μL proteinase K) and digested overnight at 37°C. The reaction was stopped by boiling samples for 10 min before performing polymerase chain reaction (PCR) amplification. The internal transcribed spacer (ITS) region of the nuclear DNA was used as a species-specific marker (Heath et al. 1995). ITS primers were purchased from Operon Technologies, Inc. Twenty-two microliters of reaction mixture containing 1 × PCR buffer, 2 mM MgCl$_2$, 0.5 μM of each primer, 0.2 mM dNTP, and 1.0 U Taq polymerase were added to 2 μL of DNA solution. The thermal cycling protocol was used as described in Heath et al. (1995). PCR-amplified ITS fragments (approximately 1250 bp) were verified by running 8 μL of the amplification product on 1% agarose gels. Successfully amplified ITS fragments were digested with the endonuclease *HhaI* (MBI Fermentas). Ten microliters of digestion mixture containing 10× enzyme buffer and 5.0 U of *HhaI* was added to 15 μl of amplification product. The *Mytilus* species-specific restriction fragment length polymorphisms (RFLPs) were then visualized on 2% agarose gels and samples were identified as *M. edulis*, *M. trossulus*, or hybrid.

Statistical Analyses

The percentage of *M. edulis* spat was determined for each of the rope segments. Hybrids were rare and were not included in the percentage calculations. Arcsin-transformed data were tested for normality with the Kolmogorov-Smirnov (K-S) test. Levene's test was used to test the homogeneity of the error variance. Normal *Q–Q* plots of the residuals were visually inspected for deviations. Transformed data were subject to analysis of variance (ANOVA).

A full factorial ANOVA was calculated from the 1998 data series collected at Parrang Cove (Table 1) with two factors, Time (six consecutive sampling periods) and Depth (1, 3, and 5 m), using a type III sums of squares. For the two collectors which were left in the water from July 24 through to October 16, a one-way ANOVA with Depth as a factor (1, 2, 3, 4, and 5 m) was performed. The results of these analyses inspired the subsequent field program to determine the temporal and spatial stability of our observations.

To determine whether there were annual variations in the species-specific settlement patterns of *Mytilus*, a three-factor, full-factorial ANOVA was calculated using type III sums of squares. Specifically, the data collected from Parrang Cove (Table 2) were analyzed to examine whether the percentage of *M. edulis* spat varied in response to Year of collection (1998, 1999, 2000), Time of collection during the spawning season (three periods of collection) and/or depth (1, 3, and 5 m). Including the 1998 data created a lack of independence between this analysis and the previous ANOVA; however, excluding the 1998 results did not change the significance of the terms and so the full analysis is presented here for its greater interpretive value.

A similar three-way ANOVA was calculated to examine whether the percentage of *M. edulis* spat varied in response to the Site of collection (i.e., Parrang Cove or Ship Harbour), Time during the season, and Depth (Table 2). Data for this analysis were collected in only 1 y, 2000.

All interaction terms and main effects were tested, however, the three-way interaction term in the last two analyses was nonsignificant and so this term was removed from the equation and the analyses were rerun, testing only main and two-way effects. Tukey-Kramer honestly significant difference (HSD) pairwise multiple comparison tests between each pair of means were performed in post-hoc analyses of all ANOVA results with a significance level of 0.05.

RESULTS

The 1998 data from Parrang Cove were analyzed prior to the 1999 field season in order to determine the design of subsequent fieldwork. The 1998 field season was designed to test the hypothesis, with higher power, that there was no significant difference in the percentage of *M. edulis* spat settling at different depths.

The arcsin-transformed percentage of *M. edulis* spat data distribution was not significantly different from a normal distribution (K-S $Z = 0.628$; $P = 0.825$). The variances were homogeneous and the residual plots supported a random distribution of the error term. The two-way ANOVA model explained 71% of the variance in the data set (adjusted $R^2 = 0.711$; $F = 5.92$; $P = 0.00$). There was no significant interaction between Depth and Time, however, the factors Depth and Time of collection were both highly significant (Table 3).

Post-hoc analyses of the data using Tukey's HSD multiple comparisons test revealed highly significant differences between the transformed percentage of *M. edulis* spat settling at 1 m depth and the percentage settling at 3 and 5 m ($P = 0.004$ and 0.000, respectively). There was no significant difference between the percentage of *M. edulis* spat settling at 3 and 5 m ($P = 0.102$). Examination of the mean values for each depth shows that there are significantly fewer *M. edulis* at 1 m, or conversely, that there are more *M. trossulus*. The nonsignificant interaction term indicates that this pattern did not change during the course of the setting season.

The percentage of *M. edulis* spat settling also showed a significant difference with Time (Table 3). The post-hoc analyses (Table 4) did not have any clear pattern in these differences other than period 4 (deployed September 4th and retrieved on the 18th) which had a significantly lower percentage of *M. edulis* across all depths than most other sampling periods (except period 2). Although no pattern of species-specific spat settlement was known to exist during the setting season, a significant Time factor causing higher percentages of one species over the other through the season can be explained by events such as differential spawning time, larval periods, and/or current changes.

Did mussel spat change their position along the ropes when allowed to stay in the water for longer periods? As in the above analysis, depth was a significant factor in the one-way ANOVA ($F = 11.95$; $P = 0.009$; power 0.93). Post-hoc analyses using Tukey's HSD test indicated that there was no significant difference ($\alpha = 0.05$) between the percentage of *M. edulis* at 1, 2, 3, or 4 m, but that significantly more *M. edulis* were found at 5 m than at these depths ($P = 0.032$, 0.008, 0.14, and 0.47, respectively). These animals were all over 9 mm in length, indicating that they had settled on the collectors early in the season. Therefore, the preference of *M. edulis* to settle at 5 m depth as observed in the newly settled spat, persists through 85 days to at least 9 mm shell length.

Annual Variation in Setting Pattern

The results from the 1998 program indicated that in the field, *M. edulis* prefers to settle at 3 and 5 m depth, while *M. trossulus* is dominant near the surface to 1 m. However, we felt that such results could not be generalized until the annual stability of the

TABLE 3.

Two-way ANOVA of the arcsin-transformed percentage of *M. edulis* spat settling at Parrang Cove, Nova Scotia, by time (six periods during set) and depth (1, 3, 5 m) with two-way interactions.

Factor	df	SS	*F*	*P*	Observed Power[a]
Time	5	1.93	8.45	0.00	1.00
Depth	2	1.64	18.02	0.00	1.00
Time × Depth	10	1.01	2.21	0.72	0.72
Residual	17	0.775			

[a] Computed at $\alpha = 0.05$.

TABLE 4.

Significant (α = 0.05) Tukey's HSD multiple comparisons test in a post-hoc analysis of the transformed mean percentage of *M. edulis* spat setting at six different periods at Parrang Cove, Nova Scotia, during the summer of 1998 (see Table 1 for dates).

(I) Set Time	(J) Set Time	Probability
1	4	0.034
2	5	0.003
	6	0.008
3	4	0.045
4	1	0.034
	3	0.045
	5	0.002
	6	0.004
5	2	0.003
	4	0.002
6	2	0.008
	4	0.004

pattern could be determined. Annual differences in the percentage of *M. edulis* spat setting at depth during three time periods in each of 3 y were examined.

The three-factor ANOVA model explained 70% of the variance in the data set (adjusted R^2 = 0.702; F = 7.66; P = 0.00) and identified a significant interaction between Year of collection and Time of collection during the spawning season (Table 5). When the adjusted mean values of the transformed data are plotted (Fig. 2), it can be seen that this interaction effect is due to a much higher percentage of *M. edulis* sampled in 1998 during the first collection period (July 14–August 15) than in a similar time period in 1999 and 2000. The second time period (sampled between August 7 and September 12) showed relatively high percentages of *M. edulis* each year, whereas in the third time period (September 4–October 10) the percentage of *M. edulis* was very low in 1998 but remained high in 1999 and 2000. These results indicate that the percentage of *M. edulis* vs. *M. trossulus* spat varies through the season and is not predicted from year to year.

Depth was the only significant main effect in this analysis (Table 5), but was never significant in an interaction. Figure 3 shows the strong change in the median value of the transformed data with depth. Post-hoc analysis using Tukey's HSD identified significant differences between all pairs of means of each depth, with the greatest percentage of *M. edulis* spat settling at the 5 m depth and the least at 1 m.

TABLE 5.

Three-way ANOVA of the arcsin-transformed percentage of *M. edulis* spat settling at Parrang Cove, Nova Scotia, by year (1998, 1999, 2000), time (three periods during set), and depth (1, 3, 5 m) with two-way interactions.

Factor	df	SS	F	P	Observed Power[a]
Year	2	0.044	0.69	0.51	0.16
Time	2	0.181	2.85	0.07	0.52
Depth	2	2.733	42.94	0.00	1.00
Year × Time	4	1.031	8.10	0.00	0.99
Year × Depth	4	0.145	1.14	0.35	0.32
Time × Depth	4	0.254	1.99	0.12	0.54
Residual	33	1.050			

[a] Computed at α = 0.05.

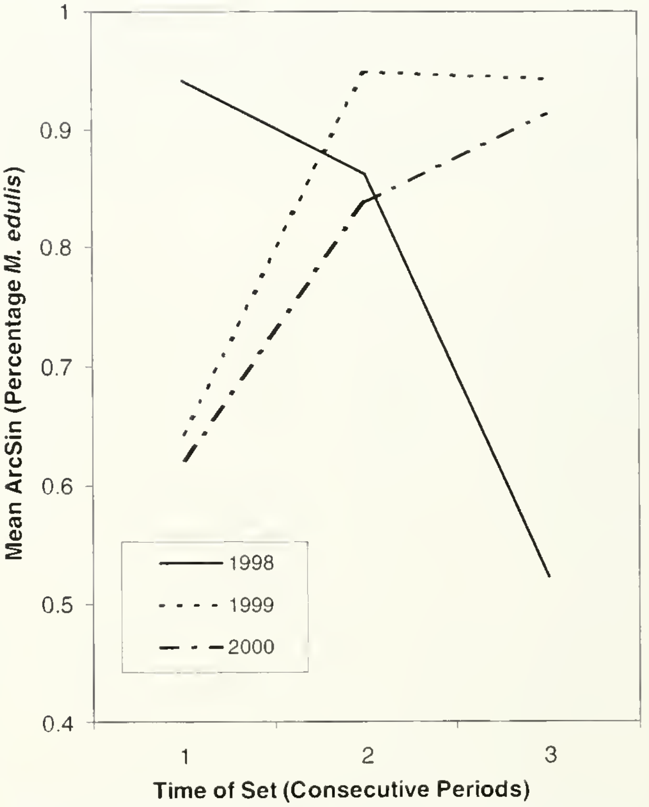

Figure 2. The interaction between year (1998, 1999, and 2000) with sampling period in the transformed adjusted mean percentage of *M. edulis* spat collected at Parrang Cove, St. Margarets Bay, Nova Scotia.

Geographic Differences

The above-described analyses confirmed that the observations first documented at Parrang Cove in 1998 were repeated in 1999 and 2000, with a greater percentage of *M. edulis* occurring at depth (5 m) and a greater percentage of *M. trossulus* occurring at the surface (1 m). The final analysis was designed to determine whether this pattern was particular to Parrang Cove, or whether it also occurred at other locations.

The three-factor ANOVA model explained 66% of the variance in the data set (adjusted R^2 = 0.660; F = 6.23; P = 0.00) and identified a significant interaction between the Site of collection and Time of collection during the season (Table 6). In all cases, there is a greater percentage of *M. edulis* spat at Parrang Cove than at Ship Harbour. However, in the first sampling period and in the last (third) sampling period, the relative difference between the sites is the same, whereas in the second period there are fewer *M. edulis* postlarvae (and therefore more *M. trossulus*) at Ship Harbour relative to Parrang Cove (Fig. 4).

Unlike the previous ANOVA, all three main effects were significant. The boxplots of the median values by depth and site are illustrated in Figure 5. As in the previous analysis, there is sig-

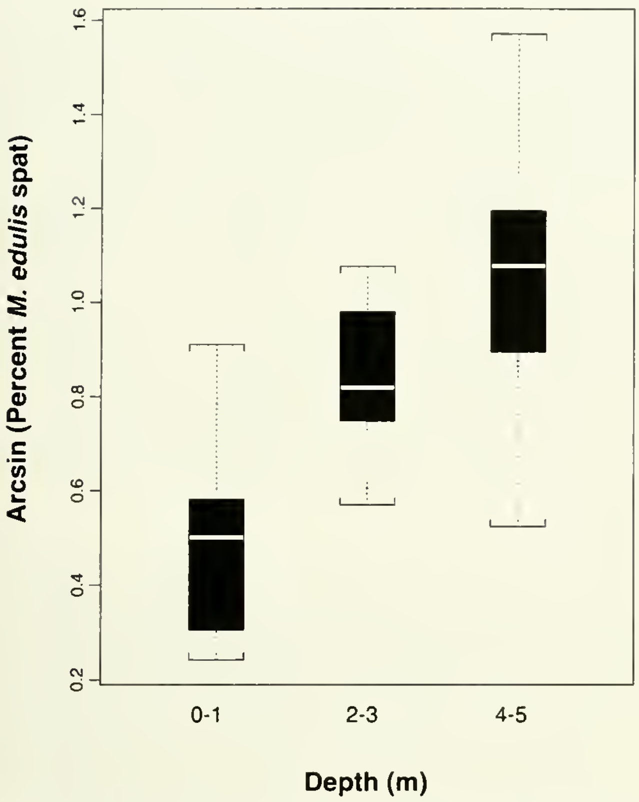

Figure 3. Boxplots (median bar, upper and lower quartiles, and outer fences marking extent of data beyond the quartiles) of the arcsin-transformed percentage of *M. edulis* spat settling at Parrang Cove, St. Margarets Bay, Nova Scotia, at three depth intervals.

nificantly more *M. edulis* spat settling at 5 m depth than at the surface meter (Fig. 5). The third time period had the largest percentage of *M. edulis* spat across all sites and depths. Therefore, as in the previous analysis, depth is an important factor in determining the percentage of *M. edulis* spat, regardless of site or time period, or year of collection.

The total numbers of postlarvae at the 1, 3 and 5 m depth intervals from a single collector rope from each site on two dates are provided in Table 7 in order to place the percentage data in perspective. As expected the total number of mussels collected was highly variable and ranged from 134 to over 10,000 per metre. The experimental design was not adequate to statistically analyze these data and so additional counts were not completed. However, it can be seen that large numbers of postlarvae did settle below the surface and that the total numbers of *M. edulis* collected at depth was consistently higher than the number setting at the surface (Table 7). *M. trossulus* was more variable being present in the highest numbers at the surface in two collections, at 5 m in one and

TABLE 6.

Three-way ANOVA of the arcsin-transformed percentage of *M. edulis* spat settling in 2000 by site (Parrang Cove, Ship Harbour), time (three periods during set), and depth (1, 3, 5 m) with two-way interactions.

Factor	df	SS	*F*	*P*	Observed Power[a]
Site	1	1.559	28.56	0.00	0.99
Time	2	0.747	6.84	0.01	0.88
Depth	2	1.199	10.98	0.00	0.98
Site × Time	2	0.434	3.98	0.03	0.65
Site × Depth	2	0.225	2.06	0.15	0.38
Time × Depth	4	0.256	1.17	0.35	0.31
Residual	36	17.853			

[a] Computed at $\alpha = 0.05$.

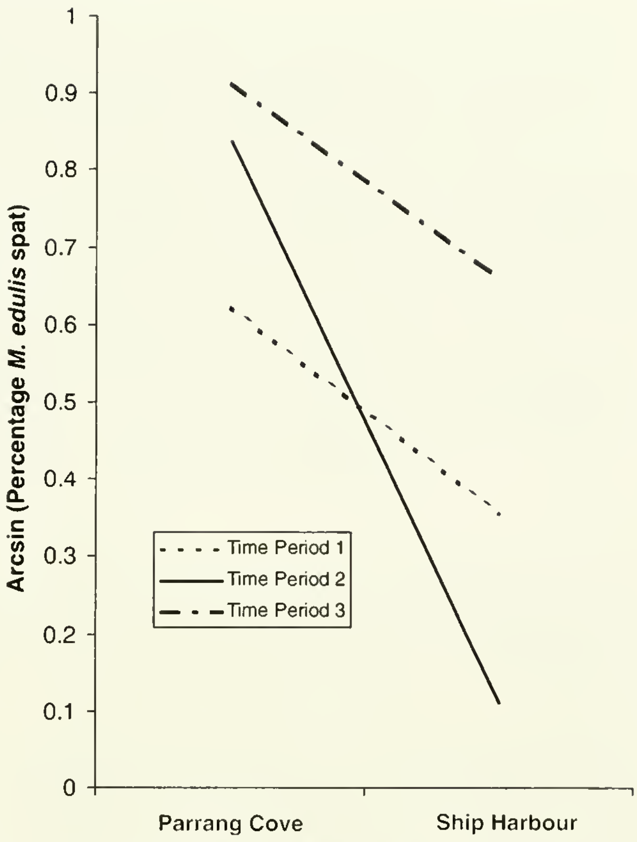

Figure 4. The interaction between site with sampling period in the transformed adjusted mean percentage of *M. edulis* spat collected at the two locations in Nova Scotia in 2000.

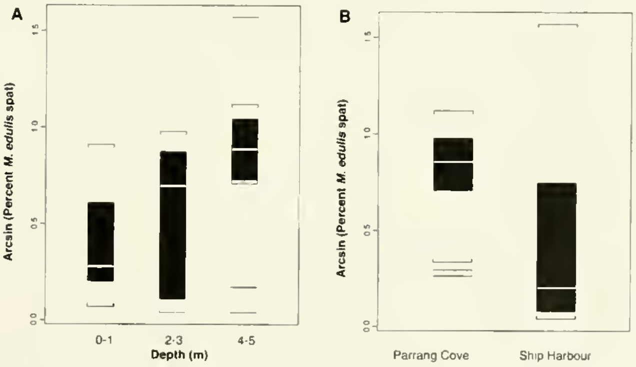

Figure 5. Boxplots (median bar, upper and lower quartiles, and outer fences marking extent of data beyond the quartiles) of the arcsin-transformed percentage of *M. edulis* spat sampled in 2000 at three depth intervals (A), and at two sites in Nova Scotia, Canada (B).

TABLE 7.

The absolute number of mussel postlarvae per 1 m rope at 1, 3 and 5 m depths collected at Parrange Cove and Sheet Harbour, Nova Scotia at two sampling dates in 2000. The percentage of mussels identified as *M. edulis* using DNA markers is indicated, as well as the estimated numbers of *M. edulis* ($N_{M\ edulis}$) and *M. trossulus* ($N_{M\ trossulus}$) postlarvae.

Date	Location	Depth (m)	N	% *M. edulis*	$N_{(M.\ edulis)}$	$N_{(M.\ trossulus)}$
Aug. 15, 2000	Parrang Cove	1	476	31.3	149	327
		3	184	61.9	114	70
		5	367	60.7	223	144
	Ship Harbour	1	569	6.7	38	531
		3	831	10.7	89	742
		5	6560	65.5	4297	2263
Oct. 10, 2000	Parrang Cove	1	6030	71.9	4336	1694
		3	10750	68.8	7396	3354
		5	7470	80.0	5976	1494
	Ship Harbour	1	741	15.2	113	628
		3	134	64.7	87	47
		5	140	100.0	140	0

homogenously distributed through the depth range in the fourth sample (Table 7).

Hybrids

Of the 2485 animals scored using the DNA technique, 8.4% (208) were hybrids. The hybrids were not present in sufficiently high numbers to permit a robust analysis of their distribution. However, hybrids were relatively abundant in the October 2 replicates sampled at Parrang Cove in 1998 (Table 1), with the percentages ranging from 5% to 58%. In these two samples, the greatest percentage of hybrids was at the surface.

DISCUSSION

The mixing of seawater due to tides, winds, currents, and upwelling, and the seasonal establishment of thermoclines, characterize inshore marine waters around Eastern Canada and in similar temperate waters elsewhere in the world where *Mytilus* is found. The larval period is therefore one of great uncertainty, where the dynamic environment must be contended with while simultaneously searching for food and avoiding predators. Under these conditions, it is remarkable that we were able to observe a consistently and significantly higher percentage of *M. edulis* postlarvae at 5 m depth. This depth preference observed in the newly settled spat persists through 85 days to at least 9 mm shell length, despite the fact that postlarval mussels are very active and have an ability to repeatedly attach themselves to various substrata prior to choosing a final settlement location (e.g., Bayne 1964; Dare & Davies 1975). Further, the total number of *M. edulis* collected at depth was greater than at the surface, to the extent that this was examined.

Pineda (2000) identified three processes affecting larval settlement in invertebrates: (1) processes influencing the larval pool; (2) physical transport; and (3) microhydrodynamics, substrate availability, and behavior. Larger-scale processes governing physical transport, and coupled to the onset and duration of spawning, likely explain the variation in the relative percentage of each species through the spawning season, across years, and between sites, observed here and elsewhere (e.g., Seed & Suchanek 1992). However, the significant difference in postlarval depth distribution between the species is consistent with a behavioral differences.

Freeman et al. (2002) observed the settlement of *M. edulis* and *M. trossulus* separately in the laboratory, under mixed water conditions and with a thermocline. In all cases the greatest percentage of postlarvae settled at the surface, however, under mixed water conditions the postlarvae of *M. edulis* also had a large settlement at 8 m, a pattern not seen in *M. trossulus*. These data support an innate behavior as opposed to a response created through interspecific competition (e.g., Petersen 1984). Furthermore, the limited data that we obtained on hybrids identified a settlement pattern similar to *M. trossulus*. We were unable to determine which species dammed these hybrids, however, the possibility of maternal inheritance of setting depth is intriguing.

Mussel larvae are known to show behavioral responses to light, gravity, pressure, and substratum (e.g., Bayne 1976; Seed & Suchanek 1992; Young 1995). To cite only a few examples: competent larvae of *M. edulis* migrate to deeper water in the North Sea (Bayne 1976) and prefer to settle subtidally below 3 m in the Wadden Sea (Pulfrich & Ruth 1993), although Dobretsov and Miron (2001) observed the opposite in the White Sea, with pediveligers migrating toward the surface to set primarily at 1.5 m. *M. galloprovincialis* Lamark, 1819 is more homogeneously distributed with depth than *M. edulis* and *M. trossulus* (as observed in this study). However *M. galloprovincialis* shows greater settlement at 5 and 10 m depth in northwest Spain (Caceres-Martinez & Figueras 1998), reflecting the pattern seen in *M. edulis*. In addition, variation in settlement depth preference of mytilid species can be influenced by the presence of phytoplankton and predators. With the literature confounded by difficulties with species identification (cf. Johannesson et al. 1990), it is premature to determine whether the observations reported here are representative of the two species.

Miron et al. (1999) have suggested that competent barnacle larvae position themselves at a depth in the water column corresponding to the position where they might settle on shore. If this were true for mussels, then photonegative and geopositive behavior would ensure a greater depth with less exposure when setting on a fixed substrate (e.g., shore, pilings). Conversely, the photopositive and geonegative behavior hypothesized for *M. trossulus* would ensure a distribution above mean low water spring tide (MLWS), with greater exposure to desiccation, freshwater, and temperature fluctuations associated with life in the upper intertidal environment. This tolerance is consistent with the more euryhaline nature of *M. trossulus*.

For aquaculturists, positioning mussel collectors on the bottom or at 5 m depth would ensure the preferential collection of *M. edulis* over *M. trossulus*, at least in Nova Scotian waters. This is in contrast to the current practice of deploying plastic mesh on headropes near the surface for several days before the anticipated onset of settlement, a practice which may favor the collection of *M. trossulus* and increase the relative proportion of this species on the lease sites over time. The absolute numbers of spat were not recorded for all collectors and density is expected to be much more variable because of large-scale processes and microhydrodynamic conditions. However, in the collectors examined, placing the collectors below the surface rarely resulted in reduced settlement, although the total number of post larvae was highly variable with respect to depth of collection. Nevertheless, we encourage mussel growers to experiment with subsurface and bottom deployment of collectors at sites where both *M. edulis* and *M. trossulus* occur.

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ROUTES OF INTRODUCTION OF THE MEDITERRANEAN MUSSEL (*MYTILUS GALLOPROVINCIALIS*) TO PUGET SOUND AND HOOD CANAL

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ABSTRACT To test alternative routes of introduction we describe the distribution of *Mytilus galloprovincialis*, *M. trossulus*, and their hybrids in Puget Sound and Hood Canal. Native mussels, *Mytilus trossulus*, dominate the blue mussel communities of Puget Sound and Hood Canal; at most sites *M. galloprovincialis* alleles were rare or absent. *M. galloprovincialis* alleles were present but uncommon (~5%) in mussel populations in the southern portion of Puget Sound, and were nearly absent in populations in the northern Puget Sound and the Hood Canal. The only locations where *M. galloprovincialis* alleles are locally common are sites where they are likely to have been repeatedly introduced. These include sites near mussel farming operations and near the Port of Seattle and Bremerton Naval Shipyards. The results indicate that both aquaculture and shipping activities have been responsible for the repeated introduction of *M. galloprovincialis* to Puget Sound. Although hybridization between *M. galloprovincialis* and *M. trossulus* occurs, there was little evidence for advanced introgression between these two species.

KEY WORDS: introductions, mussels, *Mytilus galloprovincialis*

INTRODUCTION

The invasion of nonindigenous species has become of ecological and economic concern in the last few decades (Schmitz & Simberloff 1997). The effects of species introduction range from localized and limited to those that severely degrade native ecosystems and have immense economic consequences (Geller et al. 1994; Ruiz et al. 1997; Ruiz et al. 2000). The unpredictable ecological consequence of introducing nonnative species makes such introductions generally undesirable.

Global commerce is responsible for the majority of both intentional and unintentional species introductions. In marine systems a major route of species introduction has been through the transport and discharge of ballast water from ships (Geller et al. 1994; Ruiz et al. 1997; Ruiz et al. 2000). For example, Carlton and Geller (1993) found that the ballast water from 159 cargo ships in Coos Bay, Oregon, contained a minimum of 367 marine species. A second, potentially major, source of species introductions is aquaculture endeavors, particularly farms and fisheries. The decline of traditional fisheries has lead to a rapid increase in aquaculture as a source of aquatic products (Johnson 1998); presently 20% of the world's seafood comes from aquaculture. The commercial culture of aquatic organisms has led to the intentional and accidental introduction of nonnative species in many parts of the world (Bartley & Subasinghe 1996).

One marine species that has been both intentionally and accidentally introduced by these activities is the mussel *Mytilus galloprovincialis*. This mussel has been unintentionally introduced to South Africa (Grant & Cherry 1985), the Sea of Japan (Wilkins et al. 1983), and California (McDonald & Koehn 1988). Larvae of *M. galloprovincialis* are commonly found in ballast water of transport ships (Geller et al. 1994), making unintentional introductions likely. *M. galloprovincialis* is also the subject of large aquaculture farms in Europe and has been intentionally introduced to Puget Sound and the Georgia Straits as part of culture efforts in the northeastern Pacific (Heath et al. 1995).

Mytilus galloprovincialis is a member of a complex containing two other species of blue mussel, *M. edulis* and *M. trossulus*. *M. galloprovincialis* is native to the Mediterranean and the Atlantic coast of southern Europe. *M. edulis* is native to temperate Atlantic waters of Europe and North America, and *M. trossulus* is native to cold-temperate waters of the northern Pacific and Atlantic (Gosling 1992). *M. galloprovincialis* is the most warm-adapted of the three species (Hilbish et al. 1994), which perhaps contributes to its propensity for introduction. All members of the blue mussel species complex will hybridize in nature. In particular, extensive hybridization between *M. galloprovincialis* and *M. trossulus* occurs in California (Rawson et al. 1999) and between *M. galloprovincialis* and *M. edulis* in Europe (Skibinski et al. 1983). The capacity to interbreed with congeneric species expands the possible consequences of introducing non-native species; they may genetically contaminate native species as well as ecologically displace them. The fate of hybrids, however, is uncertain. In Europe hybridization between *M. galloprovincialis* and *M. edulis* is extensive resulting in high levels of introgression between the species (Quesada et al. 1998; Rawson & Hilbish 1998). In contrast, hybridization between *M. galloprovincialis* and *M. trossulus* is prevalent, but results in very limited introgression (Rawson et al. 1999).

This study describes the distribution of *Mytilus galloprovincialis*, *M. trossulus*, and their hybrids in Puget Sound. *M. galloprovincialis* has been intentionally introduced to Puget Sound through aquaculture efforts, but also may have been repeatedly introduced via shipping activities. If *M. galloprovincialis* is present in Puget Sound as a result of shipping activities, we expect high concentrations of *M. galloprovincialis* alleles to be present around major shipping ports, particularly in the vicinity of Seattle. Alternatively, if aquaculture activities are responsible for the presence of *M. galloprovincialis*, we expect to find alleles indicative of this species in the vicinity of culture operations. In this study we also have used molecular markers that distinguish *M. galloprovincialis* and *M. trossulus* to determine whether genetic contamination of native mussels is occurring in Puget Sound and to infer the possible fate of hybrid mussels.

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METHODS AND MATERIALS

Mussels were collected in the summer of 1997 and 1998 from 28 locations in Puget Sound and Juan de Fuca Strait and five locations in the Hood Canal (Fig. 1).

Shell length of each mussel was measured from the anterior to posterior margin to the nearest 0.1 mm. We attempted to analyze both small and large mussels from each location, but this was not always possible because the size structure of the mussels varied widely among sites. We analyzed between 16 and 64 mussels from each location. Each mussel was dissected and a section from the margin of the mantle was removed and preserved in 95% ethanol. Total cellular DNA was extracted from each tissue sample as described by Rawson et al. (1999). The Glu-5′ gene was amplified using the polymerase chain reaction (PCR) as described by Rawson et al. (1996) using the primers of Inoue et al. (1995). PCR amplification of the Glu-5′ marker produces species-specific products which differ in size for *Mytilus edulis*, *M. trossulus*, and *M. galloprovinicialis* (Rawson et al. 1999). A second locus, internal transcribed spacer (ITS), was amplified using the procedures described by Rawson et al. (1999), cut with the restriction enzyme *Sau96*, and run on a 1.5% agarose gel. This produces a species-specific restriction pattern that distinguishes *M. trossulus* from *M. galloprovincialis* (Rawson et al. 1996). Mussels from three locations (Silverdale, Totten Inlet, and Edmonds) were also assayed at a third nuclear locus, Mal-I, according to the protocol given by Rawson et al. (1999).

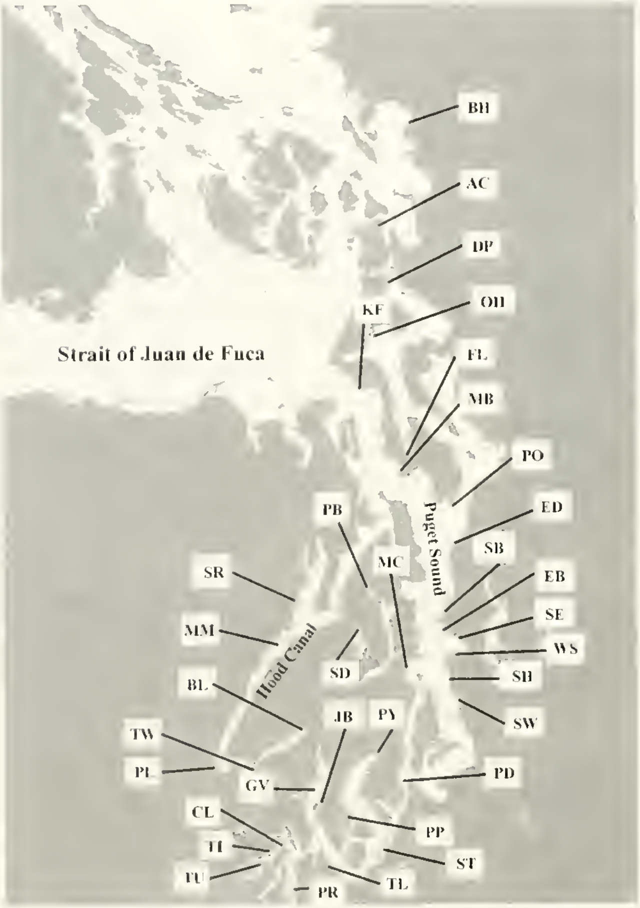

Figure 1. Location of sampling sites in Puget Sound and Strait of Juan de Fuca: Bellingham Bay = BH, Anacortes = AC, Deception Pass Marina= DP, Oak Harbor = OH, Keystone Ferry = KF, Freeland = FL, Mutiny Bay=MB, Possession Point = PO, Edmonds = ED, Poulsbo = PB, Silverdale = SD, Shilshole Bay = SB, Elliot Bay Marina = EB, Seattle Pier 91 = SE, West Seattle = WS, Manchester State Park = MC, Seahurst County Park = SH, Purdy = PY, Saltwater State Park = SW, Point Defiance = PD, Grapeview Marina = GV, Penrose Point State Park = PP, Joemma Beach State Park = JB, Carlyon = CL, Totten Inlet = TI, Taylor United = TU, Steilacoom Marina = ST, Tolmie State Park = TL, and Priest Point = PR. Location of sampling sites in Hood Canal: Seal Rock = SR, Mike's Marina = MM, Potlatch State Park = PL, Twanoh State Park = TW, and Belfair State Park = BL.

RESULTS

The *M. galloprovincialis* allele frequency for the Glu-5′ and ITS genes at each site is presented in Table 1. Alleles specific to *M. galloprovincialis* are not generally very abundant in Puget Sound. Outside of the Totten Inlet (where Taylor United, Inc., cultivates *M. galloprovincialis*) the average frequency of the *M. galloprovincialis* allele was 0.032 for Glu-5′ and 0.019 for ITS (counting only sites for which both genes were scored). The frequency of *M. galloprovincialis* alleles at Glu-5′ and ITS was highly correlated ($r = 0.775$, $P < 0.05$, sites without *M. galloprovincialis* alleles at both loci and the Taylor United site were excluded). In the central and southern Puget Sound, *M. galloprovincialis* alleles are relatively common in three locations. The Totten Inlet contained relatively high frequencies of *M. galloprovincialis* alleles. Mussels farmed by Taylor United in the Totten Inlet contained only *M. galloprovincialis* alleles at both loci. Wild mussels from a floating dock near the middle of the inlet (Totten Inlet) also had relatively high frequencies of *M. galloprovincialis* alleles. Mussels from the Carlyon Marina near the mouth of Totten Inlet, however, had low frequencies on *M. galloprovincialis* alleles, similar to that observed in other mussel populations in the southern Puget Sound (Table 1). Alleles specific to *M. galloprovincialis* were also common at Silverdale and at sites north and south of Seattle (Edmonds, Shilshole Bay, and Seahurst) (Table 1, Fig. 1). Silverdale is near the Bremerton Naval Shipyards, whereas the other three sites are near the Port of Seattle at Elliot Bay. Surprisingly, *M. galloprovincialis* alleles were absent from samples collected within Elliot Bay (Elliot Bay Marina, Seattle Pier 91, and West Seattle) (Table 1). Mussels with *M. galloprovincialis* alleles were absent from samples collected in the Hood Canal and from the northern portion of Puget Sound and Juan de Fuca Strait (Table 1).

To evaluate the extent of introgression between the two species mussels from the three sites with the highest frequency of *Mytilus galloprovincialis* alleles (Silverdale, Totten Inlet, and Edmonds) were assayed at all three genetic markers and pooled into multilocus genotypic classes. Mussels homozygous for *M. trossulus* alleles at all three loci were designated *M. trossulus*. Those homozygous for *M. galloprovincialis* alleles at all three loci were designated *M. galloprovincialis*. Mussels heterozygous for *M. trossulus* and *M. galloprovincialis* alleles at all three loci were designated F1 hybrids. Those homozygous for *M. trossulus* alleles at one locus and *M. galloprovincialis* alleles at another locus were designated F2 hybrids. Of the remaining possible genotypes, those that contained four or five *M. trossulus* alleles were designated as *M. trossulus* back-crosses and those that contained one or two *M. trossulus* alleles were designated *M. galloprovincialis* back-crosses. Sorting individuals into the categories listed above does not necessarily indicate their genealogical origin; genuine F2 and back-cross matings should generate a wide variety of multilocus

TABLE 1.

Allele frequency of Glu-5′ and ITS at each sample site in the Puget Sound and Hood Canal: Sample size (*n*) for each locus and the size range and habitat sampled are also indicated.

			Glu-5′		ITS	
Site	**Habitat**	**Length (mm)**	**Frequency**	***n***	**Frequency**	***n***
Puget Sound						
Bellingham Bay	Intertidal	25–40	0.00	22	0.00	18
Anacortes	Subtidal	>40	0.00	32	0.00	28
Deception Pass Marina	Subtidal	>40	0.00	30	0.00	28
Oak Harbor	Intertidal	35–40	0.00	32	0.00	32
Keystone Ferry	Intertidal	>35	0.00	28	0.00	32
Freeland	Intertidal	>40	0.00	26	0.00	30
Mutiny Bay	Intertidal	15–20	0.00	28	0.00	30
Possession Point	Subtidal	20–25	0.00	28		
Edmonds	Subtidal	>35	0.19	26	0.16	32
Poulsbo	Subtidal	>45	0.00	32	0.00	26
Shilshole Bay	Subtidal	<10 and >35	0.12	68		
Silverdale	Subtidal	5 to >70	0.35	126		
Elliot Bay Marina	Subtidal	<10 and >35	0.00	66		
Seattle Pier 91	Intertidal	25–35	0.00	28	0.00	28
West Seattle	Subtidal	<10	0.00	24		
Manchester State Park	Intertidal	3–35	0.00	48	0.00	20
Seahurst County Park	Intertidal	<5 and >40	0.19	32	0.04	22
Purdy	Intertidal	<10 and >40	0.04	52		
Saltwater State Park	Intertidal	<15 and 25–35	0.04	28	0.04	26
Point Defiance	Intertidal	20–40	0.07	28	0.04	24
Grapeview Marina	Subtidal	<10 and >35	0.09	32		
Penrose Point State Park	Subtidal	5–20 and >40	0.02	60	0.00	22
Joemma Beach State Park	Subtidal	10–40	0.02	58	0.00	32
Carlyon	Subtidal	35–40	0.08	26	0.07	28
Totten Inlet	Subtidal	35–50	0.21	24	0.33	26
Taylor United	Subtidal	>60	1.00	58	1.00	30
Steilacoom Marina	Both	<15 and >35	0.00	62		
Tolmie State Park	Intertidal	5–25	0.00	44		
Hood Canal						
Seal Rock	Intertidal	5–35	0.00	54	0.00	24
Mike's Marina	Subtidal	5–20 and 35–40	0.00	30		
Potlatch State Park	Intertidal	5–15 and 35–45	0.00	30	0.00	30
Twanoh State Park	Intertidal	5–15 and 40–45	0.00	32	0.00	22
Belfair State Park	Intertidal	5–10 and 35–40	0.00	26	0.00	20

genotypes, including individuals that are homozygous for alleles from one species at all three loci and mussels that are heterozygous at all three loci. It is important to note, however, that mussels homozygous for *M. trossulus* alleles at one gene and homozygous for *M. galloprovincialis* alleles at another can only be the product of F2 or other advanced introgressive crosses; they can not be the progeny of either F1 hybridization or first-generation back-crossing.

Of the 55 mussels from Silverdale, Edmonds, and Totten Inlet assayed for all three genetic markers, 51% had genotypes consistent with pure *Mytilus trossulus* and 22% appeared to be pure *M. galloprovincialis* (Table 2). Putative F1 hybrids were rare, comprising only 9% of these samples. Putative back-crosses to *M. trossulus* appear to be about four times more common than back-crosses to *M. galloprovincialis* (15% versus 4%, Table 2). No individual was observed, in either the overall sample (Table 1) or this subsample (Table 2), that had a genotype exclusively compatible with F2 or other advanced hybridization.

Silverdale was the only site with a high frequency of *M. galloprovincialis* alleles where a wide range of size classes was available for analysis (Table 2). Mussels between 5 and 60 mm shell length were similar in genetic composition; the frequency of *M. trossulus* alleles was between 0.73 and 1, depending on locus, and *M. trossulus* genotypes predominated. Mussels >60 mm shell length were dominated by *M. galloprovincialis* genotypes and allele frequencies (0.75–0.77, depending on locus) (Table 2). *M. galloprovincialis* may either grow faster or attain a larger size than *M. trossulus*, be selectively favored, or historical variation in recruitment success may have favored *M. galloprovincialis* sometime in the past, but not more recently. The presence of putative F1 and *M. galloprovincialis* back-cross genotypes among the largest mussels at Silverdale suggests the observed relationship between size and allele frequency is a long-term and recurring feature at this location and not the result of historical variation in recruitment.

DISCUSSION

Native mussels, *Mytilus trossulus*, dominate the blue mussel community in Puget Sound; at most sites *M. galloprovincialis*

TABLE 2.

Multilocus genotypes of mussels from Silverdale, Edmonds, and Totten Inlet: The number of mussels of each genotype is indicated.

	Genotype				
	Trossulus	**Back-tross**	**F1**	**Back-gallo**	**Gallo**
Silverdale, length (mm)					
0–20	3	2	0	0	0
20–40	4	3	0	0	0
40–60	7	2	1	0	1
>60	1	0	3	2	6
Edmonds	8	1	0	0	2
Totten Inlet	5	0	1	0	3

Trossulus = mussels homozygous for *M. trossulus* alleles at Glu-5′, ITS, and Mal-I, Gallo = mussels homozygous for *M. galloprovincialis* alleles at all three loci; and F1 = mussels heterozygous at all three loci. Back-tross and Back-gallo indicate mussels with genotypes consistent with *M. trossulus* back-crosses and *M. galloprovincialis* back-crosses, respectively, as specified in the text. No mussels were observed that were homozygous for *M. trossulus* alleles at one locus and homozygous for *M. galloprovincialis* alleles at another (i.e., F2 genotypes).

alleles were rare or absent. The distribution of *M. galloprovincialis* alleles varies regionally within Puget Sound. *M. galloprovincialis* alleles are present but uncommon (~5%) in mussel populations in the southern portion of Puget Sound, and appear to be nearly absent in populations in the northern Puget Sound and the Hood Canal. The only locations where *M. galloprovincialis* alleles are locally common appear to be sites where they are likely to have been repeatedly introduced. These include the Totten Inlet where Taylor United, Inc., maintains a mussel farm, and sites near the Port of Seattle and Bremerton Naval Shipyards.

Aquaculture operations appear to be the source of the high frequency of *M. galloprovincialis* alleles in mussel populations from the Totten Inlet. Taylor United, Inc., maintains a grow-out facility in the inlet that produces 1–1.5 million pounds (live weight) of *M. galloprovincialis* per year (Gordon King, pers. comm.). The frequency of alleles specific to *M. galloprovincialis* among "wild" mussels within the inlet ranged between 21% and 33%, depending on the locus assayed. However, the impact of the aquaculture operation is highly restricted; mussels sampled at the mouth of the inlet (Carlyon Beach) had a low frequency of *M. galloprovincialis* alleles that was not readily distinguished from the background frequency of these alleles found in other mussel populations in the southern Puget Sound. This result is surprising because the Totten Inlet is about 12 km long and mussel larvae could potentially disperse over much greater distances during their development in the plankton of several weeks. These results suggest that the circulation patterns in Totten Inlet retain larvae within the inlet.

Two additional lines of evidence suggest that the genetic impact of aquaculture operations on the genetics of indigenous mussel populations may be minimal. First, Taylor United has previously cultured *M. galloprovincialis* at Freeland, on Whidby Island in the northern Puget Sound from 1992 to 1998. No *M. galloprovincialis* alleles were found among wild mussels at Freeland or at any other location on Whidby Island in 1997. Second, Taylor United maintains hatchery and nursery operations for the culture of *M. galloprovincialis* in Dabob Bay at the northern end of the Hood Canal. *M. galloprovincialis* alleles were virtually absent from all sites sampled within the Hood Canal. These results indicate that either cultured mussels are unlikely to escape aquaculture operations or, if they do, they have relatively little impact on resident populations of *M. trossulus*. Surveys conducted by Taylor United indicate that in 1996 and 1997, the frequency of *M. galloprovincialis* alleles among mussels on the shore near their mussel farm on Whidby Island was 2.8% and 1.3%, respectively (Gordon King, pers. comm.). Taylor United's mussel farming operations on Whidby Island were discontinued due to high mortality rates among *M. galloprovincialis*. These results all suggest that *M. galloprovincialis* is only marginally successful in northern Puget Sound.

It is worth noting that 100% of the mussels obtained from Taylor United's grow-out operation in the Totten Inlet were homozygotes for *M. galloprovincialis* alleles at both Glu-5′ and ITS. This result indicates that Taylor United has been highly successful at maintaining pure cultures of *M. galloprovincialis* for several generations, despite the presence of resident populations of *M. trossulus* as potential sources of contamination at both the grow-out and the hatchery/nursery locations.

Shipping also appears to be an important vector for the release of *M. galloprovincialis* into Puget Sound. The two greatest concentrations of *M. galloprovincialis* alleles outside of the Totten Inlet are near major shipping areas. The highest frequencies of *M. galloprovincialis* alleles outside of the Totten Inlet were observed at the Edmonds and Shilshole Bay Marinas north of Seattle, Seahurst County Park south of Seattle, and Silverdale, near the Bremerton Naval Base. Shipping appears to be the primary mechanism responsible for the introduction of nonnative marine species throughout the world (Carlton & Geller 1993; Ruiz et al. 1997; Ruiz et al. 2000). Geller et al. (1994) documented that larvae of *M. galloprovincialis* may be transported in ballast water of ships. The occurrence of *M. galloprovincialis* alleles near major shipping terminals in Puget Sound suggests that shipping may be responsible for the repeated introduction of this species to Puget Sound. It is also suggestive that *M. galloprovincialis* alleles are absent from the Hood Canal, which does not have any major shipping activity. Notably, *M. galloprovincialis* alleles were absent from within Elliot Bay near the Port of Seattle (Elliot Bay Marina, Pier 91 and West Seattle), which may indicate that ships release their ballast water before entering Elliot Bay.

Although *M. galloprovincialis* appears to have been repeatedly introduced into Puget Sound by both aquaculture and shipping activities, the fate of these introductions is tenuous. Mussels from the southern Puget Sound with *M. galloprovincialis* alleles appear to mostly be the result of back-crosses with *M. trossulus*. There was little evidence for pure *M. galloprovincialis* mussels outside of Totten Inlet and Dyes Inlet (Silverdale). Even in the three areas with the highest frequency of *M. galloprovincialis* alleles there was no evidence for advanced introgression, as indicated by the presence of genotypes that are exclusively compatible with being the progeny of F2 hybridization. These results indicate that when pure *M. galloprovincialis* are introduced, they occasionally hybridize with *M. trossulus* and then F1 hybrids may subsequently back-cross to *M. trossulus*. Continued hybridization is not apparent within Puget Sound. Rawson et al. (1999) examined hybridization between *M. galloprovincialis* and *M. trossulus* in California. They also found that even though mussel assemblages often contained high frequencies of both species, hybrids were relatively uncommon and there was little evidence for advanced levels of introgression. Although the mechanism is presently unclear, the results of

Rawson et al. (1999) and of this study indicate that the "genetic pollution" of native populations of *M. trossulus* by introduced *M. galloprovincialis* is limited. Further work is necessary to examine the mechanism(s) that determine the genetic fate of *M. galloprovincialis* alleles introduced by hybridization to resident populations of *M. trossulus*. It should also be noted that although introgression between these two species appears to be limited, *M. galloprovincialis* has been successfully introduced in California and in the Sea of Japan, and the ecological impact of these introductions on native mussel populations is presently unclear.

ACKNOWLEDGMENTS

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TEMPORAL AND SPATIAL VARIABILITY IN DREDGING INDUCED STRESS IN THE GREAT SCALLOP *PECTEN MAXIMUS* (L.)

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ABSTRACT Temporal and spatial variability in the stress caused to the great scallop *Pecten maximus* by dredge capture was determined using the adenylic energetic charge (AEC) of the striated muscle as an index. Samples were taken on a seasonal basis from dredge captured scallops of a single fishing ground in the north Irish Sea. All samples were taken from individuals under the minimum legal landing size. On one date samples were taken from scallops from three separate grounds with contrasting bottom characteristics. At all dates dredge captured scallops had a significantly reduced level of AEC compared to control scallops. This reduction was greatest in February when glycogen levels in the muscle tissue were shown to be at a minimum. There was little or no variability among fishing grounds in the effect of dredge capture on AEC levels. An additional aim of this study was to determine if scallops that encounter dredges but are not captured suffer a reduction in AEC levels. Scallops collected from dredge tracks immediately following the passage of dredges showed a significant reduction in AEC compared to control, scallops collected from outside the tracks. However, the reduction in AEC was not as great as in captured scallops.

KEY WORDS: scallop, *Pecten maximus*, dredging effects, adenylic energetic charge, seasonal effects, glycogen

INTRODUCTION

Most fishing techniques aim to capture a single or limited number of target species over a minimum size. This size may be dictated by markets or by government legislation. In scallop dredging, as in many other fisheries, a proportion of the captured target species is discarded owing to their small size. These undersized discards may be damaged during capture, for example suffering chipped valve margins or separation of the hinges, or may suffer stress from fatigue and desiccation. The level of mortality of these discarded scallops is unknown but may be considerable. Several studies have speculated on the levels of dredging induced mortality in relation to scallop size, sorting time and conditions on deck (Medcof & Bourne 1964), substrate type (Naidu 1988), catch weight (Chapman et al. 1977) and type and performance of gear (Gruffydd 1972).

Damaged scallops that are left on the seabed, or are discarded, are likely to show high mortality owing to the aggregation of predators to dredge tracks (e.g., Caddy 1973; Kaiser 1994; Medcof 1964) and to damaged bycatch (Veale et al. 2000). However, the majority of undersized discarded scallops show no signs of external physical damage. In the Irish Sea Jenkins et al. (2001) showed that less than 10% of scallops (*Pecten maximus*) encountering dredges showed any signs of damage while Shepard and Auster (1991) estimated that damage to the scallop *Placopecten magellanicus*, that encounter dredges but are not captured, can vary between 7% and 25% depending on substrate type. Although discarded scallops may have low levels of damage, they are likely to be highly stressed from the process of capture. Little is known regarding the effects of fishing induced stress on subsequent survival of scallops.

Dredging induced stress can be assessed in scallops using a variety of biochemical indicators. Maguire et al. (in press, a) discussed the usefulness of various techniques for stress assessment in scallops and found Adenylic Energetic Charge (AEC) to be the most effective in the measurement of acute dredging stress. AEC was first proposed as a stress index by Atkinson (1968) and is defined by the ratio:

$$\mathrm{AEC} = (\mathrm{ATP} + 0.5\mathrm{ADP}) \div (\mathrm{ATP} + \mathrm{ADP} + \mathrm{AMP})$$

where: ATP = adenosine tri-phosphate, ADP = adenosine diphosphate and AMP = adenosine mono-phosphate.

The AEC ratio varied from 0 to 1 i.e., 0 (all nucleotides were energy deficient AMP molecules) or 1 (all nucleotides were energy rich ATP molecules). In optimal conditions animals typically displayed AEC levels >0.8 while under stress conditions values ranged from 0.5 to 0.75. Such animals had a reduced growth rate and did not reproduce, but recovered when returned to optimal conditions. Severely stressed animals yielded values of <0.5 and these animals had a negative scope for growth and did not recover (Duncan 1993; Ivanovici 1980). Subsequently many studies have been carried out using AEC as a stress index in scallops, for example *P. magellanicus* (de Zwaan et al. 1980; Livingstone et al. 1981) and *P. maximus* (Fleury et al. 1997; Maguire et al. 1999a; Maguire et al. 1999b). Maguire et al. (in press a) used AEC as a stress index to investigate the effect of dredge capture on undersized scallops. They found no difference in AEC levels between different lengths of tow (15, 30 or 45 minutes) but found that emersion following dredging had an added stress effect. In this study we aimed to investigate different aspects of dredging on scallops i.e., effects of different seasons, ground types and to assess the stress caused to scallops that come in contact with dredges but remain uncaught on the seabed.

We aimed to examine dredging induced stress in the great scallop *Pecten maximus* on a seasonal basis to determine if seasonal reproductive cycle in this species affected the extent to which it is negatively affected by dredge capture. Seasonal variability in AEC levels has been examined in a number of invertebrate species including the crayfish, *Procambarus acutus acutus*

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(Dickson & Giesy 1982), the oysters *Ostrea edulis* and *Crassostrea gigas* (Moal et al. 1989b; Moal et al. 1991), the mussel *Mytilus edulis* (Zaroogian et al. 1982) and the clam *Cardium* sp. (Picado et al. 1988). Such variability has generally been associated with the reproductive cycle. Seasonal changes in biochemical composition of the scallop *P. maximus* have been well documented (Ansell 1978; Maguire & Burnell 2001). Carbohydrate reserves are built up during periods of greater food availability during the summer and used up in the winter. In *P. maximus* the main period for gonadal proliferation takes place between November and February by the mobilization of glycogen and protein reserves from the adductor muscle and lipid from the digestive gland. *P. maximus* may spawn over a long period from spring to autumn (Ansell 1978). Gametogenesis represents a period of high-energy demand in scallops and when external food supplies are limited, gamete production occurs at the expense of biochemical components in somatic tissues (Calow 1985). In this study on each sampling day we measured the glycogen content of both the striated adductor muscle and the gonad in order to assess the reproductive state of the scallops. Brokordt et al. (2000) showed that the reproductive state of the scallop *Chlamys islandica* affected its ability to escape, presumably as a result of changes in the energetic state of the individual. Therefore, it would be reasonable to hypothesize that seasonal variability in the energetic state of scallops would affect the degree to which they are negatively affected by dredging.

In addition to the major aim of this study, the determination of temporal variability in dredging induced stress, two further objectives were pursued. Initially, field trials were conducted at three different fishing grounds to assess the degree to which dredging induced stress varies spatially. Fishing grounds were specifically chosen with contrasting bottom characteristics. Diving was undertaken to collect scallops that encountered dredges but were not captured. AEC was measured to determine the extent to which these scallops were negatively affected by this experience.

MATERIALS AND METHODS

Seasonal Field Trial

The field trial was carried out on the commercial scallop ground known as the Chickens, off the south west of the Isle of Man in the North Irish Sea (53°58.75'N, 04°52.71'W). The scallops were caught using a Newhaven type spring-loaded dredge (width – 75 cm) with a toothed cross bar (tooth spacing – 66 mm; tooth length – 100 mm), a collecting bag made from case hardened steel rings (diameter – 70 mm) and a net mesh cover (mesh size – 100mm). Eight dredges were towed at a speed of 5 kph for forty-five minutes on 4 dates, March 15th, June 5th, October 6th (2000), and February 14th 2001. On each date two tows were made. At the end of each tow at least 15 scallops below the minimum legal landing size (<110 mm shell height) were taken from two random dredges and samples of muscle and gonad taken for AEC and glycogen analysis. Each scallop was opened and two small portions of the striated adductor muscle and one portion of gonad weighing approximately 0.2 g were removed using a scalpel. Samples were wrapped separately in foil and immediately placed in liquid nitrogen.

On each date 10 undersized scallops were taken from the dredges and transferred alive to Port Erin Marine Laboratory and placed in tanks containing running seawater. Samples of muscle were taken one week later as described above. These samples acted as controls for AEC analysis. A preliminary experiment showed that the AEC levels of post-dredged scallops returned to normal after 24 hours (at most) of recovery in seawater (Maguire et al., in press b). Scallops placed in tanks for a full week had made a complete recovery and thus this protocol was adopted for the control animals in this study.

Comparison of Scallops from Different Grounds

This field trial was carried out in June 2000 on three commercial scallop fishing grounds off the Isle of Man (Fig. 1); Chickens (53°58.75'N, 04°52.71'W), Laxey (54°13.50'N, 04°21.38'W) and Bradda Inshore (54°06.5'N, 04°47.76'W). The substratum on all grounds was generally coarse sand or gravel, often overlain with pebbles, cobbles and dead shell. The abundance of pebbles and cobbles, as indicated by the number caught during dredging varied greatly among the three grounds sampled. At Laxey there were relatively few and the substratum was predominantly sandy with areas of dead maerl. The Chickens and Bradda Inshore grounds were both made up of variable gravel sediment with large numbers of pebbles and cobbles at Bradda Inshore and intermediate amounts at Chickens.

Scallops were caught using the same method as above. Samples of muscle for AEC analysis were taken from 15 undersized scallops from random dredges from two tows.

Stress in Non-Captured Scallops

In June 2000 dredging was undertaken on the Bradda Inshore scallop ground as part of a study into the impacts of dredging on benthic megafauna (see Jenkins et al. 2001 for details). Divers collected scallops that had encountered dredges but had not been captured by following the tracks on the seabed formed from the passage of the dredge. Forty-two scallops from within the dredge tracks (impacted scallops) and 14 scallops from outside the tracks (control scallops) were collected between 15 and 45 minutes after the passage of the dredges. Dredging also took place and two tows were carried out. For each tow, 15 undersized animals were removed from the dredge bags. On surfacing all scallops were im-

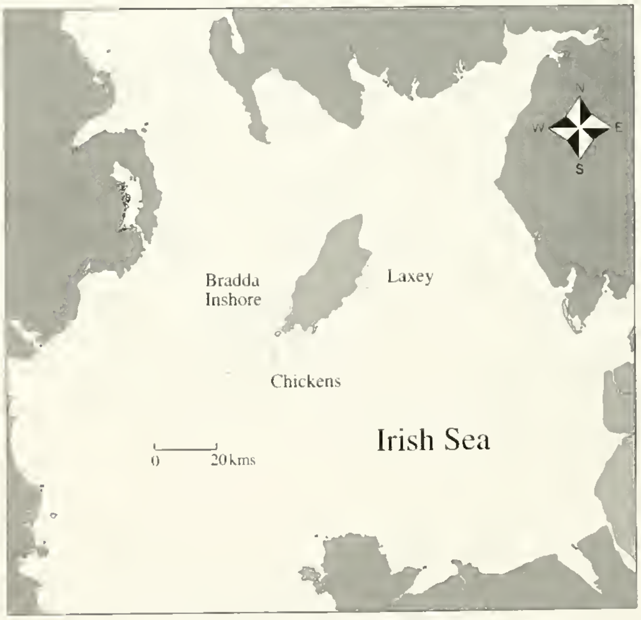

Figure 1. Map of dredged sites.

mediately opened and samples of striated adductor muscle taken for AEC analysis.

Biochemical Analyses

All samples were stored in liquid nitrogen until analysis. Adenylic Energetic Charge (AEC) of the striated adductor muscle was determined using the technique of Moal et al. (1989a). Maguire et al. (1999b) conducted an experiment to assess the effect of stress on the AEC level of both the smooth and striated muscle of the king scallop. A greater significant AEC decline was shown between treatments in the striated muscle. For glycogen analysis samples were withdrawn from the liquid nitrogen and freeze dried using a HETOSICC CD 53-1 freeze dryer. The glycogen content was analyzed using a miniaturization of the Dubois et al. (1956) method.

Statistical Analyses

After testing for normality, non-parametric data were normalized by log transformation or arcsine square root transformation. One-way analyses of variance (ANOVAs) were used to test significant differences between treatments and *a posteriori* Tukey test was used to contrast treatments. The level of significance was set at 0.05.

RESULTS

Seasonal Field Trial

Figure 2 shows the effect of dredging on the AEC level of scallops from the Chickens scallop fishing ground off the coast of the Isle of Man. The AEC level decreased significantly in the dredged scallops from the control samples at each sampling period ($P < 0.001$). However, the AEC levels of the control samples varied throughout the year but not significantly. The highest AEC levels in the control animals were found in October (0.90 ± 0.01) and the lowest levels were found in February (0.77 ± 0.05). Because of this seasonal AEC variation in the control animals the % AEC decrease from the control to dredged scallops was calculated for each season. The AEC reduction was highest in February (44.8% ± 3.3) and June (43.4% ± 2.9) and significantly less ($F_{136,3} = 3.476$, $P < 0.05$) in March (33.6% ± 2.8). The lowest absolute value of AEC in the striated muscle of scallops following dredging was also in February (0.43 ± 0.03) and the highest was in October (0.53 ± 0.01). The AEC level reached in scallops sampled in February was significantly different ($F_{136,3} = 4.942$, $P < 0.05$) from all the other dates sampled except for June. No difference in the AEC levels of dredged scallops was found between any of the other sampling dates.

The percentage of glycogen content in the striated muscle of the animals showed a similar pattern to the AEC results found in the control animals (Fig. 3). The lowest glycogen levels were found in the scallops sampled in February (4.38% ± 0.27), with levels over three times higher in October ($F_{165,3} = 83.32$, $P < 0.001$). The % glycogen content in the gonad also varied significantly throughout the year ($F_{156,3} = 331.42$, $P < 0.001$). The glycogen levels in the gonad ranged from a minimum in October (0.30% ± 0.05) to a maximum in June (10.33% ± 0.43).

Sea water temperatures decreased from a maximum on August 29th 2000 (15.7°C) to a minimum value on the 17th of January 2001 (7.3°C). On the specific sampling days the temperatures are shown on Figure 2.

Comparison of Scallops from Different Grounds

There was no difference in the AEC levels of scallops dredged at different sites in June (Fig. 4). The AEC level varied in scallops from 0.46 to 0.48 on all three grounds.

Stress in Non-captured Scallops

Scallops that came in contact with the dredge but remained uncaught had a significantly lower AEC level than the control scallops ($t_{69} = 7.401$, $P < 0.001$). However, the reduction in AEC was not as great as in captured scallops. Figure 5 showed that the AEC level in captured scallops was significantly lower ($t_{55} = 6.869$, $P < 0.001$) than in the non- captured scallops.

DISCUSSION

The results from this study suggest that the AEC response to dredging of undersized scallops is different according to the season of the year. Lowest AEC levels in dredged and control scallops

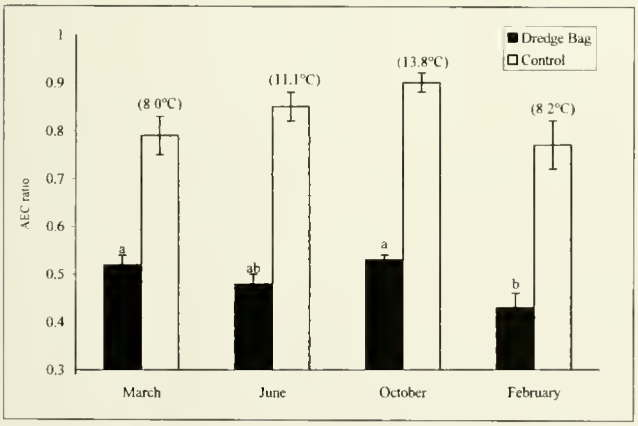

Figure 2. The seasonal effects of dredging on the AEC level (mean ± s.e) of scallops from Chickens (March 2000 to February 2001). Sea water temperatures shown in parenthesis. Any two means sharing a common letter between each treatment are not significantly different at $P < 0.05$ (Tukey test).

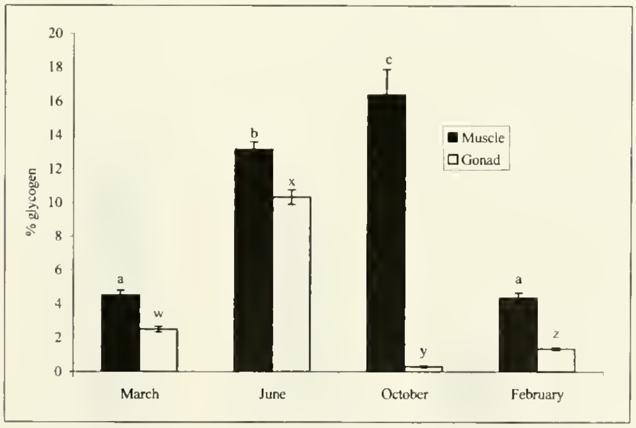

Figure 3. The seasonal % glycogen content (mean ± s.e) of scallops from Chickens (March 2000 to February 2001). Any two means sharing a common letter between each treatment are not significantly different at $P < 0.05$ (Tukey test).

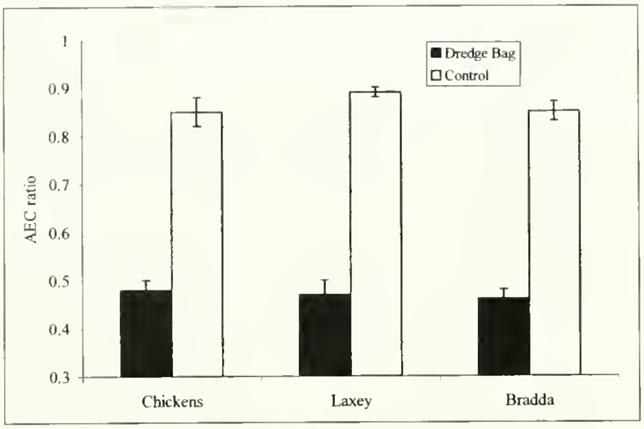

Figure 4. The effects of dredging on the AEC ratio (mean ± s.e) of scallops from three sites off the Isle of Man (June 2001).

were found in February and highest levels in October. This coincided with the troughs and peaks of the glycogen content in the adductor muscle of the test animals. Reserves are built up during periods of greater food availability in the summer. However, energy requirements for maintenance are high therefore rapid utilization of reserves takes place during the winter. Many demands are placed on the metabolism of scallops during the winter from the stresses imposed from internal physiological drives such as gonadal proliferation. However, during unfavorable winter conditions these energy demands may be insufficient to support gonadal development (Ansell 1978). The greatest percentage of AEC decrease from control to dredged scallops was also in February (44.8%). Therefore, the additional stress effect of dredging at the end of winter had a greater negative effect on the AEC level of scallops whose energy reserves were already low. Similarly, Brokordt et al. (2000) showed that the mobilization of reserves had a detrimental impact on the escape response of the Iceland scallop, *Chlamys islandica*.

Moal et al. (1989b), also found that the AEC response to the stressor aerial exposure was different according to the time of year in the oyster *Crassostrea gigas*. The animals were subjected to a three hour emersion period in January, May, and July. Lowest AEC values were found in July and these levels were inversely correlated with temperature. Similarly, in our study a large % AEC decrease from control to dredged animals was recorded in June (43.4%) and October (41.1%) when temperatures were highest. The June sample coincided with the spawning season of the scallop population in the Isle of Man. Moal et al. (1991), also found low AEC levels during the summer in the farmed oysters *C. gigas* and correlated this decrease with the reproductive state of the oysters.

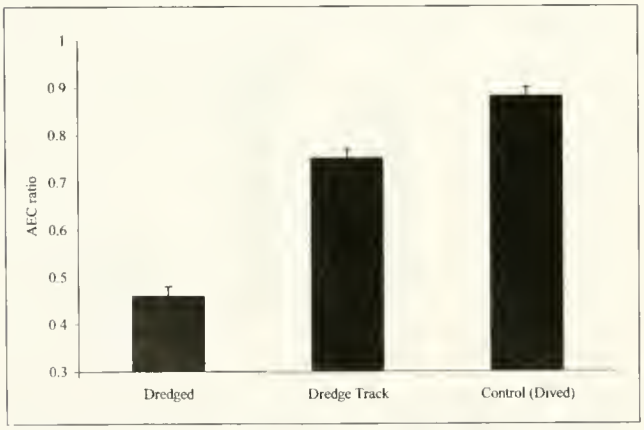

Figure 5. Comparison of the AEC levels (mean ± s.e) of dredged scallops and scallops left behind on the dredge track from Bradda in June.

Many authors have suggested that AEC levels in molluscs below a value of 0.5 result in a negative scope for growth, which would eventually lead to mortality even if the animals were transferred to optimal conditions (Ivanovici 1980; Duncan 1993). However, in this study scallops dredged in February and June had AEC levels <0.5. However, no mortality was recorded in the control scallops. Similarly in a previous study by Maguire et al. (1999a), juvenile scallops were transported in polystyrene boxes for 12 hours and the AEC level of the spat decreased form 0.88 to 0.42. Recovery of these animals was initially rapid and AEC levels had increased to 0.68 after only two hours in optimal conditions and had returned to normal after 24 hours. In addition dredge captured scallops that are returned to the laboratory and maintained in running seawater showed consistently low levels of mortality (Jenkins, unpublished data). Although the stress and subsequent reduction in AEC levels in dredge captured scallops may not lead directly to mortality, such scallops may be more susceptible to predation (Thompson et al. 1980) through a reduction in their escape response or inability to recess (Minchin et al. 2000). Maguire et al. (in press a) found that the AEC levels of dredged scallops (30 minute tow length) had returned to normal after 3 days of recovery but had not recessed into the sediment in that time period. Jenkins and Brand (in press) have shown that the escape response in *P. maximus* was significantly reduced following simulation of dredging for up to 24 hours. Such observations suggest that predator induced mortality may be significant for stressed discards.

In general, the percentage of glycogen content of these Isle of Man scallops was quite low in the striated adductor muscle ranging from 4.4% to 16.4%. In a study of a population of scallops in Bantry Bay, Ireland the glycogen content ranged from 16.5% to 45.9% (Maguire & Burnell 2001). Ansell (1978) studied the storage of reserves in the adductor muscle of some scallop populations in the U.K. and found that the % glycogen content generally varied from 2.2% to 24.0%, but in one population the glycogen content was as high as 39%. He also suggested that glycogen content could vary between sites and between different years.

There was no difference in the AEC values between the three different sites studied. The effect of the dredging process was the dominant stressor on the animals so much so that the subtle differences between the sites had little added impact on the AEC decrease. Similarly, Verschraegen et al. (1985) found no difference in the AEC levels of the polychaete species *Nereis diversicolor* and *Nephtys* sp. between sites in the Western Scheldt estuary, Belgium. Also, Zaroogian et al. (1982) found no difference in AEC levels of the mussel *Mytilus edulis* in two sites.

Scallops that came in contact with the dredge but remained uncaught and were found on the dredge track were stressed (AEC level = 0.75). Although the AEC level was not as low as in captured scallops it is important to demonstrate that the impact of dredging is not confined solely to those scallops landed on deck. Owing to the relatively low efficiency of scallop dredges (Dare et al. 1993) far more scallops will encounter dredges and remain on the seabed than are captured. These non captured animals which include all sizes, may suffer enhanced mortality due to dredge induced stress in the same way as undersized discards. Also, it was interesting to note that the collection and transportation of scallops from the sea-bottom to the surface did not induce stress in the control animals.

CONCLUSIONS

1. AEC levels in dredged and control animals, and % glycogen in the striated muscle of dredged scallops, were lowest in February and highest in October.
2. The largest % AEC decrease from control to dredged scallops was also in February and the smallest decrease was in March.
3. There was no difference in the AEC level of dredged scallops from three sites with different ground types around the Isle of Man.
4. Scallops that came in contact with the dredge but remained uncaptured on the dredge track had a reduced AEC level but this level was significantly higher than the AEC level of captured animals.

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REDUCED GROWTH OF ICELAND SCALLOPS *CHLAMYS ISLANDICA* (O.F. MÜLLER) CULTURED NEAR THE BOTTOM: A MODELLING STUDY OF ALTERNATIVE HYPOTHESES

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ABSTRACT Slow growth of *Chlamys islandica* held in pearl nets near the bottom is usually attributed to poor seston quality owing to higher concentration of resuspended inorganic particles near the bottom. In a recent contribution, we hypothesized that current speed variations in boundary layers and feeding behavior may also result in slower growth of Iceland scallops kept in pearl nets near the bottom, independently of resuspension effects. Here we present computer simulations of growth of scallops kept in pearl nets at two population densities and various heights above the bottom. Our simulations suggest that resuspension may account for experimental results but that food depletion within the pearl nets is required. Our simulations also suggest that a direct positive effect of current speed on clearance rate or physiological regulation of clearance rate, both coupled with seston depletion within pearl nets, may both explain experimental patterns. Based on the profile of growth across height, our results also provide a criterion for interpreting results of experiments designed to identify the actual control mechanism.

KEY WORDS: *Chlamys islandica*, density dependence, growth, scallop, seston quality

INTRODUCTION

Iceland scallops (*Chlamys islandica* O.F. Müller) cultured in pearl nets typically exhibit reduced growth rate near the bottom (Wallace & Reinsnes 1984; Wallace & Reinsnes 1985; Thorarinsdóttir 1994). Because reduced seston quality dramatically reduces assimilation efficiency of Iceland scallops (Vahl 1980), near-bottom growth reduction is usually attributed to low seston quality due to local resuspension. Resuspended particles indeed tend to be of poor trophic quality as compared to planktonic food, the stronger the resuspension forces the larger the difference (Demers et al. 1987; Thomsen & Gust 2000).

Boundary layers are zones of strong change in current speed with respect to distance from the interface between fluids and solid surfaces. They are universal features of flow near such discontinuities. Bottom resuspension results from shear in the benthic boundary layer (BBL; e.g., Muschenheim 1987; Thomsen & Gust 2000). Therefore if organisms are held across a resuspension gradient near the bottom, they will also experience a current speed gradient. It follows that the seston quality hypothesis cannot be invoked as the sole explanation to near-bottom vertical differences in scallop growth without implying that the scallops were also held across a current speed gradient and that current speed effects were negligible.

Fluid movement and associated physical processes are fundamental to the biology and ecology of aquatic organisms (Vogel 1981; Wildish & Kristmanson 1997). Trophic dynamics of scallops are no exception. For instance, Wildish et al. (1992) reported that current speed affects growth in a non-monotonous way through its effect on clearance rate. At low current speed, clearance rate increases with current speed until a maximum is reached at about 10 cm s^{-1}. Higher current speed results in clearance rate inhibition. These patterns are reflected in growth (e.g., Wildish et al. 1987; Cahalan et al. 1989; Eckman et al. 1989; Wildish et al. 1992; Wildish & Saulnier 1992) but in pearl net culture, they may interact with seston depletion within the pearl nets (Claereboudt et al. 1994b). Furthermore clearance rate may respond to temporal variability in phytoplankton concentration and current speed, indicating an effect of feeding history (Pilditch & Grant 1999). Therefore near-bottom effects may be ascribed to direct and indirect effects of current speed as much as to sediment resuspension.

In a recent study of growth and survival of Iceland scallops kept in pearl nets, we tested the effect of site, of height above the bottom (0.1 m versus 2.0 m) and of group size within the pearl nets (Fréchette & Daigle, in press). Our analysis also included fluctuating asymmetry as a proxy of individual effects. The group size treatment allowed testing for containment effects within the pearl nets. Available data showed no evidence of phytoplankton vertical depletion in the water column. However, we found slower growth near the bottom. This is similar to previous reports (Wallace & Reinsnes 1984; Wallace & Reinsnes 1985; Thorarinsdóttir 1994). We also found that at 2.0 m above the bottom there was a group size effect, but not at 0.1 m. High density groups grew slower than low density groups. This is consistent with food depletion within the pearl nets and has been observed repeatedly in growth experiments (Parsons & Dadswell 1992; Claereboudt et al. 1994a; Claereboudt et al. 1994b). The reasons for the absence of a group size effect at 0.1 m, however, are not clear. Food within the pearl nets was presumably more depleted at 0.1 m height, down in the BBL, where current speed is slower than at 2.0 m height. This should have generated a group size effect at 0.1 m as found at 2.0 m.

The goal of the present study was to explore three hypotheses for the group size*height interaction found in the growth experiment. We modeled growth according to various control factors: (1) with increasing inorganic particle concentration near the bed, due to resuspension; (2) with a direct positive effect of current speed on clearance rate; (3) with an indirect effect of current speed on clearance rate because of physiological regulation of clearance rate in response to more intense phytoplankton depletion in the pearl nets held near the bottom. To simplify matters, we modeled the effect of each factor separately.

METHODS

We modeled soft tissue growth of individual scallops contained in hypothetical pearl nets, as a function of non-dimensional current speed, U_z/U_2, with U_z and U_2 being respectively current speed at

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heights z and 2.0 m above the bottom. We assumed that the benthic boundary layer extended to 2.0 m above the bottom and that U_z increased logarithmically with height (Grant et al. 1984). A first set of simulations was made assuming that no seston depletion occurred in the pearl nets. In the second set of simulations, we assumed that seston depletion occurred in the pearl nets. Outside the pearl nets, phytoplankton concentration was assumed to be constant across heights (see Fréchette & Daigle, in press). We assumed that there were no interactions between individual scallops otherwise than through their effect on bulk food concentration in the pearl nets. We modeled flesh growth as the balance between energy intake and energy losses, based on the Scope for Growth approach (Bayne & Widdows 1978), although more mechanistic approaches to this problem are possible (e.g., van Haren & Kooijman 1993).

The numerical model has two state variables, scallop flesh mass (m, g) and phytoplankton concentration in the hypothetical pearl nets (P, J L^{-1}). We assumed that survivorship was 60% and 80% in pearl nets held at 0.1 m and 2.0 m, respectively (Fréchette & Daigle, in press). Mortality was spread evenly through time, one individual at a time. Flesh mass growth ($\mathrm{d}m/\mathrm{d}t$) is driven by net energy balance, with

$$\mathrm{dm/dt} = \mathrm{c_1} \cdot [(\mathrm{c_2} \cdot \mathrm{CR} \cdot \mathrm{P} \cdot \mathrm{AE}) - \mathrm{R}] \qquad (1)$$

where c_1 is a conversion factor from J to mass (c_1 = 0.00051; Dauvin & Joncourt 1989), c_2 is a parameter accounting for flow effects on clearance rate (see later), CR is clearance rate (L day^{-1}), AE is assimilation efficiency (dimensionless) and R is respiratory losses (J day^{-1}). To model phytoplankton in the pearl nets, we assumed that pearl nets were analogous to flow-through chambers, with flow rate being directly proportional to current speed. It is assumed finally that mixing is complete and that water displacement by scallops has negligible effect on residence time. Phytoplankton conservation in the pearl nets is given by

$$\mathrm{dP/dt} = (\mathrm{V} \cdot \mathrm{c_3})^{-1} \cdot [\mathrm{c_3} \cdot \mathrm{c_4} \cdot \mathrm{v} \cdot (\mathrm{P_i} - \mathrm{P_s}) - \mathrm{N} \cdot (\mathrm{P_s} \cdot \mathrm{c_2} \cdot \mathrm{CR})] \qquad (2)$$

where $\mathrm{d}P/\mathrm{d}t$ is the rate of change of phytoplankton concentration in the pearl nets, V is the volume of the hypothetical pearl nets, c_3 and c_4 are parameters (see later), P_i and P_s are phytoplankton concentration (J L^{-1}) at the inflow and the outflow of the pearl nets, respectively, N is the number of scallops in the pearl nets. In all simulations, P_i = 15.6 J L^{-1}. Assuming that the energy content of phytoplankton is 10.4 J mg^{-1} (Bayne & Widdows 1978, Thompson 1984), this specifies a particulate organic matter (POM) concentration equal to 1.5 mg l^{-1}, constant along the vertical. Under the assumption of complete mixing within the pearl nets, P is estimated by P_s (Northby 1976).

Growth was simulated for 360 days with no seasonal signal in P_i. Temperature was not taken into account. The values of V (40 L), c_2, c_3, c_4 and basic flow rate v (1000 L day^{-1}) were set arbitrarily to provide the desired conditions in the pearl nets–either seston depletion or no depletion, and flow rate variations as a function of height. The parameters c_2 and c_4 are the ratio of current speed at height z to current speed at 2 m above the bottom. c_2 and $c_4 \in \{0.1, 0.2, 0.3, \ldots, 1\}$, depending on the situation modeled. They are used to mimic the effect on flow rate of change in current speed with height in the benthic boundary layer.

In the first set of runs where phytoplankton depletion in the pearls nets is negligible, c_3 = 1000. In the second set of runs, where depletion occurs, c_3 = 1. To model the impact of a positive effect of current speed on CR, we assumed that c_2 and c_4 increase proportionally to current speed (Wildish et al. 1992) and ran the model with c_2 and c_4 equal to 0.1, 0.2, . . ., 1 successively. Clearance rate (l day^{-1} ind.$^{-1}$) was given by $CR = 24 \cdot 3.09 \cdot m^{0.60}$ after Vahl (1980).

To model the expected results of an indirect effect of current speed, we set c_2 = 1 and c_4 equal to 0.1, 0.2,. . ., 1 as above. Clearance rate was given by $CR = 24 \cdot (5 \cdot \exp(-0.4 \cdot P\ ) + 3.09 \cdot m^{0.60})$. The exponential part of the equation allows realistic mimicry of physiological regulation of clearance rate in response to phytoplankton concentration, at least as observed in *Placopecten magellanicus* (Bacon et al. 1998) and *Argopecten irradians* (Cahalan et al. 1989). In the cases of direct and indirect effects of current speed, AE = 0.5.

To model the effect of resuspension, we first constructed a suspended inorganic sediment (PIM) concentration profile above the bed, based on Rouse's theory, according to which we have $C = C_a \cdot (a/z)^q$, where C is sediment concentration (mg l^{-1}) at height z above the bed, C_a is a reference concentration at a reference height a (C_a = 1 mg l^{-1}; a = 200 cm), and $q = w_s/\kappa U_*$, where w_s is sediment mean fall velocity (w_s = 0.05 cm s^{-1}), κ is von Karman's constant (0.41) and U_* is friction velocity (U_* = 1 cm s^{-1}) (see e.g., Muschenheim 1987). Assuming that velocity above the scallop bed followed the law of the wall, with $U_z = U_* \kappa^{-1} \ln(z/z_0)$ (e.g., Grant et al. 1984), and estimating z_0 = 0.2 cm by means of Nikuradse's parameterization of bed roughness ($z_0 = k_b \cdot 30^{-1}$, where k_b = 7 cm, the approximate size of scallops and pebbles recovered from scallop dredge tows on the donor site of our growth experiment), we constructed an hypothetical flow profile above the bed. We then found the heights at which we had $U_z/U_2 \in \{0.1, 0.2, \ldots, 1\}$ and deduced the corresponding PIM concentration profile. Knowing PIM as a function of U_z/U_2 (POM is assumed to be constant across heights), we determined EA at height as $EA = \sin^2(139.6 - 2.14 \cdot \arcsin(1 - (PIM/(PIM + POM)))^{0.5})$, as modified from Vahl (1980). In all cases, respiration is given by $R = (155.2\ \mu\mathrm{l}\ \mathrm{O_2} \cdot \mathrm{m}^{0.80}) - 19.9 \cdot 10^{-3}\ \mathrm{J}\ \mu\mathrm{lO_2}^{-1}$ (Vahl 1978).

RESULTS

Results of the growth simulations with and without depletion in the hypothetical pearl nets are shown in Figure 1 and Figure 2, respectively. All results are reported as a function of U_z/U_2, which scales as ln z under the assumption of a logarithmic current profile above the bed. In all cases, growth at 2.0 m above bottom did not vary because this was the reference level, and was assumed to have constant current speed and suspended sediment concentration. Growth at height z, however, decreased with decreasing U_z/U_2. Without food depletion in the hypothetical pearl nets, growth decreased in a curvi-linear fashion with U_z/U_2, with upward concavity for both resuspension and a direct effect of current speed on clearance rate as control mechanisms. Because there was no food depletion to generate group size effects, data for different group sizes overlap in Figure 1. Therefore there was no interaction between height above bottom and group size. With food depletion in pearl nets, however, growth patterns were richer (Fig. 2). For U_z/U_2 = 1, growth at height z was higher than at 2.0 m because mortality at the lower level was more severe than at 2.0 m and therefore per capita food availability was higher. Resuspension and a direct effect of flow on clearance rate resulted in a curvilinear decrease of growth as U_z/U_2 decreased, with upward concavity

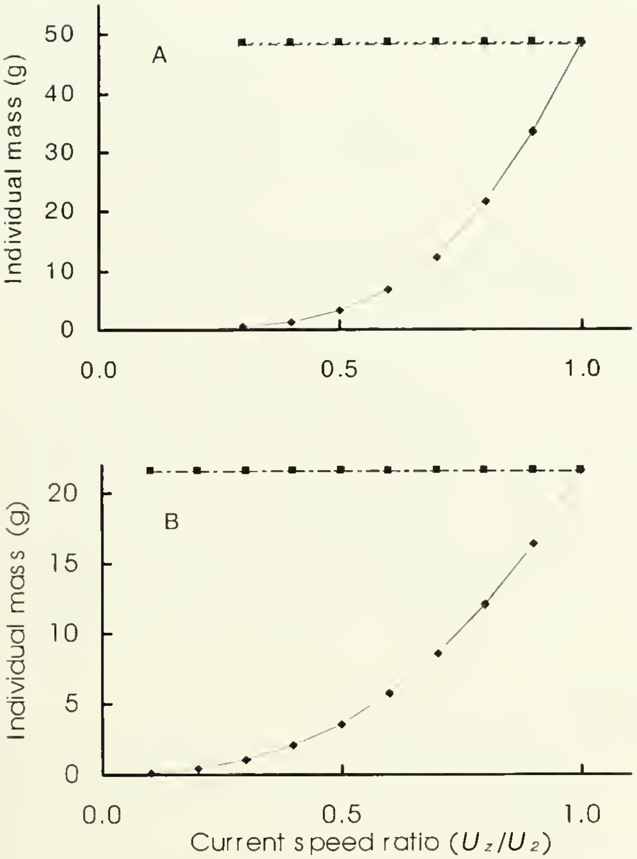

Figure 1. Simulated soft tissue growth without phytoplankton depletion in the hypothetical pearl nets. Diamonds: height z above bottom; squares: 2 m above bottom; solid symbols: 5 individuals per hypothetical pearl net; empty symbols: 10 individuals per hypothetical pearl net. Here empty symbols are hidden by solid symbols. A: effect of particle resuspension at the bottom. B: effect of positive relation between current speed and clearance rate.

(Fig. 2A, B). An indirect effect of current speed, however, generated a response with downward concavity (Fig. 2C). In all three cases, the difference between group sizes decreased with decreasing U_z/U_2.

DISCUSSION

Our simulations indicate that decreasing U_z/U_2 ratio resulted in a height effect on simulated growth. However, phytoplankton depletion within the hypothetical pearl nets was required to generate a height*group size interaction similar to that found in the actual growth experiment (Fréchette & Daigle, in press). The chances of occurrence of a significant height*group size interaction increased with decreasing height z because the effect of group size on scallop growth decreased with decreasing current speed. The lower the U_z/U_2 ratio, therefore, the better the simulations mimicked the experiment. The key factors implied here are seston depletion within the pearl nets and vertical differences in current speed owing to the BBL.

Our simulations show that the three mechanisms tested might have generated the height*group size interaction. Vahl (1980) reported that seston quality had a strong negative effect on growth of Iceland scallops. According to our simulations, higher *PIM* near the bottom, coupled with homogeneous *POM* concentration in the vertical and with phytoplankton depletion within the pearl nets (Claereboudt et al. 1994a; Claereboudt et al. 1994b), may have generated the height*group size interaction (Fig. 2A). Because within pearl net phytoplankton depletion is required, this mechanism is different from the hypothesis of a straightforward negative effect of poor seston quality on assimilation efficiency which is usually invoked to explain near-bottom effects on growth of Iceland scallops held in pearl nets (Wallace & Reinsnes 1984, 1985; Thorarinsdóttir 1994). Clearly different group sizes should be used routinely in growth experiments to test for containment effects.

The second mechanism tested, that is, a direct positive effect of higher current speed on clearance rate (Wildish et al. 1992), coupled with seston depletion within the pearl nets, also resulted in

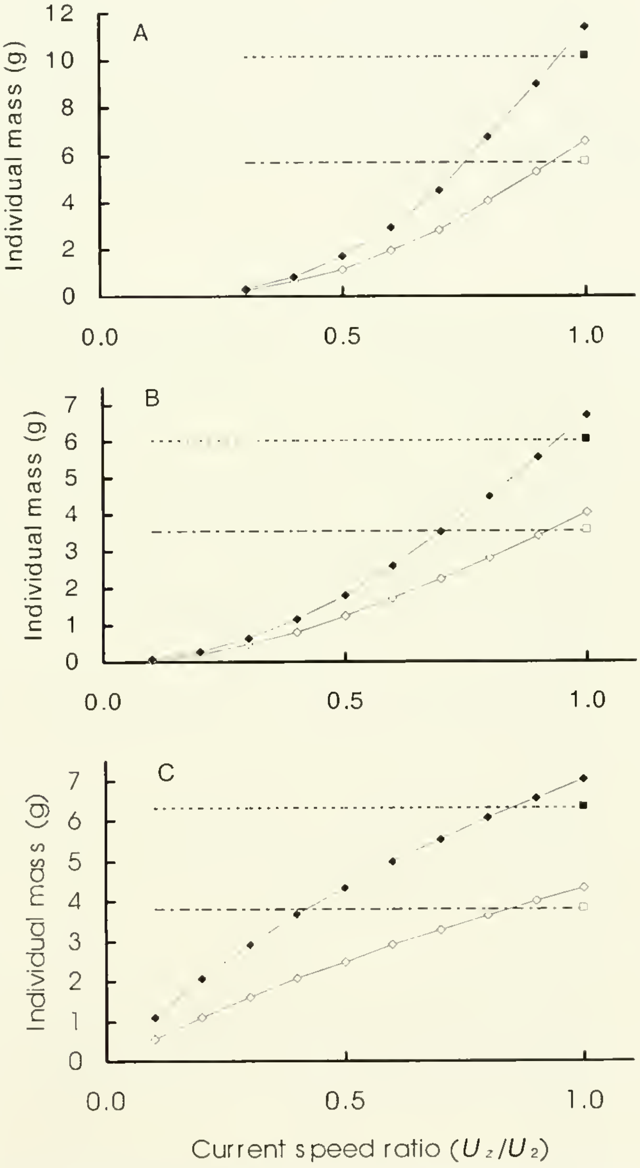

Figure 2. Simulated soft tissue growth with phytoplankton depletion in the hypothetical pearl nets. Symbols as in Figure 1. A: effect of particle resuspension at the bottom. B: effect of positive relation between current speed and clearance rate. C: effect of clearance rate regulation in response to seston concentration within the hypothetical pearl nets.

a height*group size interaction (Fig. 2B). It has been argued, however, that the positive relationship between clearance rate and current speed may be an artifact of flow removing seston depletion envelopes around individuals (Lenihan et al. 1996). There is indeed evidence that phytoplankton concentration may be structured in space (cm scale) by exhalent jets of suspension feeders (Monismith et al. 1990; O'Riordan et al. 1993; O'Riordan et al. 1995). In addition, Claereboudt et al. (1994b) found no evidence of positive effect of current speed on sea scallop growth. Although the ongoing considerations provide little support for a positive response of clearance rate to current speed, it should be noted that Butman et al. (1994) found such a positive response in a mussel population contained in a flume.

The third mechanism tested, that is, an indirect effect of flow mediated through changes in phytoplankton concentration within pearl nets coupled with clearance rate regulation (clearance rate is assumed to increase with decreasing phytoplankton concentration; e.g., Bacon et al. 1998), also resulted in a height*group size interaction. The relation between growth and U_r/U_2 was curvilinear, with downward curvature. In the case of resuspension and of a direct effect of flow, the relation was also curvilinear, but with upward curvature. Therefore, assuming that regulation of clearance rate in Iceland scallops resembles that of sea scallops and bay scallops, results of a growth experiment with different heights tested may provide information as to whether the height*group size interaction was attributable mainly to physiological regulation or to environmental forcing. We did not study the effect of inhibitory high flows on clearance rate (Eckman et al. 1989; Wildish et al. 1992) because it was inconsistent with lower growth near the bottom.

In summary, our simulations suggest that the original seston quality hypothesis alone is insufficient to account for the decrease in growth of scallops near the bottom. An additional factor is required, that is, seston depletion within the pearl nets and its interaction with the BBL. Furthermore, two other mechanisms, independent of resuspension, may explain the growth pattern in the BBL. Both require food depletion within the pearl nets and interaction with the BBL. The first mechanism is a direct positive effect of higher current speed on clearance rate and the second mechanism involves clearance regulation in response to food depletion. Because our simulations allow predictions about the profile of growth across height, growth experiments may provide evidence on the factor actually causing near bottom reduction in growth, provided that the processes simulated above act independently.

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ULTRASTRUCTURAL CHARACTERISTICS OF SPERMATOGENESIS IN DIPLOID AND TRIPLOID CATARINA SCALLOP (*ARGOPECTEN VENTRICOSUS* SOWERBY II, 1842)

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ABSTRACT Spermatogenic stages in diploid and triploid catarina scallop (*Argopecten ventricosus*) were studied by light and electron microscopy at two different ages. In nine month old triploid scallops, a reduced number of spermatogonia and primary spermatocytes were seen in the male acini, and although no spermatids were found, a few spermatozoa were observed. All triploid spermatogenic stages and their nuclei were larger in diameter than those in diploids. Ultrastructure analysis reveled that spermatogenesis in triploids was almost completely arrested early, at the primary spermatocyte stage, indicating the halt occurs during the prophase stage of meiosis I, before the first reductional division. At age 12 months a large number of hemocytes invade some of the remaining male acini of triploids, which possibly have a trophic role after development is halted. At this age, the male part of the gonad in most analyzed scallops is completely replaced by female acini, with some developing oocytes. Checkpoint mechanisms recently described as "meiotic checkpoints" are proposed as possibly being involved in the partial or total sterility resulting from the triploid condition.

KEY WORDS: *Argopecten ventricosus*, sterility, EM, triploid, pectinid, spermatogenesis, meiotic-checkpoint

INTRODUCTION

Triploid sterility has been reported for a number of mollusk species, but in most cases the sterility is not reflected in a complete lack of gametogenic stages, but rather on a delayed process and a reduced number of fully developed gametes (Allen et al. 1986; Komaru & Wada 1989, 1990; Allen 1987; Allen & Downing 1990; Guo & Allen 1994b; Cox et al. 1996; Eversole et al. 1996; Kiyomoto et al. 1996; Ruiz-Verdugo et al. 2000). Whereas the most common characteristic in triploid mollusks has been reported to be the reduced development of the gonad and gametes, the reversal of gonad sex in a known functional hermaphrodite has been only reported to occur in catarina scallop (Ruiz-Verdugo et al. 2000).

In as much as several studies comparing diploids and triploids have been done on gametogenic stages by light microscopy, partial or total ultrastructure analysis of abnormal gametogenesis in polyploid mollusks has been done only for triploid Pacific oyster *Crassostrea gigas* (Komaru et al. 1994), triploid Sydney rock oyster *Saccostrea commercialis* (Cox et al. 1996), and triploid and tetraploid Mediterranean blue mussel *Mytilus galloprovincialis* (Komaru et al. 1995; Kiyomoto et al. 1996). Spermatogenesis in diploid catarina scallop *Argopecten ventricosus* at the ultrastructure level has not been studied before, although it has been done for other pectinid species, *Pecten maximus* (Dorange & Le Pennec 1989a; Beninger & Le Pennec 1991).

The catarina scallop, *Argopecten ventricosus*, is a functional hermaphrodite pectinid in which the gonad is divided into two parts, one female and one male. When triploidy is induced in this species, a gradual suppression of functional hermaphroditism has been noted to occur, with only female gametogenic stages developing in the male part of the gonad in one-year-old scallops (Ruiz-Verdugo et al. 2000). As reported for oysters (Guo & Allen 1994a) and Manila clams (Utting et al. 1996), as well as for this particular species (Ruiz-Verdugo et al. 2001), the number of oocytes formed was reduced when compared to diploids. In a continuance of those studies with triploid catarina scallop, we report here the effects of triploidy on spermatogenesis, studied as the comparative structure and ultrastructure of 9-month-old and 12-month-old diploid and triploid catarina scallops.

MATERIALS AND METHODS

Scallops

Diploid and triploid catarina scallops about nine months age (6 cm in shell length), kept in a growout area at Bahía Magdalena in Baja California Mexico, were transported to the Genetic Laboratory of CIBNOR (Centro de Investigaciones Biológicas del Noroeste), and placed under maturation conditioning for 20 days. A second group of scallops that were not conditioned for maturation in the laboratory was sampled from the field area at age12 months. The triploid scallops were produced using cytochalasin-B (0.5 mg/L) as described by Ruiz-Verdugo et al. (2001), and the diploids were from a control group the same age.

Maturation Conditioning

Each ploidy group was stocked (five scallops per 40-L tank) and kept under standardized controlled maturation conditions during 20 days (Ramírez et al. 1999). Feeding consisted of adding a mixture of *Isochrysis galbana*, *Monochrysis lutheri*, and *Chaetoceros muelleri* at a total concentration of 4×10^6 cells / scallop / day. Water temperature was kept at 19–20°C, salinity at 36‰, and dissolved oxygen from 7–9 mg / L. The maturation room was provided with a 12-hour light and 12-hour dark photoperiod.

Sampling and Histology for Light Microscopy

After the 20-day maturation-conditioning period, the gonads from 20 scallops per ploidy group were fixed in buffered formaldehyde (10%), dehydrated in a graded ethanol series, and embedded in paraffin. Duplicated sections, 5 μm thick, were obtained from each gonad, and stained with hematoxilin-eosin (H&E) and Feulgen.

Spermatogenic stages in diploid and triploid scallops were measured using image analyses. Images were taken with an Olympus BX-41 microscope, with an integrated camera (CoolSNAP-Pro). The images were analyzed with SigmaScan Pro 5, obtaining an area by digitalizing contours and estimating diameters from the

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area for cytoplasm and nucleus of each spermatogenic stage. To correct for scale differences, for each microphotograph evaluated the image analysis program was calibrated accordingly to the microscope objective used for those images. For each ploidy group and stage (spermatogonium, spermatocyte, spermatid, and spermatozoa), 30 cells were measured.

Diameters of each spermatogenic stage for the two ploidy groups were analyzed with a single factor (ploidy) ANOVA, and mean differences tested with the F-test (Neter et al. 1985). Significance was set at $P < 0.05$.

To establish the impact of triploidy on both nuclei and cell size, a ratio between nucleus diameter and total cell diameter was estimated by dividing the nucleus diameter by the total cell diameter. Additionally, the increase in area (Area = 3.1416 × radius^2) in triploids was estimated for each cell and nuclei from the following relationship; [(Area in 3n × 100 / Area in 2n) − 100].

Electron Microscopy

Five gonad samples from each ploidy group were taken 20 days after laboratory conditioning (9-month old) as well as five from each ploidy group from 12-month-old field scallops.

The methodology described by Komaru et al. (1994) was followed for transmission electron microscopy (TEM). Gonad samples (2 mm^2) were fixed with a 4% gluteraldehyde solution in 0.1 M Sorensen's phosphate buffer, pH 7.5, for two hours at 4°C. The pieces were then washed in Sorensen's buffer solution three times for 30 minutes, and post-fixed in 1% osmium tetraoxide (OsO_4) in the same buffer for one hour, at 4°C. Samples were then dehydrated in ethanol (25, 50, 70, 95, and 100%) for 20 minutes in each concentration, and twice in 100% propylene oxide for 20 minutes. They were embedded according to the protocol of the commercial Kit Embed 812 Electron Microscopy Sciences (Luft 1961). Sections of 900Å were obtained with an ultramicrotome (Reicher, Model OMU3), mounted on a grid and stained with 2% uranyl acetate for 20 minutes and lead citrate for five minutes (Komaru et al. 1994). Photo microscopy was done using a JEOL JEM-1200EXII TEM.

Diameters of cell and nuclei of spermatogenic stages captured by photographic prints of TEM were measured after scanning the photomicrographs. To correct for amplifications, the size bar of each EM photomicrographs was used as a reference. All measurements were done using the image analyzer SigmaScan Pro 5. The number of stages measured varied, and depended on the number found in photomicrographs (6 spermatogonia, 15 spermatocytes and spermatids, 30 spermatozoa nucleus, mitochondria, and flagella, and 21 for acrosome length and invaginations in spermatozoa). The low number of captured spermatogenic stages in the triploids did not allow for measurement.

Scanning electron microscopy (SEM) gonad samples (4 mm^2) were fixed, post-fixed, and dehydrated in the same manner. Samples were dried in a SAMDRI-PVT-3P critical point dryer and coated with gold in an EDWARDS S150B sputter coating system (Komaru et al. 1994). Photo-microscopy was done using a JEOL JSM-5410LV SEM. All electron microscopy work was done at the Institute of Cellular Physiology in the National Autonomous University of Mexico (UNAM).

RESULTS

Gonad structure of nine-month diploid and triploid scallops are presented in Figure 1. In diploid scallops the characteristic synchronized development of sperm and oocytes in the gonad was seen (Fig. 1A). Compared to diploids (Fig. 1C), gametogenesis in triploids was retarded (Fig. 1B), and spermatogenesis was largely abnormal with only a reduced number of acini showing development of spermatogenic stages (Fig. 1D).

A summary of differences in mean size of spermatogenic stages between the two ploidy groups is in Table 1. The acini of triploid scallops were significantly larger (55%, $P = 0.01$) than those in diploids. There were also significant differences between ploidy groups in the diameter of all spermatogenic stages, as well as in their nucleus. Triploid scallops not only had larger nuclei area in spermatogonia and spermatocyte than diploid scallops (37% and 112% larger area in 3Ns, respectively), but also more cytoplasm area (78% and 119% more in 3Ns). Spermatids were not found in triploids, and comparative measurements between ploidy groups were not possible, but the few spermatozoa found indicated that triploids had a 50% larger area than diploids (Table 1). The nucleus/total cell ratio of spermatogonia was smaller for triploids (0.67) than diploids (0.76), whereas for spermatocytes it was approximately the same ratio for both ploidy groups (0.88 for 2Ns, 0.86 for 3Ns).

Diploid Male Gonad Structure and Ultrastructure

The details of the male part of the gonad of a diploid scallop are shown in Figure 2 and Figure 3. Male germinal cells were grouped in acini ranging from 120 to 200 μm in diameter. Each acinus contained a variety of developing stages distributed in a centripetal pattern from the inner acinus wall to the lumen (Fig. 2A). The spermatogonia (Spg) are oval shaped, and located nearest to the inner wall, whereas the spermatocytes (Spc) are smaller and loose from the acinus wall, positioned between the Spg and the lumen of the acinus. Mature spermatozoa (Spz) are almost exclusively confined to the central lumen of the acinus (Fig. 2A). The Spg had a diameter in ultrastructure measurements of 6.95 μm, and a nucleus 4.39 μm. The Spc appeared to be more numerous than the Spg (Fig. 2B), with a diameter approximately 3.92 μm, and a nucleus 2.93 μm. The Spc cytoplasm contained a complement of organelles, Golgi bodies (Gb) and mitochondria (Mi), very similar to those in the spermatogonia. The nucleus of primary spermatocytes (Spc1) exhibits scattered electron-dense chromatin (Fig. 2C), and its cytoplasm contains some Mi and Gb. Spermatids (Spm) in their early stages are spherical cells about 2.63 μm in diameter by ultrastructure measurements, with a dense nucleus, also spherical, about 1.95 μm in diameter (Fig. 2D). In the cytoplasm of the maturing Spm, Mi can be seen in what will be the basal pole of the future Spz.

The Spz have a proximal acrosome (Ac), nucleus (Nu), and a maximum of five cross-sectioned Mi in its distal part (Fig. 3A), where the flagellum (Fl) emerges. The anterior part of the spermatozoa, where the acrosome is, appears slightly denser than the nucleus. The ultrastructure of the spermatozoa in diploid *Argopecten ventricosus* can be divided into three main components: a sperm head consisting of the Ac and Nu, a middle piece consisting of two centrioles and the mitochondria, and a tail or flagella. The acrosome is invaginated toward the nuclear surface forming a conical structure about 0.51 μm in height, and it is composed of an outer layer with electron dense material and an inner layer of electron lucent material (Fig. 3A). The spherical electron-dense Nu is 1.82 μm in diameter and presents an anterior

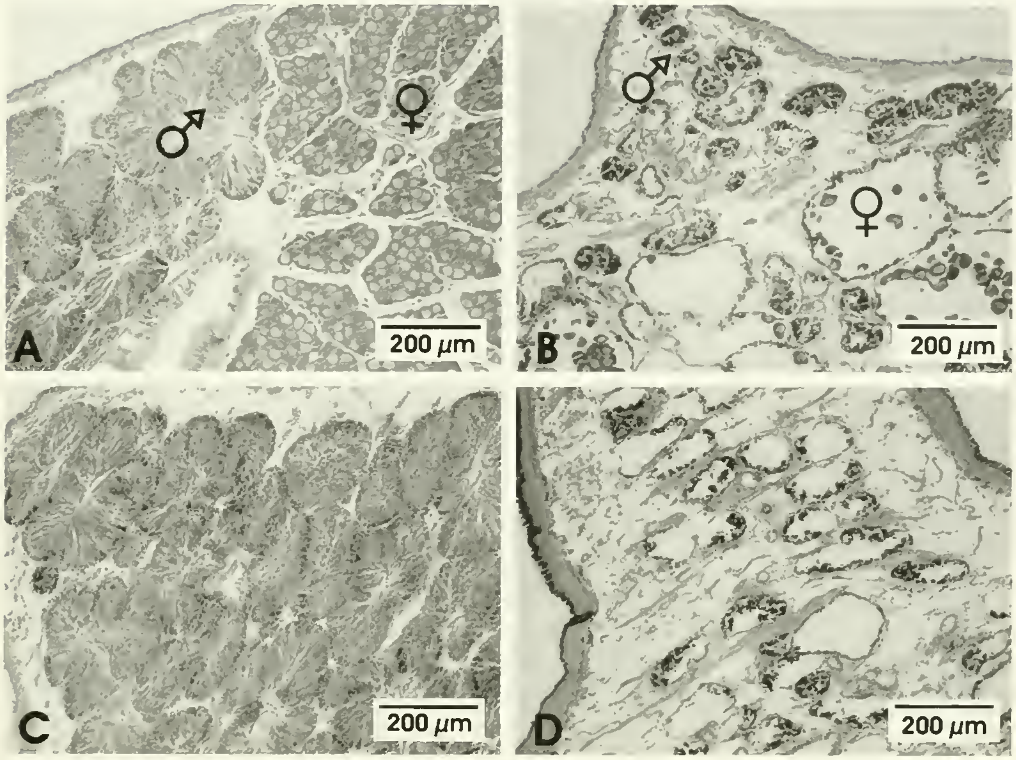

Figure 1. Diploid and triploid light photomicrographs. A. Diploid female and male gonads in advanced stages of gametogenesis. B. Triploid female and male parts of the gonad at 9 months old. C. Diploid male portion of the gonad in advanced stage of spermatogenesis. D. Triploid male part of the gonad with few spermatogenic stages developing.

invagination (Ai) 0.23 μm in depth (Fig. 3A), and a posterior invagination (Pi) 0.29 μm in depth (Fig. 3B). The distal mid-part of the spermatozoa head contains a ring of three to five cross-sections of mitochondria, each about 0.76 μm in diameter around two centrioles (Fig. 3B, 3C). The proximal centriole (Pc) is joined to the nuclear envelope by a satellite body (not shown) found in the post-nuclear fosse. The distal centriole (Dc) is joined and forms the basal body of the Fl, which has a diameter of 0.31 μm. Granules of glycogen (Gi) are detected between the mitochondria cross-section (Fig. 3B). Transversal cuts of the flagellum show the classical structure of nine external microtubule doublets (Md) and two internal single microtubules (Fig. 3D).

TABLE 1.

Mean diameter (SD) of spermatogenic stages ($n = 30$) in diploid and triploid catarina scallop *Argopecten ventricosus* measured by image analyses—light microscopy, and percent increase area in triploids with regard to diploids.

Spermatogenic stages	Diameter (μm) Diploid	Diameter (μm) Triploid	Percent area increase in 3N
Acinus^1	167 (36)^a	259 (47)^b	141
Spermatogonia^2	6.30 (0.43)^a	8.41 (1.64)^b	78
Spermatogonia nucleus^2	4.81 (0.36)^a	5.62 (1.11)^b	37
Spermatocyte^2	3.81 (0.31)^a	5.64 (0.44)^b	119
Spermatocyte nucleus^2	3.34 (0.32)^a	4.86 (0.36)^b	112
Spermatid^3	2.62 (0.25)	n.a.	
Spermatozoa^3 head	1.69 (0.08)^a	2.07 (0.15)^b	50
Number of sperm/ 1000 μm^2	250,000	2–3	

Superscript numbers in spermatogenic structures indicate magnification used for measurements: 1 = 4×; 2 = 10×; 3 = 100×. n.a. = not available for measurements. Different letters between ploidy groups indicate significant differences ($P < 0.05$).

Triploid Male Gonad Structure and Ultrastructure of Nine-Month-Old Scallops Conditioned in the Laboratory

Male acini of triploids were larger than in diploids, with a diameter varying from 197 to 387 μm. On the acinus wall dark colored cells representing Spg can be seen, and detached from the acinus wall some Spc were present. In some of the triploid scallops, Spz were seen sparsely distributed in the lumen of the acinus when using the Feulgen stain (Fig. 4A), and also when using scanning electron microscopy (Fig. 4B). The overall structure of the spermatozoa produced by a triploid catarina scallop appears to be similar to those produced by a diploid.

The spermatogonia were oval, and had the chromatin uniformly dispersed in small aggregations, with numerous mitochondria in the cytoplasm (Fig. 4C). As in diploids, the primary spermatocyte had a smaller size than the spermatogonia, and their cytoplasm contains a complement of organelles very similar to the spermatogonia (Fig. 4D). In the nucleus, the chromatin, set out in clusters, indicates the spermatocytes were in zygotene-pachytene stages of prophase I. There was no evidence in transmission electron microscopy of secondary spermatocytes and spermatids, although as already stated, some spermatozoa were observed by scanning electron microscopy (Fig. 4B).

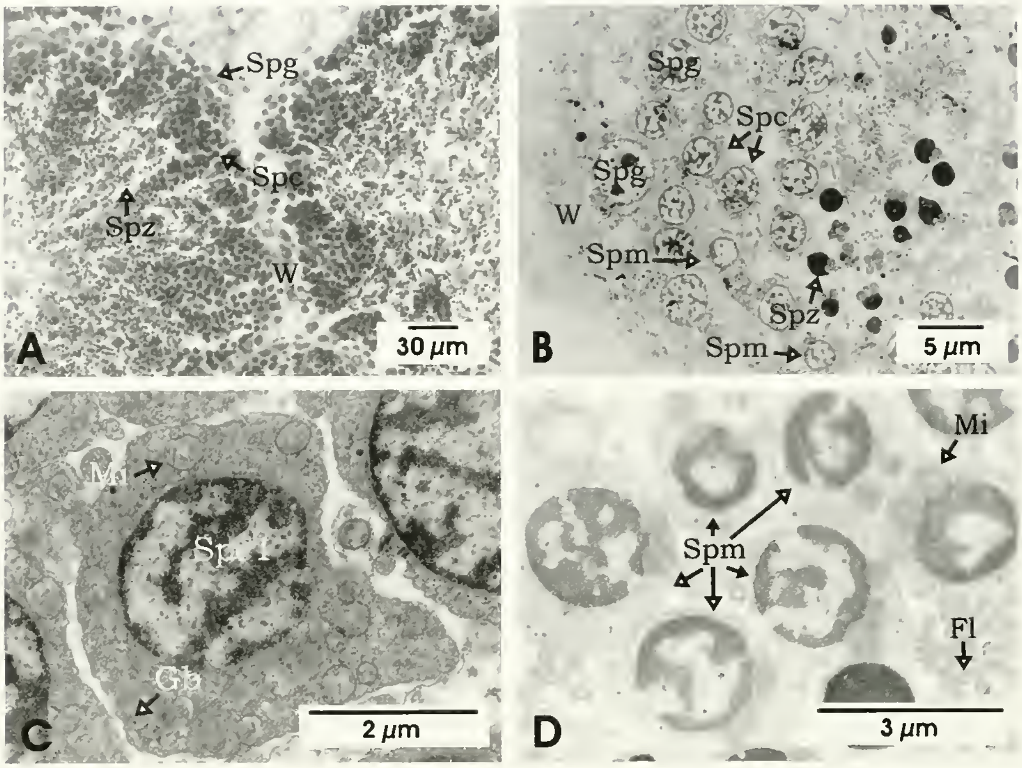

Figure 2. Diploid gonad structure and ultrastructure. A. Light microscopy (LM) of male acinus with spermatogonia (Spg) attached to the wall (W), spermatocytes (Spc), and spermatozoa (Spz). B. TEM of spermatogenic stages: Spg, Spc, spermatid (Spm), and Spz. C. TEM of primary spermatocyte with multiple mitochondria (Mi) and Golgi bodies (Gb). D. TEM of Spm with mitochondria at posterior pole, transversal cuts of flagella (Fl).

Triploid Male Gonad Structure and Ultrastructure of Field Scallops Sampled at 12 Months Old

The male part of the gonad in some triploid catarina scallops at age 12 months had a mixture of male and female acini, with both male and female gametogenic stages occurring in different acinus. However, most of the male germinal cells were in the process of degeneration. Large numbers of hemocytes were invading the male acini (Fig. 5A, 5B), which were characterized by the presence of degenerating spermatogonia and primary spermatocytes (Fig. 5C). The hemocytes were of the basophilic granulocyte type, with a not centrally positioned oval-rounded nucleus 4.5 μm in diameter, condensed chromatin, and an entire cell approximately 8 μm in diameter (Fig. 5D).

In most triploid scallops a gonad sex change occurred, as the male part of the gonad (the most distal part) was completely suppressed and female germinal structures were replacing the male germinal structures (Fig. 6A). Oocytes developing in the male part of the gonad of triploids (Fig. 6B) were similar to those found in the female part of the gonad of a diploid (Fig. 6C). They contained numerous vitelline inclusions, cortical granules and a vitelline coat.

DISCUSSION

The ultrastructure of diploid spermatogenesis was similar to that described by Dorange and Le Pennec (1989a) for *Pecten maximus*, with some size and morphology differences which is not surprising, as closely related species are known to have similar but not identical ultrastructure of gametic stages (Le Pennec & Beninger 1997).

Differences in cell size between triploid and diploid mollusks have been reported only for egg size (Guo & Allen 1994a, 1994c; Eversole et al. 1996; Utting et al. 1996; Ruiz-Verdugo et al. 2001) or sperm size (Komaru et al. 1994), but in triploid fish species an increase in size of different cell types has been reported (Valenti 1975; Ueno 1984; Small & Benfey 1987; Aliah et al. 1990). In the present study not only spermatogonia, but also all other spermatogenic stages measured indicated a larger cell size in triploids than diploids. Furthermore, an increase in spermatogenic stage size was not necessarily a correlate of increase in nucleus size. For example, there is an expected increase in the nucleus/total cell ratio of spermatocytes when compared to that same ratio in spermatogonia occurring as a consequence of nuclear distention (Dorange & Le Pennec 1989a). However, whereas the increased ratio in spermatocytes was observed in this study for both ploidy groups, the nucleus-total cell ratio in spermatogonia was smaller for triploids than diploids, and this occurred despite the larger nucleus in triploid spermatogonia than in diploids. This, as well as the increase in area estimated for triploid spermatogonia cell and nuclei indicates that a larger increase in cell size than nucleus size occurs in triploid spermatogonia of catarina scallop, and might be caused by a larger number of organelles (mitochondria, endoplasmic reticulum, ribosomes) than those found in diploids, as previously proposed by Guo and Allen (1994a) to explain the increased egg size of triploid oysters. These authors proposed that the increase egg size could be a consequence of a larger nucleus requiring a larger cytoplasm,

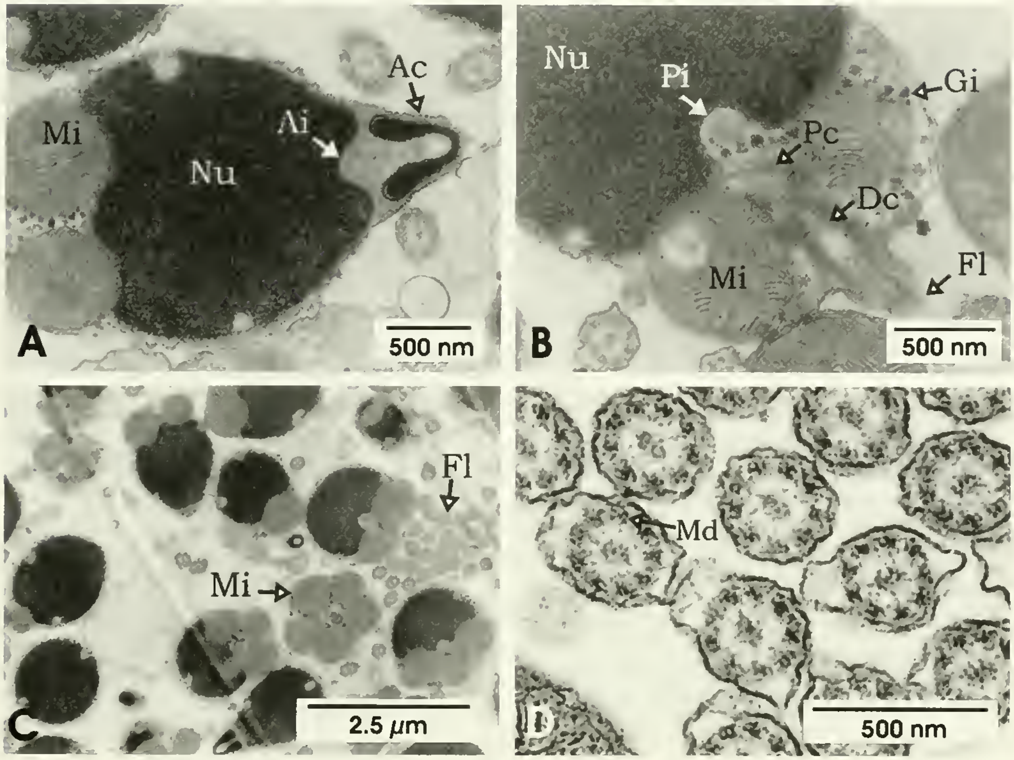

Figure 3. Diploid spermatozoa ultrastructure. A. TEM of section through a spermatozoon head, showing acrosome (Ac), anterior invagination (Ai), nucleus (Nu), and two cross-sections of mitochondria (Mi). B. TEM section of the spermatozoa mid-part; posterior invagination (Pi), proximal centriole (Pc), distal centriole (Dc), glycogen granules (Gi), mitochondria (Mi) and flagella (Fl). C. TEM of transverse section through the mid-part of spermatozoa showing five cross-sections of mitochondria at most. D. TEM of transverse section through flagella showing the classical structure of 9 external microtubule doublets (Md), and 2 internal.

such that allocation of nutrients and organelles is in adequate proportions during cell divisions. Unfortunately, because of the few spermatogenic stages found in triploids when electron microscopy was done, quantification of the number of organelles could not be accomplished.

The spermatozoa of triploid catarina scallop was morphologically similar to that in diploids, although as shown by Komaru et al. (1994) for the Pacific oyster, it showed a larger head than that in diploids. The diameter differences observed between diploid and triploid spermatozoa of catarina scallop correspond to an area for triploid sperm of 1.5 times that of a diploid sperm, and is most probably a consequence of an increased amount of DNA in the sperm produced by triploids. Komaru et al. (1994) found an increase area of 1.4 times in sperm from triploids when compared with diploids, and the increase in area in triploid spermatozoa of the Pacific oyster has been shown to agree with an increased amount of DNA (1.5 of that in sperm from diploids) by flow cytometry (Allen 1987; Guo & Allen 1994a). In spite of the size increase, the spermatozoa of triploid catarina scallop had the same number of cross-sectioned mitochondria than diploids. A larger number of organelles in gametic cells have been demonstrated only for tetraploid Mediterranean blue mussel, but not for sperm produced by triploid Pacific oyster. That is, Komaru et al. (1995) found that spermatozoa from tetraploid Mediterranean blue mussels had from 5 to 7 mitochondria with a mode of 6, whereas that from diploids had 5. Contrary to that, spermatozoa from triploid Pacific oyster, although larger than that in diploids, had the same number of mitochondria as diploids (Komaru et al. 1994).

The observation of spermatozoa in triploid catarina scallop, even if in low numbers was important because previously, Ruiz-Verdugo et al. (2000) were unable to observe sperm in triploids from this same species, but their histology techniques were limited to H&E staining and light microscopy. In the present study, the use of the Feulgen stain for light microscopy allowed for the finding of some spermatozoa in isolated acinus, an observation that was corroborated when spermatozoa were also found by scanning electron microscopy. However, even if present, the rare occasions in which spermatozoa were found indicates that the triploid condition in catarina scallop results for the most part in an arrest of spermatogenesis early during the process of gamete formation, at the primary spermatocyte stage. That is, the arrest occurs at meiosis I, when the secondary spermatocyte would be produced by the first meiotic reduction (Beninger & Le Pennec 1991). The nearly complete arrest of meiosis at the primary spermatocyte stage has been previously noted by ultrastructure analysis in other bivalves such as *Saccostrea commercialis* (Cox et al. 1996) and *Mytilus galloprovincialis* (Kiyomoto et al. 1996). Contrary to that, in triploid *Crassostrea gigas* spermatogenesis has been reported to be reduced, but not arrested at the primary spermatocyte as spermatid and spermatozoa production occurs (Allen & Downing 1990), the last one being capable of fertilizing eggs (Guo & Allen 1994a).

The finding that in older triploid catarina scallop the male portion of the gonad was replaced with female acini is similar to that reported by Ruiz-Verdugo et al. (2000) after studying the annual gametogenic cycle of diploids and triploids for this scallop. The few gametic stages still present in the male acini of scallops

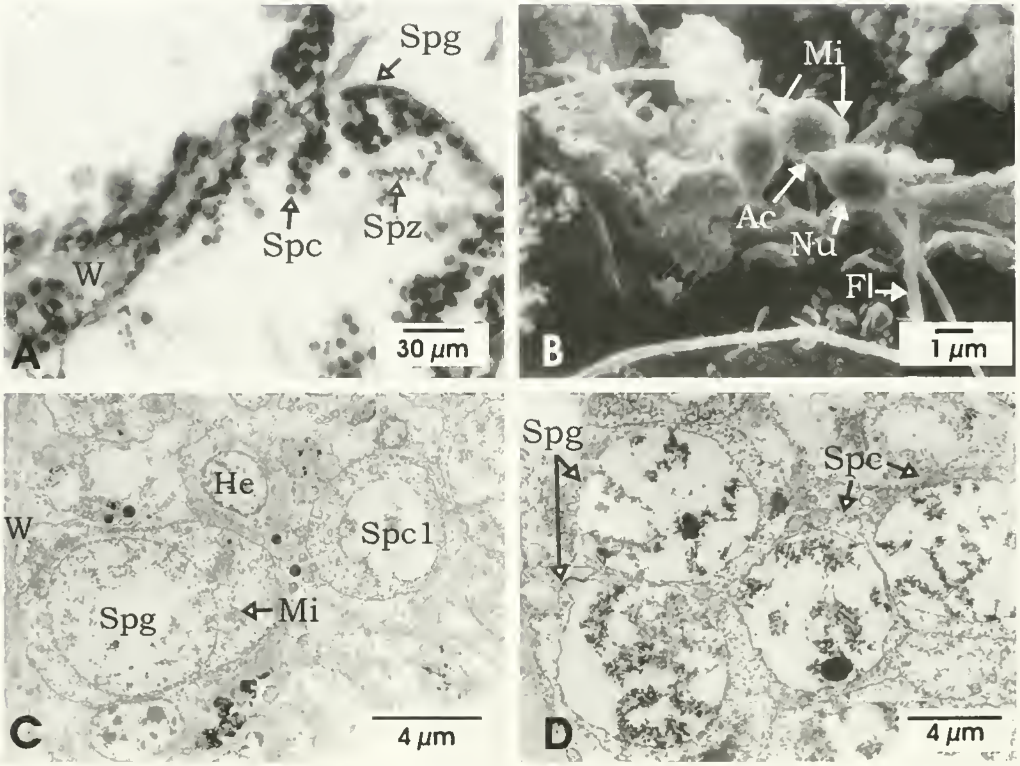

Figure 4. Triploid structure and ultrastructure of male part of the gonad. A. LM of section through a male acinus with few spermatogonia (Spg) by the wall (W), spermatocytes (Spc), and spermatozoa (Spz). B. Scanning electron microscopy (SEM) of spermatozoa found in a male acinus, with electron dense nucleus (Nu), acrosome (Ac), mitochondria (Mi) at the base, and flagella (Fl). C–D. TEM of section through a male acinus with spermatocytes (Spc1), spermatogonia (Spg), and haemocyte (He).

12 months old were being lysed and possibly recycled through phagocytic activity, as demonstrated by the presence of large numbers of hemocytes, which are known to play a role in phagocytosis of degenerating pectinids gametogenic structures (Dorange & Le Pennec 1989a, 1989b; Beninger & Le Pennec 1991). The hemocytes were the typical ones described for other bivalves such as *Mya arenaria* and *Mytilus edulis* (Cheng 1981).

With this study we have demonstrated that the male gonad of triploid catarina scallop is almost but not completely sterile, as the lack of male gametes occurs until after spermatogenesis begins and is arrested, observing later oogenesis in male acini. The cause of the reversal of sex in male acini of catarina scallop is not known, but might be a consequence of the arrest of meiosis signaling, possibly through a neuroendocrine mechanism, an arrest of synthesis or release of some type of maleness factor required for spermatogenesis. Neuroendocrine factors are known to be necessary for development of male and female gonad in some mollusks (Feral et al. 1987), or only needed for development of the male gonad in functional hermaphrodites for which female gonad results from auto-differentiation (Gomot & Griffond 1993). The existence of those neuroendocrine factors has been demonstrated by isolation in other mollusks (van Minnen et al. 1989), but not in pectinids.

With regard to the arrest of meiosis, it has generally been presumed that the primary cause for triploid sterility, measured as inability to form viable and large number of gametes as in diploids, is the inability of chromosomes to pair and complete synapses because of multivalent formation. However, it has been demonstrated at least for one species that this is not the cause of sterility. Guo and Allen (1994a) established that for the Pacific oyster, *Crassostrea gigas*, the normal synapse of chromosomes and normal segregation was not a necessary event to obtain mature gametes in triploids, as they observed that the extra set of chromosomes segregated randomly in spawned eggs from triploids. To explain the sterility induced in triploids it is interesting to point to new research being done in the molecular genetic field. During the last decade a series of "housekeeping" mechanisms or "checkpoints" operating in the cell cycle during meiosis have been described for a number of experimental organisms. Their function is to detain further development when problems during replication, recombination, or segregation are present in a cell, and it is possible to explain some of the abnormal events occurring in triploids gametogenesis by considering them. These checkpoints act by means of protein complexes signaling abnormalities in chromosome behavior, and effector proteins acting on those signals to delay or arrest the meiotic process (Roeder & Bailis 2000). For example, among the described checkpoint mechanisms for meiosis is a "S-phase or premeiotic replication checkpoint," which functions on detecting chromosomal lesions after replication, but before recombination (Murakami & Nurse 1999). A second checkpoint mechanism, the "recombination or pachytene checkpoint," is known to act on gametogenic cells that have not completed correctly the recombination between sister chromatids during meiosis I, or in which the synaptotemal complex is defective, and it will arrest meiosis at pachytene (Murakami & Nurse 1999, 2000; Roeder & Bailis 2000; Tarsounas & Moens 2001). A third checkpoint mechanism, the "metaphase checkpoint," has been described for arresting meiosis in metaphase I, by blocking the metaphase to anaphase transition when chromosomes are misaligned on the spindle apparatus. An interesting aspect of this checkpoint mecha-

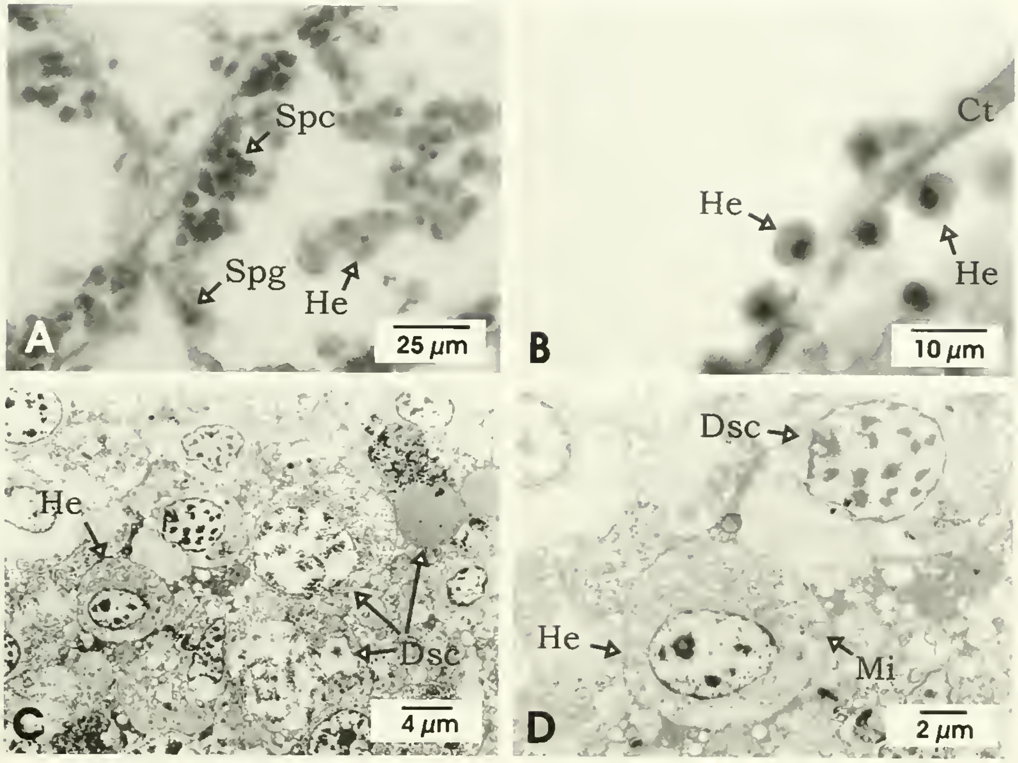

Figure 5. Triploid structure and ultrastructure showing degeneration of male spermatogenic stages. A. LM of section through a male acinus with spermatogonia (Spg) and spermatocytes (Spc) closest to the wall, and acinus filled with haemocytes (He). B. LM of connective tissue (Ct) at acinus wall with He. C. TEM of Spc in degeneration process (Dsc), surrounded by multiple He. D. TEM at larger amplification of He and Dsc.

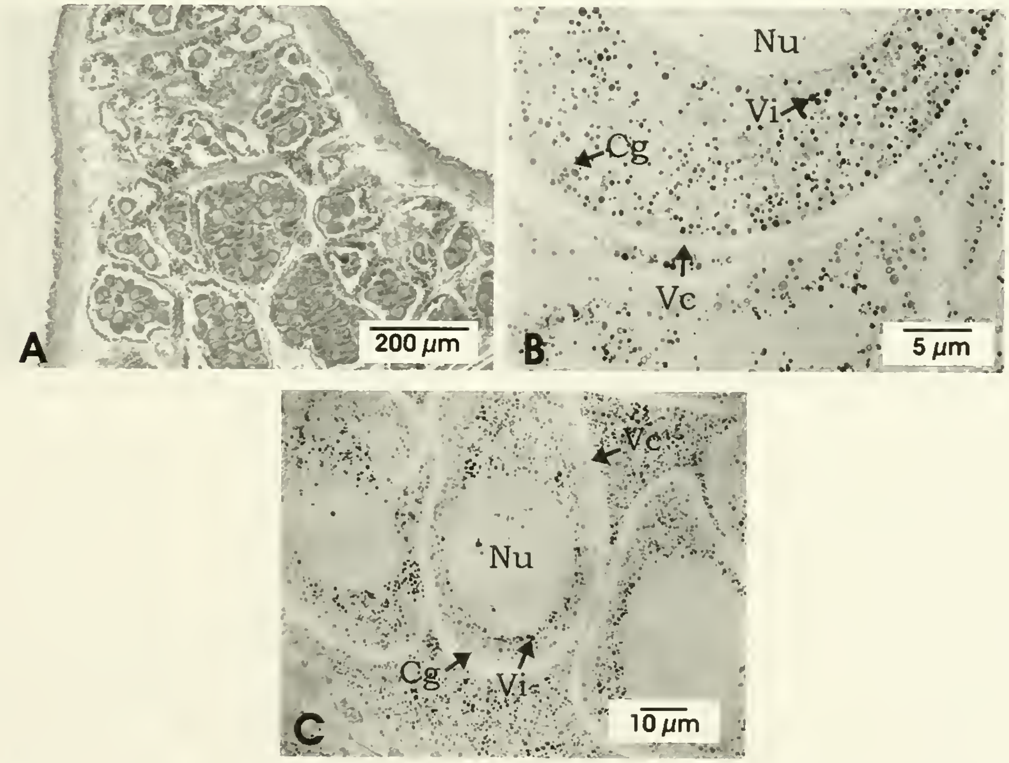

Figure 6. Gonad structure and ultrastructure of 12-mo old scallops. A. LM of male part of gonad in triploid occupied with female acini with oocyte development. B. TEM of oocytes in male part of the gonad of a triploid, showing nucleus (Nu), vitelline inclusions (Vi), cortical granules (Cg), and vitelline coat (Vc). C. TEM of oocytes from a diploid scallop with the same structures than that in triploids.

nism is that it is known to operate in mammalian males, halting spermatocytes at metaphase I, but oocyte formation is not affected or detained even when missegragation will result (for review see: Roeder & Bailis 2000). By considering the possible phenotypic effects of these three checkpoints on gametogenesis, we can parallel some of the known events in triploid gametogenesis. For example, the delay in the onset of gametogenesis known to occur in both sexes of triploids (Allen et al. 1986; Allen 1987; Allen & Downing 1990; Guo & Allen 1994b; Cox et al. 1996; Eversole et al. 1996; Kiyomoto et al. 1996; Komaru & Wada 1989, 1990; Ruiz-Verdugo et al. 2000) can be explained by the first checkpoint mechanism, the "replication checkpoint." If we can presume that DNA replication in triploids will have a larger probability of errors needing correction because more DNA is present, that could result in a delayed onset of gametogenesis. The delay reported to occur in triploid gametogenesis and not necessarily on their growth can be explained by a difference between meiosis and mitosis: the S-phase in meiosis usually takes from two to five times longer than the S-phase in mitosis (Murakami & Nurse 2000). The second checkpoint mechanism, the "pachytene checkpoint", agrees well with the observation that spermatogenesis is arrested at the pachytene-zygotene stage of prophase in meiosis I, at the primary spermatocyte, and also agrees with the fact that a lower number of vitellogenic and mature oocytes are formed in triploid catarina scallop than diploids (Ruiz-Verdugo et al. 2000, 2001), as it is known that in diploid scallops only oocytes that complete the pachytene stage and enter the diplotene stage will begin the process of vitellogenesis and become mature (Dorange & Le Pennec 1989b; Beninger & Le Pennec 1991). Further research on oocyte type frequencies in diploid and triploid mollusks are necessary to understand precisely at which stage oogenesis is halted.

In conclusion, this study has established spermatogenetic differences between diploid and triploid catarina scallop *Argopecten ventricosus*, pointing toward the halting of spermatogenesis occurring during prophase of the first meiotic division. Future studies are necessary to establish the level of ploidy and functionality of the few produced male gametes in triploids.

ACKNOWLEDGMENTS

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REPRODUCTIVE CYCLE OF *SPONDYLUS CALCIFER* CARPENTER, 1857 (BIVALVIA: SPONDYLIDAE) IN THE "BAHÍA DE LORETO" NATIONAL PARK, GULF OF CALIFORNIA, MEXICO

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ABSTRACT The reproductive cycle of *Spondylus calcifer* from Bahía de Loreto, Gulf of California, was studied from January 1998 to March 1999. Microscopic analyses established that this species is gonochoric. The size at spawning occurred at 113-mm in shell height. *S. calcifer* exhibits a short spawning period from August to October followed by a long inactive period from November to February. Increase in water temperature initiates the gonadal maturation process, while decrease in water temperature inhibits gametogenesis. The threshold water temperature for spawning in *S. calcifer*, is 29 C. Nutrient reserves from the digestive gland are mobilized and used by the gonad during the developing stage. Meanwhile, reserves stored in the muscle are used only after gamete production has started, helping to support the energetic output during the ripe and spawning stages. In Bahía de Loreto, the period of highest phytoplankton abundance in bottom waters coincides with the onset of gametogenesis in *S. calcifer*. This suggests that *S. calcifer* also depends on food intake for oocyte growth.

KEY WORDS: spawning season, histology, energy transfer, *Spondylus calcifer*, Gulf of California

INTRODUCTION

Spondylus calcifer Carpenter, 1857 commonly named "Burra clam," belongs to the Spondylidae Family. It is geographically distributed from the Gulf of California to Peru. This clam can be found from intertidal to subtidal zones, attached by the right valve to exposed boulders or under rock ledges (Skoglund & Mulliner 1996). At present in Loreto, Baja California Sur, Mexico, this species is not commercially exploited; however divers capture it (only in small quantities) for human consumption year around.

This clam is the largest species of the genus *Spondylus*. *S. calcifer* is considered in danger of extinction (Baqueiro et al. 1982), and is currently protected by Mexican laws (Norma Oficial Mexicana 1994). Despite the above, there are no published records on its biology. Few articles have been published about the genus *Spondylus*, most focusing on the taxonomy, anatomy, morphology, habitat, and other topics (Yonge 1973; Dakin 1928 a,b; Mata et al. 1990; Parth 1990; Okutani 1991; Skoglund & Mulliner 1996). There is only one study about the reproductive cycle and spawning season of *Spondylus leucacanthus* (Villalejo-Fuerte & García-Domínguez 1998).

The present study describes the reproductive cycle throughout 15 months, and the spawning season of *S. calcifer*, from histological analysis and measurements of the volumetric fraction of oocyte. Additionally, the relation between the reproductive cycle and gonadic, digestive gland and muscle yield indexes is analyzed.

MATERIALS AND METHODS

Sampling was conducted in Bahía de Loreto, Gulf of California, Mexico (25°48′54″, 111°15′45″) (Fig. 1). Fifteen to 30 specimens of *S. calcifer* were collected monthly, from January 1998 to March 1999, by scuba diving at 10-m depth. Shell height and wet weight of the gonad, digestive gland, muscle and total soft body were recorded for each clam after fixation in a neutral 10% formalin solution prepared with sea water. Water temperature was recorded at each sampling time. The photosynthetic pigment concentration (mg chlorophyll/m^3) in Bahía de Loreto from January 1998 to March 1999 was obtained from Seawifs Project, NASA/Goddard Space Flight Center, and this was considered as an estimation of the food availability for the clams.

Because *S. calcifer* does not exhibit sexual dimorphism, individual sex was determined through histological analysis. The sex ratio for the total sample was obtained. The null hypothesis of a 1:1 sex ratio was established and its significance was tested using a chi-squared analysis (Sokal & Rohlf 1979).

For histological studies, gonads were dehydrated in an alcohol series and embedded in paraplast. Sections (7 μm) were placed on slides and stained with hematoxylin-eosin (Humason 1979).

The classification of gonadal development was similar to that of *S. leucacanthus* (Villalejo-Fuerte & García-Domínguez 1998), which includes five development stages: undifferentiated, developing, ripe, spawning, and spent. To facilitate the description of the reproductive cycle, the monthly relative frequencies for all gonadal development stages were calculated.

The size at spawning in the population is defined as the smallest length at which 50% of females and males are spawning (Somerton 1980). The size at spawning was estimated as the shell height at which the 50% of cumulative frequency of clams in the spawning stage was attained.

Three indexes were calculated: gonad index (GI), digestive gland index (DGI) and muscle yield index (MYI). Each index was calculated by dividing the gonad, digestive gland or muscle wet weight (respectively) by the total soft body wet weight, and expressing the results as a percentage (Sastry 1970).

Furthermore, the monthly proportion occupied by developing and mature oocytes combined (volume fractions) were estimated by stereology (Lowe et al. 1982; MacDonald & Thompson 1988). The gamete volume fraction (GVF) was obtained from point counts in a Weibel eyepiece graticule mounted in an ocular microscope and applied to a gonadal section (100X). Three replicates were done for each gonad. The algorithm of Lowe et al. (1982) was applied: GVF = No. of positive counts/total points

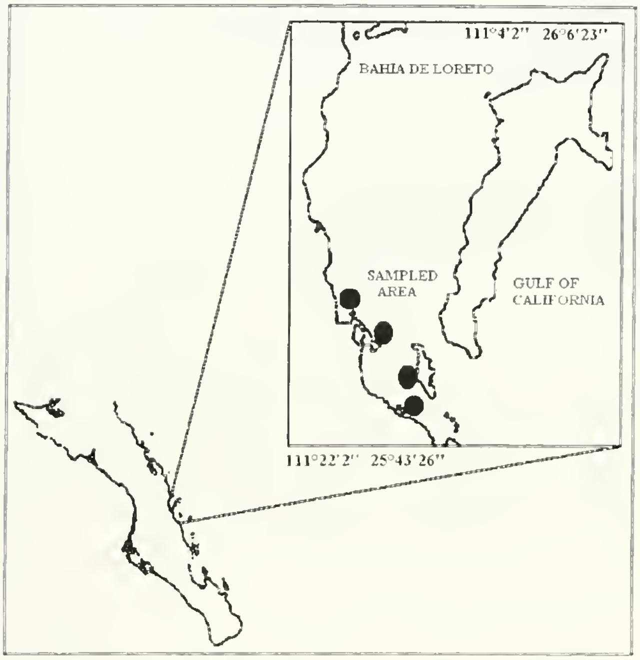

Figure 1. Location of sampling station in Bahía de Loreto, Gulf of California, Mexico.

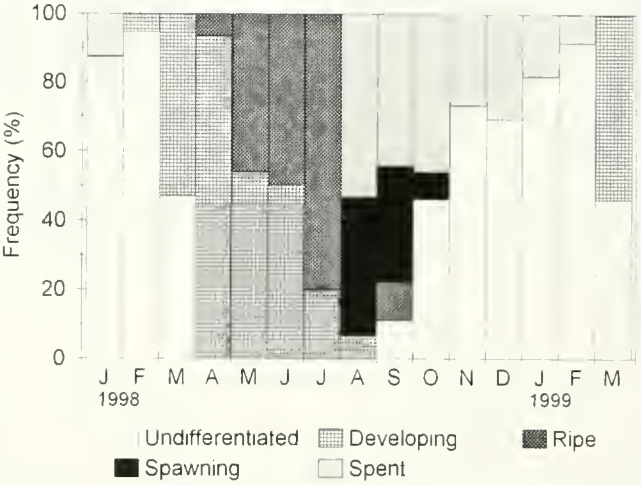

Figure 2. Reproductive cycle of *Spondylus calcifer* in Bahía de Loreto, Gulf of California. Relative frequency of gonadal stages between January 1998 and March 1999. Observations of males and females are combined.

counted X 100. GVF was calculated as the sum of the values for developing and mature oocytes in all females. The monthly mean GVF values were plotted.

A Spearman-rank correlation analysis was used to investigate the relationship between monthly mean values of GI, DGI, MYI, GVF, water temperature, and photosynthetic pigment concentration. As GI, DGI, and MYI are percentage values, the arcsine transformation (Sokal & Rohlf 1979) was used to attain data normality and homoscedasticity for statistical analysis.

RESULTS

A total of 223 clams were captured, of which 27% were females and 32% were males. The remaining (41%) were undifferentiated. The sex ratio for the total sample was 0.8 F:1 M and did not significantly differ from the expected ratio of 1:1 ($P > 0.05$). Shell height ranged from 70.5 mm to 165.2 mm, with the mode at 115 mm.

The reproductive cycle of *S. calcifer* was remarkably seasonal (Fig. 2). In January and February of both years, the majority of clams was inactive. Gametogenesis started in February in a small proportion (5.3%) of the clams. By March 52.9% of the clams were in the development stage. Development continued until August with the highest proportion occurring in April (93.7%). Ripe clams were found from April (6.2%) to July when the highest proportion was observed (80.0%). A small proportion of ripe clams was found in September (11.1%). Spawning was found in a higher proportion during August and September (40.0% and 33.3%, respectively) and dropped drastically in October (7.7%). From November to February, only undifferentiated and spent stages occurred.

Figure 3 shows that the size at spawning in the population of *S. calcifer* is 113-mm in shell height; however, individual organisms may begin spawning at 86 mm in shell height.

The GI shows its peak values during May, June, and July, coinciding with the occurrence of the highest frequencies of clams in the ripe stage. The GI declined from August to October, coinciding with the spawning season. A period of low values occurred from November to March, coinciding with the highest frequencies of spent and inactive stages (Fig. 4a).

The DGI shows high values in February, declining from March to June leave as is coinciding with the developing and ripe stages. In August and September (months in which the highest proportions of clams in spawning stage occur), the DGI values decrease. Afterwards, from October to March, DGI values start to rise again, coinciding with a reproductive inactivity of the specimens (Fig. 4b).

The MYI values decreased according to the maturation process, beginning the decrease in April, two months after the onset of gonadal development (February). The lowest MYI value was found in January 1999 and the higher value was found in March 1999 (Fig. 4c).

The volumetric fraction of gametes reflected the gonad devel-

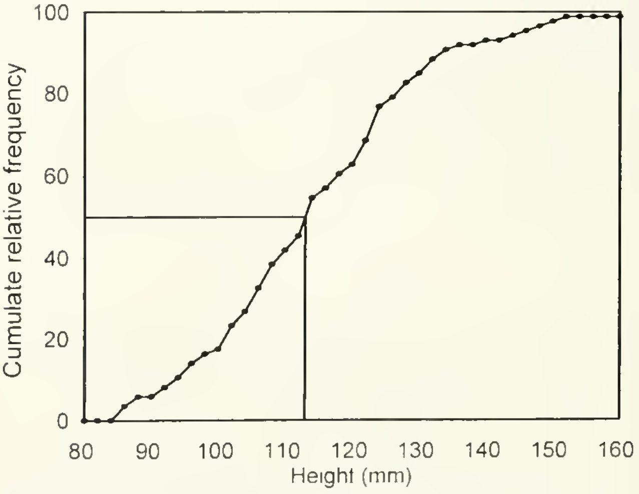

Figure 3. Size at spawning of 50% of the population of *Spondylus calcifer* in Bahía de Loreto, Gulf of California, Mexico.

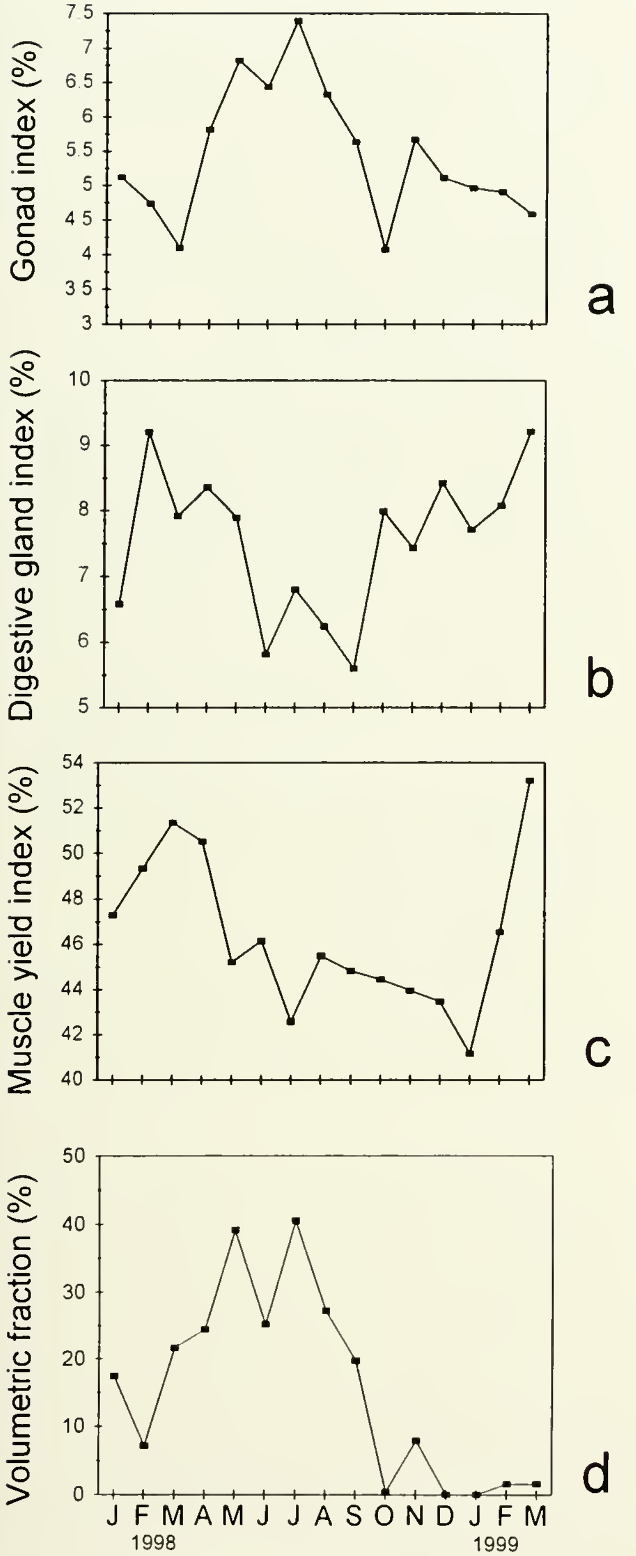

Figure 4. Variation in the mean values of a) gonad index, b) digestive gland index, c) muscle yield index, and d) oocyte volumetric fraction.

opment stages. Values increased when development and ripe stages were present, and they decreased concurrently with the spawning stage. Low values occurred when the inactivity and spent stages prevailed (Fig. 4d).

Seawater temperature varied seasonally from 19°C to 29°C during the study period. Temperature increased from March to August, reaching its peak (29°C) from August to October, then decreasing from November to February. The lowest temperature was recorded in February, both years (19.5°C and 19°C) (Fig. 5).

Photosynthetic pigment concentration (mg chlorophyll/m^3) in Bahía de Loreto was greater in the colder months than in the warmer ones. The maximum value was in March 99 (2.1 mg chlorophyll/m^3) and the minimum was in September (0.2 mg chlorophyll/m^3) (Fig. 6).

GI and GDI values were negatively correlated, with statistical significance ($P < 0.05$). GVF had a significant positive correlation ($P < 0.01$) with GI and a significant negative correlation with photosynthetic pigment concentration ($P < 0.05$). GDI showed a significant negative correlation with temperature ($P < 0.05$) and a significant positive correlation with photosynthetic pigment concentration ($P < 0.01$). The photosynthetic pigment concentration showed a significant negative correlation with temperature ($P < 0.01$). No significant correlation was found between MYI and any other variable ($P > 0.05$).

DISCUSSION

In the Gulf of California low densities of *S. calcifer* have been reported, ranging from 1 clam per 100 m^2 (from 1 to 25 m deep) to 1–5 clams per 25 m^2(>25 m depth) (Baqueiro et al. 1982). In this study, the number of organisms captured per month corresponds to these low population densities.

The histological analysis revealed that *S. calcifer* is a gonochoric species as no hermaphrodite or sex-reversal specimens were collected. However hermaphroditism has been reported in 3.8% of clams studied for *S. leucacanthus* from the same locality (Villalejo-Fuerte & García-Domínguez 1998). The sex ratio of *S. calcifer* was not different from 1:1, as also found for other bivalves like *Mercenaria mercenaria* (Heffernan et al. 1989), *Venus striatula* (Gaspar & Monteiro 1998), and *Megapitaria squalida* (Villalejo-Fuerte et al. 2000). No references regarding the sex ratio for this species were found in the literature. However, *S. leucacanthus*, a related and sympatric species, presents a sex ratio different from parity (Villalejo-Fuerte & García-Domínguez 1998).

The size at spawning in *S. calcifer* occurs at 113-mm in shell height, although specimens may start to spawn at 86-mm in shell height. In contrast, in the sympatric species *S. leucacanthus*, the size at spawning is 75-mm in shell height with some spawning specimens being as small as 40-mm in shell height. This difference in size at spawning may result from *S. calcifer* being a the larger species within the genus *Spondylus*.

The reproductive cycle of *S. calcifer* was remarkably seasonal. *S. calcifer* exhibits an annual reproductive cycle with a short spawning period from August to October followed by a long inactive period largely during the winter (November–February). The reproductive cycle of *S. calcifer* was similar to the one described for *S. leucacanthus* (Villalejo-Fuerte & García-Domínguez 1998). In both species, recruitment occurs once per year with a similar short spawning period. These characteristics correspond to a conservative reproductive strategy, similar to the one reported for *S. leucacanthus* (Villalejo-Fuerte & García-Domínguez 1998).

Gametogenic cycles are generally ruled by external environmental factors that may trigger and synchronize the "timing" of the different stages (Lubet 1983). The synchronization of the

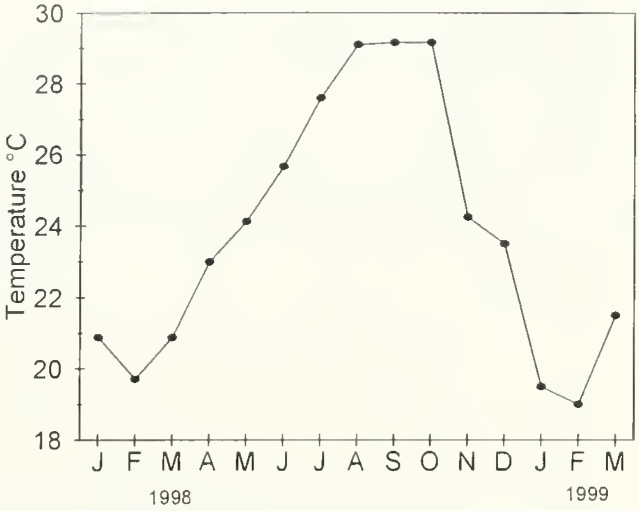

Figure 5. Variation of surface temperature from January 1998 to March 1999 in Bahía de Loreto, Gulf of California, Mexico.

gonadal cycles in a population is probably also the result of some kind of exogenous regulation (Gallardo 1989). Most studies consider that temperature is the important environmental factor in the regulation of bivalve reproduction (gametogenesis and spawning) (Giese and Pearse 1974; Sastry 1979; Barber & Blake 1981). However, in this study temperature was not correlated with GI. Nevertheless, the increase in water temperature coincided with the beginning of gametogenesis. The above suggests that the rise of water temperature may be responsible for triggering the gonad maturation process, however, laboratory studies are need to confirm it. In this respect, it had been previously observed that sudden increases in water temperature appear to be the final cue for stimulating maturation and ovulation in some fish species (Bye 1990).

In *S. calcifer* the inactive period (undifferentiated and spent stages) occurs from November to February, when water temperature drops (from 29°C in October to 19°C in February). Thus, it is reasonable to state that the decrease in water temperature inhibits gametogenesis, but newly laboratory studies are necessary to confirm this conclusion. Similarly, a cooler temperature inhibits gametogenesis in *Spisula solidissima* (Kanti et al. 1993). On the other hand, spawning in *S. calcifer*, only occurs when water temperature is at least 29°C (August to October). This fact suggests that 29°C is the threshold water temperature at which spawning occurs in *S. calcifer*. A threshold temperature for spawning has also been reported for some oyster species (Burrell 1985). In contrast, in other clam species, like *Paphies donacina* and *Cerastoderma edule* spawning occurs within a relatively wide temperature range (Navarro et al. 1989; Marsden 1999).

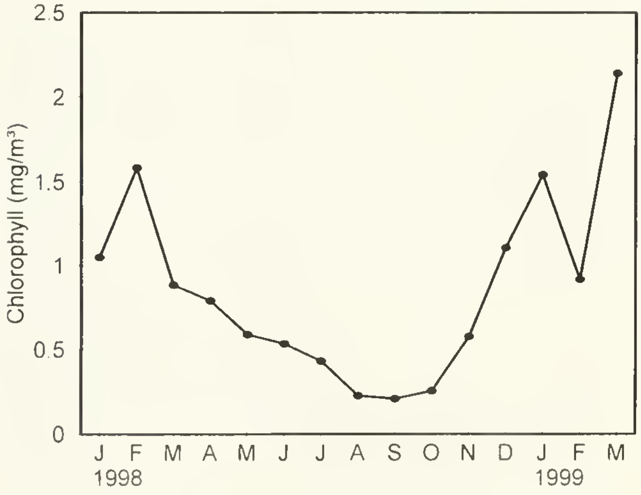

Figure 6. Variation of photosynthetic pigments concentration from January 1998 to March 1999 in Bahía de Loreto, Gulf of California, Mexico.

The bivalve gamete production is strongly influenced (set in a seasonal context) by environmental factors such as temperature but also by food availability (MacDonald & Thompson 1985). However, both factors may be strongly related. In this work, the photosynthetic pigments concentration was negatively correlated with temperature. Then the higher food availability for clams (expressed as photosynthetic pigment concentration) were during the cold months coinciding with the resting period. On the other hand, the spawning season of *S. calcifer* coincide with the lowest food availability (August to October). A relation of spawning time and food availability was found in *Chlamys amandi* (Jaramillo et al. 1993), whereas *Hinnites giganteus* showed no correlation between food availability and spawning (Malachowski 1988).

The DGI had a significant positive correlation with photosynthetic pigment concentration, then the higher food availability and the higher DGI values were during the inactive period of reproduction. The trend observed for DGI suggests that a period of accumulation of nutrient reserve in the digestive gland takes place during the inactive period. These nutrient reserves are mobilized and utilized by the gonad during the developing stage. The significant negative correlation between DGI and GI confirms the above for *S. calcifer*. In mollusks, it has been observed that the onset of the oocyte growth phase is dependent upon the accumulation and transfer of nutrient reserves from the digestive gland to the gonad (Sastry 1968; Gabbott & Bayne 1973).

Despite MYI and GI not being correlated, MYI values start to decrease in April, just two months after gametogenesis had started. This suggests that there is a delayed in energy transfer from muscle to gonad. The reserves accumulated in the muscle are used up after gamete production had started, helping to support the energetic output during the ripe and spawning stages. Similarly, in *Argopecten irradians* it appeared that lipids from the digestive gland were used to fuel the beginning of gametogenesis whilst adductor muscle reserves become important later on to complete gonadal development (Barber & Blake 1981). A relationship between MYI and GI has been observed in *Pecten maximus* (Comely 1974; Faveris & Lubet 1991), *Argopecten irradians* (Sastry 1966), *Patinopecten yessoensis* (Mori 1975), *Placopecten magellanicus* (Robinson et al. 1981), and *Argopecten circularis* (Villalejo-Fuerte & Ceballos-Vázquez 1996).

However, in pectinids and other bivalves, it has been proposed that oocyte growth is dependent on two factors: food intake and energetic storage in specialized organs (Sastry 1963, 1966, 1968; Barber & Blake 1983). In *P. magellanicus* and *A. circularis*, the energy for gamete maturation comes from both stored reserves and ingested food (Thompson 1977; Robinson et al. 1981; Luna-González et al. 2000).

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BACULOVIRUS-LIKE PARTICLES IN EPITHELIAL CELL OF DIGESTIVE DIVERTICULA OF THE SCALLOP, *PATINOPECTEN YESSOENSIS*

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ABSTRACT Virus-like particles were encountered in one epithelial cell of digestive diverticulum in a clinically healthy scallop, *Patinopecten yessoensis*. Virions, showing typical rods, were found in the cytoplasm without marked cellular degenerative change and were recognized as ovoid or spherical bodies, depending on sectional orientations. The envelope was approximately 12 nm in thickness and the space between the envelope and nucleocapsid was about 10 nm. Average length and diameter of the complete virions were 520 and 130 nm, respectively. No occlusion bodies were observed. From this morphological evidence, it was considered that the virus particles observed in one epithelial cell of digestive diverticulum in the scallop are likely to be a species of nonoccluded, baculovirus type C, which are morphologically very similar to the baculovirus associated with white spot syndrome (WSS) and baculoviral mid-gut gland necrosis (BMN) in shrimp.

KEY WORDS: bivalve, *Patinopecten yessoensis*, baculovirus, white spot syndrome (WSS), baculoviral mid-gut gland necrosis (BMN)

INTRODUCTION

A number of infectious diseases have been observed in bivalves such as oysters, clams, mussels, and scallops. Because bivalve production represents a considerable proportion of fisheries industry in South Korea, much attention is now being paid to the cause of the massive deaths occurring in bivalve farms along the southern coast. To date, diseases have been reported from 63 species of parasites, 7 strains of bacteria, 2 strains of fungi, and 3 strains of rickettsia or chlamydia. More recently, five kinds of viral diseases were reported from oysters (Fisher 1988; Sindermann 1990; Couch & Fournie 1993; Faisal & Hetrick 1994; Woo 1995). There are two other kinds of diseases with unknown cause. For most of the diseases cited, little is known regarding pathologic effects on the host and methods of prevention and control.

Virus particles were, by chance, encountered in one epithelial cell of digestive diverticulum during a study on the relationship of histological structures in digestive diverticula to nutrient accumulation. The morphological characteristics of the particles are described in comparison with other virus particles and discussed with respect to pathogenic potential.

MATERIALS AND METHODS

Thirty scallops were sampled every month from March 1982 to December 1983 from a bottom culture farm in Abashiri waters of Hokkaido, Japan. Each individual, ranging from 8.5 to 11.2 cm in shell height and from 62.9 to 197.4 g in total weight, was clinically healthy. For transmission electron microscopy, all scallops were necropsied and their digestive diverticula were carefully removed, then diced into 1-mm cubes. All tissue cubes were prefixed with a 5% glutaraldehyde in 0.1 M cacodylate buffer (pH 7.4, 4°C). After postfixation with 0.2 M cacodylate buffered 2% OsO_4 for about an hour, tissue blocks were dehydrated in a graded series of ethanol and embedded with Epon 812. Ultrathin sections were obtained using an ultramicrotome (Porter Blum II, Agwam, Massachusetts, USA) and stained with uranyl acetate and lead citrate. The grids were examined with a transmission electron microscope (JEOL 100B, Tokyo, Japan) under an accelerating voltage of 80 kV.

RESULTS

As shown in Figure 1, one epithelial cell containing viral particles corresponded to type I of the 3 types of cells that have been classified by Chang et al. (1989). Because of the shortness of apico-basal length, it appears to be embedded between the other types of cells. Cytoplasm of the virus-containing cell was completely occupied basally by a densely packed mass of rough endoplasmic reticulum (RER) and apically by a round area containing a number of viral particles.

A great number of virions had a fence-like array, which again formed several layers, roughly in parallel. Virions showed typical rods in shape but, depending on the sectional orientation, they were ovoid or spherical (Fig. 2). Virions were present among a great number of small vacuoles that appeared to be a network of proliferated, tubular smooth endoplasmic reticulum (TER). A layer of variably sized, unidentified vacuoles surrounded the virus-containing area (Fig. 3). The vacuoles, ranging from 520 to 1050 nm in diameter, were spherical in shape and clearly limited by double membranes. They also had thread-like or fuzzy materials with high electron density. No other morphological abnormalities were found in this virus-infected cell.

Each virion had a nucleocapsid and an envelope of unit membrane that was quite similar morphologically to baculovirus (Fig. 4). The average thickness of the envelope was 12 nm and the average distance from the nucleocapsid was 10 nm. The average length and diameter of virions were 520 and 130 nm, respectively. No occlusion bodies were observed.

DISCUSSION

There have been few reports on viral diseases in bivalves, and most of these were reported for oysters. Since the first identification of birnavirus from clam, *Tellina tenuis* (Hill 1976), retrovirus-like particles from clam *Mya arenaria*, iridovirus-like particles from oyster *Crassostrea angulata*, and herpes-like particles from

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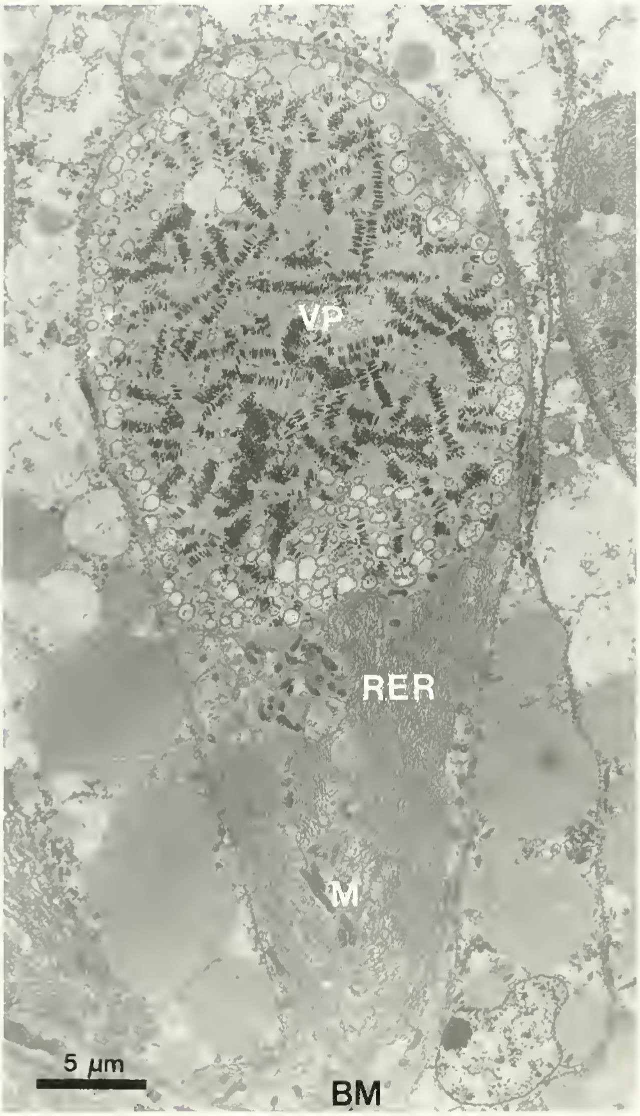

Figure 1. Virus-infected type I cell in digestive diverticula epithelium. This cell was very similar to pancreatic exocrine cells of mammalian pancreas. Cytoplasm near the basement membrane (BM) of the cell contained many large flattened cisternae of the rough-endoplasmic reticula (RER) and relatively few mitochondria (M). The apical part of the cytoplasm was without marked degenerative change, but the cell was filled with numerous viral particles (VP).

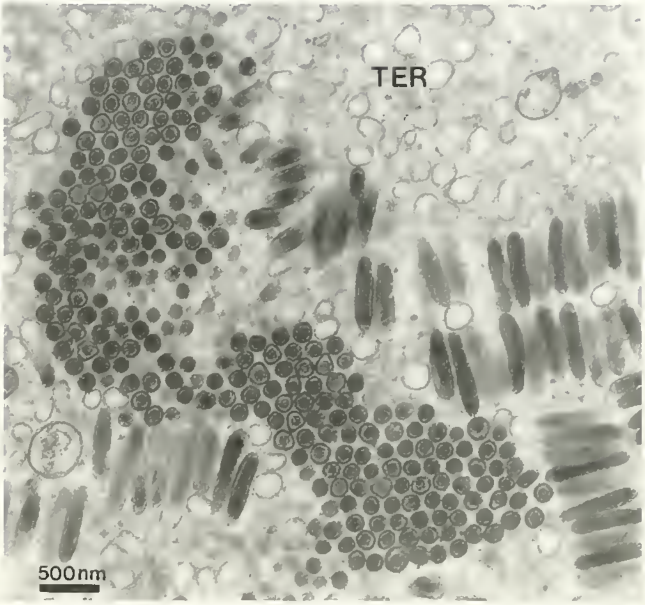

Figure 2. Fence-like array of viral particles. Numerous viral particles surrounded by tubular smooth endoplasmic reticula (TER) were rod-shaped, spherical, or ovoid in morphology, because of the difference in viewing orientation.

C. virginica have been reported (Perkins 1993). Since oyster velar virus disease (OVVD) caused by iridovirus was reported from farmed *C. gigas* (Elston & Wilkinson 1985; Sindermann & Lightner 1988; Bower et al. 1994), descriptions followed on small and nonenveloped virus particles from digestive diverticulum of pearl oyster *Pinctada maxima* (Pass et al. 1987), green lip mussel *Perna canaliculus* (Jones et al. 1966), herpes-like particles from hemocytes of flat oyster *Ostrea angasi* (Hine & Thorne 1977) and enterovirus or calicivirus-like particles from digestive diverticula of scallop *Pecten novaezelandiae* and toheroa *Paphies ventricosum* (Hine & Wesney 1977).

Virus particles in this study were morphologically different from those particles previously reported, but were quite similar to baculovirus that has not been described from bivalves. In general, baculoviruses are rod-shaped DNA viruses, which are known to be infective only in invertebrates. Baculoviruses are divided into the three subgroups of nuclear polyhedrosis virus (NPV, type A), granulosis virus (GV, type B), and nonoccluded virus (NOV, type C) (Sindermann & Lightner 1988; Faisal & Hetrick 1994; Levy et al. 1988).

From shrimps, six kinds of baculoviruses, including baculovirus penaei disease (BP virus disease), monodon baculovirus disease (MBVD), baculoviral mid-gut gland necrosis (BMN), plebejus baculovirus disease, and yellow-head disease (YHD), have been described, although their pathogenesis is still poorly understood (Faisal & Hetrick 1994). The causative agent responsible for white spot syndrome (WSS), which was responsible for mass mortality of shrimps in Taiwan during the last 7 years, was identified to be a species of baculovirus (Wang et al. 1997).

All baculoviruses reported in shrimps range from 150 to 310 nm in length and 33 to 74 nm in diameter. Virions in the scallop were very similar in size to causative viruses responsible for BMN and MBVD in shrimps. The finding that they did not form occlusion bodies is consistent with the viruses responsible for BMN and WSS in shrimps. The causative virus of BMN belongs to NOV type C that is found only within the hypertrophied nucleus. BMN frequently brings about mass mortality in an early larval stage of shrimp. Histologically, BMN is characterized by necrosis of epithelial cells in the hepatopancreas and mucosal membranes with a variety of nuclear changes, including severe hypertrophy, margination and loss of chromatin, and loss of nucleoli (Momoyama & Sano 1988; Momoyama & Sano 1989). In contrast, WSS is characterized by homogeneous nuclei with a variable degree of nuclear hypertrophy in nearly all kinds of tissue except for hepatopancreatic epithelium (Wang et al. 1997).

Even though grossly and microscopically no marked lesions were found in association with the presence of viral particles in the scallop, it was considered that the viral particles in this animal should be carefully monitored for the pathogenic potential.

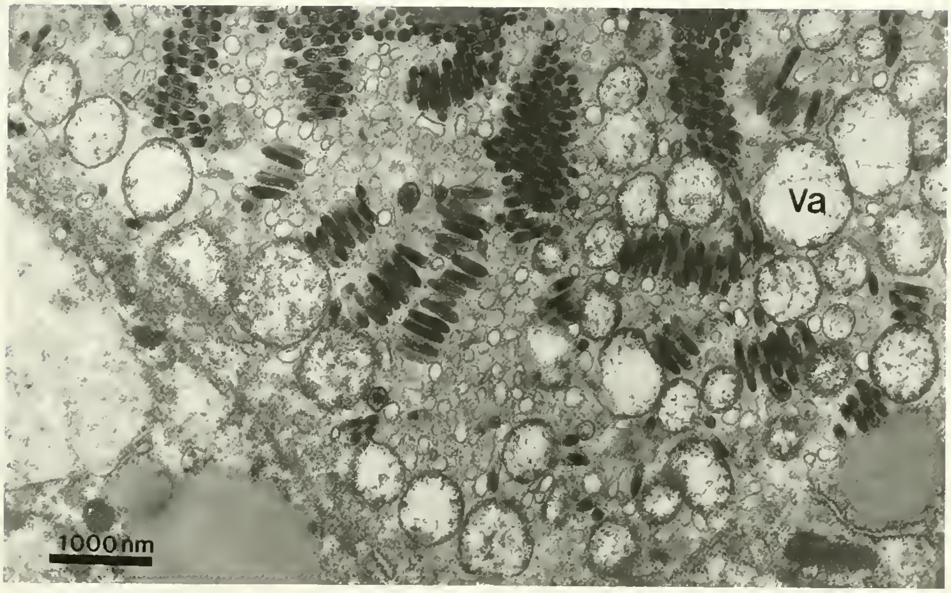

Figure 3. The cluster of viral particles was surrounded by unidentified vacuoles (Va).

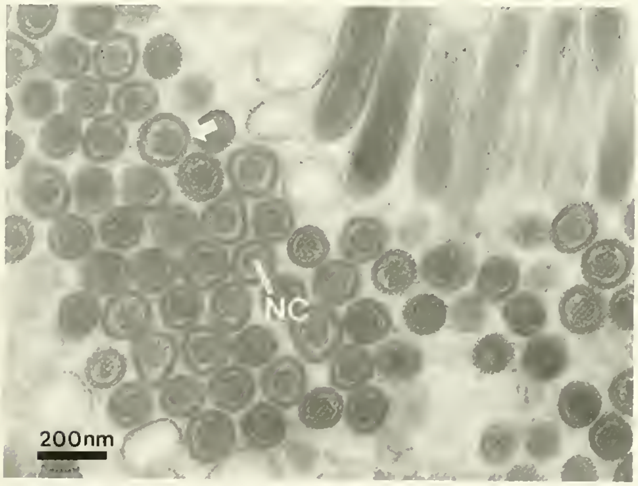

Figure 4. Higher magnification of Figure 2. Single envelope (arrow) and a central nucleocapsid (NC) were recognized in each viral particle. The clear spaces between envelopes and nucleocapsids were recognizable from all orientations of viral particles.

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HAPLOSPORIDIUM COSTALE (SEASIDE ORGANISM), A PARASITE OF THE EASTERN OYSTER, IS PRESENT IN LONG ISLAND SOUND

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ABSTRACT A haplosporidian parasite, *Haplosporidium costale* (seaside organism or SSO), is associated with high mortalities of eastern oysters (*Crassostrea virginica*) in seaside bays of Virginia and Maryland. Its presence in Long Island Sound has been tentatively suggested in several publications for the last 50 y. Positive identification of *H. costale* and differentiation from another haplosporidian parasite, *Haplosporidium nelsoni* (MSX), from histological sections is difficult and requires the presence of spores. We detected *H. costale* spores in 4 out of 5010 (0.08%) oysters collected from Long Island Sound in 1997–1999. *In situ* hybridization using an oligonucleotide DNA probe designed to detect small subunit ribosomal DNA from Virginia's *H. costale* reacted positively with tentative *H. costale* plasmodia in 5 oysters from Long Island Sound. In each case there was a coinfection of *H. nelsoni*. In Virginia and Maryland, *H. costale* has historically sporulated in all infected animals in May–June. In Long Island Sound, the rare sporulating cases were detected in October–December, suggesting a different infection cycle.

KEY WORDS: *Haplosporidium costale*, eastern oyster, *Crassostrea virginica*, *in situ* hybridization, Long Island Sound

INTRODUCTION

A haplosporidian parasite, *Haplosporidium costale*, was identified as the causative agent of seaside organism (SSO) disease, resulting in high mortalities of eastern oysters (*Crassostrea virginica*) on the Atlantic coast of Virginia (Wood & Andrews 1962). Prevalence, mortality and infection cycle of *H. costale* have been thoroughly studied in Virginia and Maryland, but information from other geographical locations is scarce. In seaside bays of the Delmarva Peninsula, the first plasmodia can be detected in the epithelia of digestive tubules in April–May. Plasmodia rapidly proliferate in May and sporulate synchronously. Peak mortality of the oysters is in May–June, when dying oysters release spores in seawater to initiate a new infection cycle. New infections remain subpotent until spring of the following year. Mortality of oysters has been 20% to 50% annually in the seaside bays of Virginia (Andrews 1988; Andrews & Castagna 1978).

Reports of *H. costale* distribution north of Virginia's Atlantic coast are inconsistent. Several reports suggest the presence of plasmodia resembling *H. costale* in eastern oysters in Long Island Sound. According to Andrews (1984, 1988), SSO disease ranges from Cape Charles, Virginia, to Maine, but is important only in high-salinity bays (>25‰) from Cape Henlopen, Delaware, to the Virginia capes. He stated that the pathogen is regularly present from New York to Massachusetts, but mortality has not been a serious problem. However, a mortality event in Long Island Sound in 1953 may have been caused by *H. costale* (Andrews 1988). Sampling locations, dates, prevalences, or possible presence of spores were not mentioned in these reports.

Newman (1971) studied 1,337 oysters from New Haven Harbor, Connecticut, from 1966 to 1967. He found five specimens (0.4%) infected with plasmodia morphologically similar to *H. costale*, with one of the oysters moribund with heavy infection. No sporulation was detected. Meyers (1981) described haplosporidia-like plasmodia (4%) in juvenile oysters ($n = 68$) collected from Oyster Bay, north shore of Long Island, New York, from 1975 to 1976. He did not find similar organisms in adult oysters ($n = 145$). No effort was made to distinguish between different haplosporidian species because sporulating stages were not present. In addition, plasmodia morphologically identical to *H. costale* were observed in oysters transplanted from the vicinity of New Haven, Connecticut, to Tomales Bay, California, in 1967–1968 (Katkansky & Warner 1970). Six specimens with tentative *H. costale* infection (four moribund and two living) were reported, one with spores. Total number of oysters studied was not mentioned.

Reports of tentative *H. costale* infections in Long Island Sound reviewed above are based on histological examinations. Reliable diagnosis of *H. costale* on histological sections is nearly impossible when sporulating forms are not present. *H. costale* plasmodia can be easily misdiagnosed as *Haplosporidium nelsoni* (MSX), another haplosporidian oyster parasite enzootic to the area (for review, see Ford & Tripp 1996). During routine monitoring for oyster diseases in Connecticut, we found several oysters with spores and plasmodia consistent with descriptions of *H. costale*. We used a DNA probe, designed to detect *H. costale* from Virginia as the *in situ* hybridization (ISH) probe for these specimens to verify the presence of *H. costale* in Long Island Sound.

MATERIAL AND METHODS

The State of Connecticut, Department of Agriculture, Bureau of Aquaculture routinely receives oyster samples for histological diagnosis from Connecticut's commercial oyster companies. Most of the seed originates from natural seed beds, though some hatchery-raised seed is also used. Every oyster is transplanted an average of four times before it is marketed, which exposes it to possible parasitic infections in several different sites. Seventeen oyster samples were studied in 1997, 63 in 1998, and 87 in 1999. Each sample consisted of 30 oysters, for a total of 5,010 oysters. Samples represented the entire Connecticut shoreline and the north shore of Long Island, New York. Of the 167 samples, 20 originated from New York. Fifty-six of the samples originated from oyster nursery systems (5 from upwellers, 51 from suspended cultures), 30 from off bottom cultures, and 81 from natural oyster beds. Tissues were fixed in Davidson's fixative in 20‰ artificial seawater. Six-micrometer-thick paraffin sections were stained with hematoxylin-eosin. Samples with *H. costale* or *H. nelsoni* prespores and spores were stained also with Ziehl and Harris' hematoxylin according to Farley (1965).

The ISH procedure was conducted with 27 oysters, two with presumed *H. costale* spores and 25 oysters with haplosporidian plasmodia. Specimens were selected for ISH as follows: (1) specimens with haplosporidian plasmodia from samples in which *H. costale* spores were detected; (2) specimens with small plasmodia with central nucleoli, morphology that is considered to be characteristic for *H. costale*; and (3) specimens with plasmodia in the stomach, intestine, or digestive tubule epithelia, locations that are considered to be characteristic for *H. costale*. For the ISH procedure, 6-μm-thick sections from these oysters were deparaffinized and ISH was performed on consecutive sections as previously described (Stokes & Burreson 1995; Stokes & Burreson 2001). Two commercially synthesized digoxigenin-labeled oligonucleotide DNA probes were used: a 22-base oligonucleotide (SSO1318) specific for *H. costale* (Stokes & Burreson 2001) and a 21-base oligonucleotide (MSX1347) specific for *H. nelsoni* (Stokes & Burreson 1995). A negative control was performed by substituting DNA probes with distilled water during hybridization.

RESULTS

Haplosporidium costale was detected in three locations on Connecticut's shoreline: Norwalk, Branford, and Clinton (Fig. 1). *H. costale* was diagnosed in seven different specimens either by the presence of spores or by a positive ISH result. There were 17 oysters with haplosporidian spores among the 5,010 oysters studied (0.3%). Four oysters had a mixture of *H. costale* and *H. nelsoni* spores (0.08%). The remaining 13 contained only *H. nelsoni* spores. Locations, dates, and seed origin of oysters with spores are listed in Table 1.

H. costale and *H. nelsoni* spores differed in size, form, and location in oyster tissues. *H. costale* spores (3 × 4 μm), the sporoplasm of which stained bright red with acid-fast stain, were detected throughout the connective tissue. Prespores, which did not retain acid-fast stain, occurred inside sporocysts throughout the tissues. *H. costale* spores were found between vesicular connective tissue cells surrounding the digestive diverticula (Fig. 2A), in connective tissue of the gills, in the adductor muscle, in the heart, between neurosecretory cells in the ganglia, and between kidney tubules. On rare occasions, *H. costale* spores were detected inside digestive tubule or digestive duct cells, in the lumens of the digestive tubules and intestine, in epithelial cells of the intestine, or in the follicles.

H. nelsoni spores (5 × 7 μm), which also stained bright red with acid-fast stain, were in most cases restricted to digestive epithelial cells (Fig. 2B). However, during intense sporulation in four specimens there was an overspill to digestive duct cells and the connective tissue surrounding the digestive tubules. In cases with

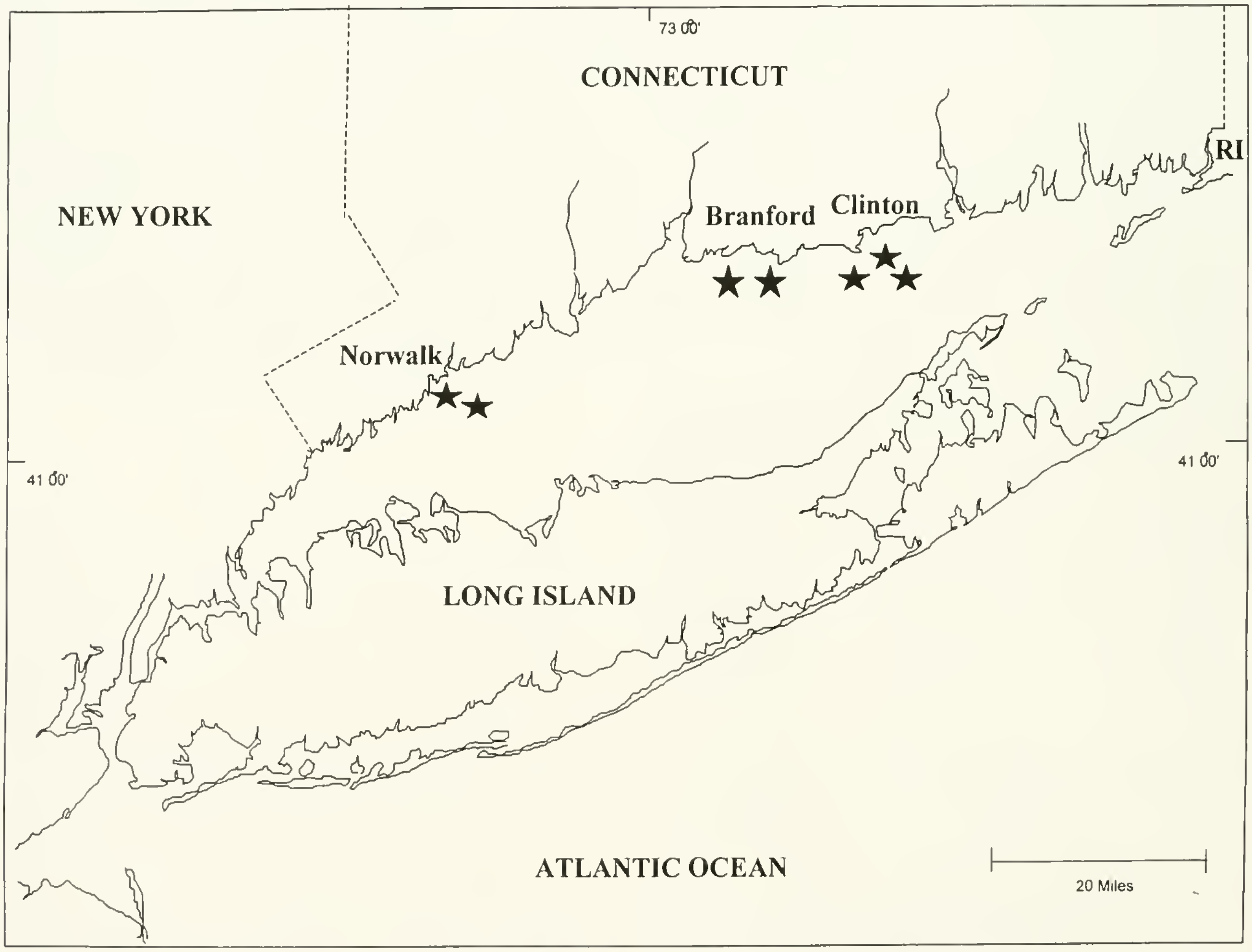

Figure. 1. A map of sampling stations with a positive diagnosis for *H. costale* in Long Island Sound.

TABLE 1.

Haplosporidium costale (SSO) and _Haplosporidium nelsoni_ (MSX) spores in eastern oysters in Long Island Sound.

Sampling Date	Sampling Site	Sampling Location	Shell Length (mm)	Spore Type	Seed Origin
09.28.97	Guilford, East River	41°16.05′N:72°39.62′W	104	MSX	Natural
10.14.97	Clinton, Cedar Island mud flat	41°15.97′N:72°32.00′W	78	MSX	Natural
10.14.97	Clinton, Cedar Island mud flat	41°15.97′N:72°32.00′W	101	MSX	Natural
12.17.97	Norwalk 1131	41°02.42′N:73°25.25′W	75	MSX	Natural
12.18.97	Norwalk 162	41°03.36′N:73°25.12′W	69	MSX	Natural
01.08.98	West Haven Lot 1B	41°15.47′N:72°55.26′W	42	MSX	Hatchery raised
09.18.98	Stratford 709	41°07.41′N:73°09.13′W	70	MSX	Natural
09.22.98	Milford 612	41°11.10′N:73°00.05′W	73	MSX	Natural
10.17.98	Branford 316	41°15.42′N:72°44.40′W	75	SSO, MSX	Natural
10.17.98	Branford 168	41°15.77′N:72°45.95′W	42	MSX	Natural
10.17.98	Branford 179	41°15.73′N:72°45.63′W	64	SSO, MSX	Natural
11.21.98	West Haven Lot 1B	41°15.47′N:72°55.26′W	48	MSX	Hatchery raised
12.03.98	Clinton, Cedar Island Marina	41°16.05′N:72°32.10′W	24	SSO, MSX	Hatchery raised
09.22.99	Clinton, Cedar Island Marina	41°16.05′N:72°32.10′W	76	MSX	Hatchery raised
09.22.99	Clinton, Cedar Island Marina	41°16.05′N:72°32.10′W	33	MSX	Hatchery raised
09.22.99	Clinton, Cedar Island Marina	41°16.05′N:72°32.10′W	26	MSX	Hatchery raised
11.16.99	Clinton, Cedar Island Marina	41°16.05′N:72°32.10′W	29	SSO, MSX	Hatchery raised

extremely heavy sporulation, phagocytosed *H. nelsoni* spores were observed in the vascular system hemolymph sinuses and veins, in the mantle lobes and the gills, and being carried through stomach or mantle epithelia via diapedesis. Spores that occurred in the connective tissue were usually surrounded by aggregates of granular hemocytes. *H. nelsoni* spores were detected inside the lumens of digestive tubules, digestive ducts, and the intestine. *H. nelsoni* prespores, which did not retain stain in acid-fast reaction, were detected exclusively inside digestive cells.

It was as probable that spores would be found in oysters originating from natural set as in oysters originating from hatchery-raised seed ($\chi^2 = 0.68$ [not significant] [NS]). Oysters with spores were detected in most areas of Connecticut's shoreline. No sporulating specimens were detected in the north shore of Long Island, New York. The size of an oyster with spores did not differ significantly from the average size of the sample from which it was taken ($t = 0.08$ [NS]). The size of an oyster with spores also did not differ significantly from the average sizes of all oysters sampled for this study ($t = 0.97$ [NS]).

ISH results with *H. costale* and *H. nelsoni* DNA probes are summarized in Table 2. Five specimens had mixed infections, the rest were infected only with *H. nelsoni*. Two specimens with mixed infections had both types of spores present; three had only plasmodia. (An additional two specimens were diagnosed positive for both *H. costale* and *H. nelsoni* on the basis of the presence of spores [Table 1], with seven positive specimens altogether.) The two specimens with both spore types (Branford lots 179 and 316) that were subjected to ISH had prominent *H. costale* infections. Approximately 90% of the plasmodia hybridized with the *H. costale* probe and 10% hybridized with the *H. nelsoni* probe in these samples (Fig. 3). Two other specimens with mixed infections (Clinton and Norwalk 1131) had very light *H. costale* infections (Fig. 4). More than 99% of the plasmodia hybridized with the *H. nelsoni* probe and the very rare *H. costale* plasmodia would not have been detected without the probe. *H. costale* plasmodia in the

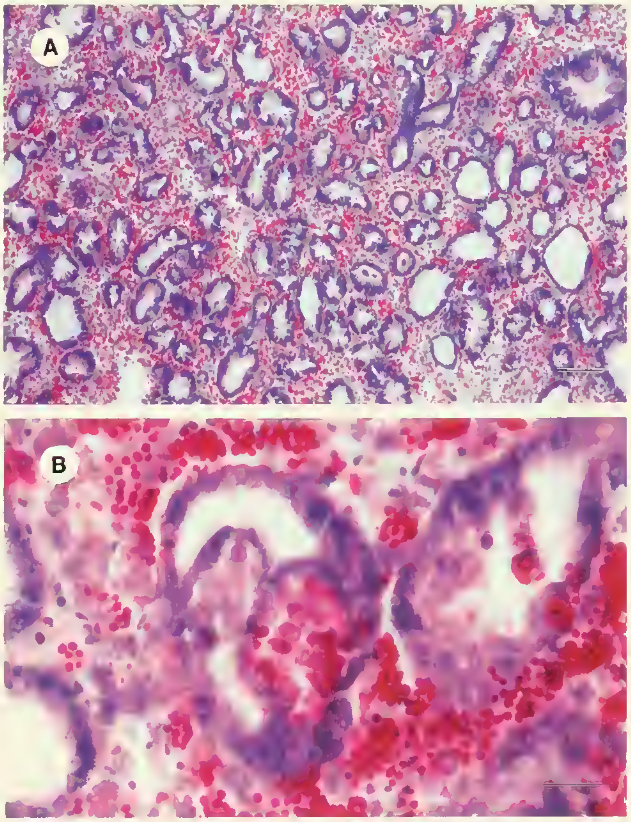

Figure 2. Sporulation of *H. costale* and *H. nelsoni* in Long Island Sound. (A) Digestive diverticulum of an oyster filled with acid-fast *H. costale* spores (Ziehl and Harris' hematoxylin). Scale bar 100 µm. (B) Cosporulation of *H. costale* and *H. nelsoni*. Small *H. costale* spores occur in the connective tissue surrounding digestive tubules; larger *H. nelsoni* spores occur inside digestive tubule. This is the same specimen as in item (A) (Ziehl and Harris' hematoxylin). Scale bar 20 µm.

TABLE 2.

ISH of *Haplosporidium costale* (SSO) and *Haplosporidium nelsoni* (MSX) in eastern oysters in Long Island Sound.

Sampling Date	Sampling Site	Sampling Location	Shell Length (mm)	ISH with SSO and MSX Probes (Positive Result)	Location of Plasmodia
09.16.97	Norwalk Natural Bed	41°04.85′N:73°23.55′W	110	MSX	Plasmodia throughout the tissues
10.14.97	Clinton, Cedar island mud flat	41°15.97′N:72°32.00′W	113	SSO, MSX	MSX plasmodia (>99%) throughout the tissues, rare SSO plasmodia (<1%) in the gills
12.17.97	Norwalk 1131	41°02.42′N:73°25.25′W	80	SSO, MSX	MSX plasmodia (>99%) throughout the tissues, rare SSO plasmodia (<1%) in the gills and mantle
12.17.97	Norwalk 162	41°03.36′N:73°25.12′W	66	SSO, MSX	MSX plasmodia (40%) throughout the tissues; prespores in digestive tubules; SSO plasmodia (60%) throughout the tissues
12.17.97	Norwalk Manresa Island	41°04.42′N:73°24.55′W	70	MSX	Plasmodia in the intestine, digestive duct and epibranchial chamber epithelia; rare plasmodia throughout the tissues
10.17.98	Branford 316	41°15.42′N:72°44.40′W	68	MSX	Plasmodia in the intestine and stomach epithelia; some plasmodia throughout the tissues
10.17.98	Branford 316	41°15.42′N:72°44.40′W	75	SSO, MSX	MSX spores and plasmodia (10%) in the digestive tubules; SSO spores and plasmodia (90%) everywhere in the connective tissue
10.17.98	Branford 168	41°15.77′N:72°45.95′W	41	MSX	A few plasmodia in stomach epithelium; several plasmodia throughout the tissues
10.17.98	Branford 168	41°15.77′N:72°45.95′W	41	MSX	A few plasmodia in stomach epithelium; several plasmodia throughout the tissues
10.17.98	Branford 168	41°15.77′N:72°45.95′W	60	MSX	A few plasmodia in the stomach, intestine, digestive tubule, and duct epithelia; several plasmodia throughout the tissues
10.17.98	Branford 179	41°15.73′N:72°45.63′W	62	MSX	Plasmodia exclusively in the stomach, intestine, digestive duct, and digestive tubule epithelia
10.17.98	Branford 179	41°15.73′N:72°45.63′W	70	MSX	Plasmodia throughout the tissues
10.17.98	Branford 179	41°15.73′N:72°45.63′W	64	SSO, MSX	MSX spores and plasmodia (10%) in the digestive tubules; SSO spores and plasmodia (90%) everywhere in the connective tissue
11.10.98	Stony Brook Harbor, NY	40°54.30′N:73°10.70′W	123	MSX	Plasmodia throughout the tissues
11.10.98	Stony Brook Harbor, NY	40°54.30′N:73°10.70′W	127	MSX	A few plasmodia in gill epithelia, several plasmodia throughout the tissues
11.10.98	Stony Brook Harbor, NY	40°54.30′N:73°10.70′W	107	MSX	Gaper; plasmodia throughout the tissues
11.12.98	Oyster Bay, H, NY	40°54.33′N:73°30.22′W	76	MSX	Rare plasmodia exclusively in the stomach, digestive tubule, and duct epithelia
11.12.98	Oyster Bay, H, NY	40°54.33′N:73°30.22′W	87	MSX	A few plasmodia in gill epithelia; several plasmodia throughout the tissues
11.12.98	Oyster Bay, W, NY	40°52.59′N:73°32.11′W	92	MSX	Rare plasmodia exclusively in stomach epithelium
11.12.98	Oyster Bay, W, NY	40°52.59′N:73°32.11′W	84	MSX	Rare plasmodia exclusively in digestive duct epithelia
12.01.98	Milford 305	41°11.15′N:73°04.80′W	78	MSX	Plasmodia throughout the tissues
12.01.98	Milford 305	41°11.15′N:73°04.80′W	24	MSX	Plasmodia throughout the tissues
12.15.98	Northport Bay, NY	40°55.87′N:73°22.87′W	92	MSX	Rare plasmodia exclusively in the stomach and gill epithelia
11.17.99	Clinton, Cedar Island Marina	41°16.05′N:72°32.10′W	67	MSX	Plasmodia throughout the tissues
11.17.99	Clinton, Cedar Island Marina	41°16.05′N:72°32.10′W	65	MSX	Plasmodia throughout the tissues
12.07.99	Oyster Bay, J, NY	40°53.50′N:73°30.23′W	90	MSX	Very rare plasmodia exclusively in intestine and gill epithelia
12.07.99	Oyster Bay, J, NY	40°53.50′N:73°30.23′W	68	MSX	Plasmodia in the stomach, digestive tubule, and gill epithelia; some plasmodia throughout the tissues

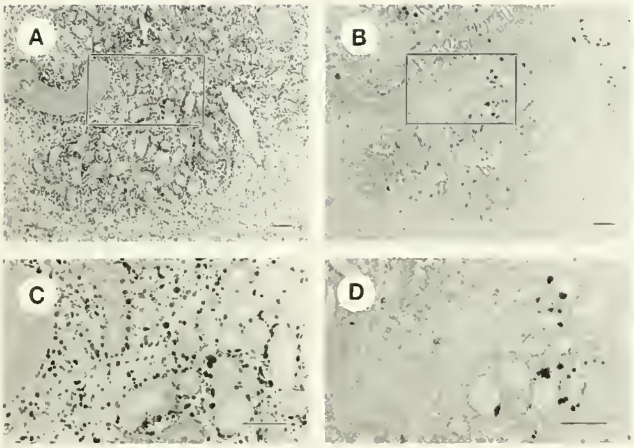

Figure 3. ISH of *H. costale*- and *H. nelsoni*-infected oyster from Long Island Sound. This is the same specimen as in Figure 2. (A) *H. costale* DNA probe hybridizing with *H. costale* plasmodia and prespores in the connective tissue surrounding digestive tubules. (B) *H. nelsoni* DNA probe hybridizing with *H. nelsoni* plasmodia and spores in digestive tubules. (C) Higher magnification of bracketed area in item (A). (D) Higher magnification of bracketed area in item (B). Scale bars 100 µm.

light infections were detected in the gills and the mantle, and in the heavy infections, were detected throughout the tissues.

ISH of an oyster with both *H. costale* and *H. nelsoni* spores is illustrated in Figure 3. *H. costale* probe hybridized to plasmodia and prespores scattered in the connective tissue surrounding the digestive diverticula (Fig. 3A and C), in the gills, and the mantle, whereas *H. nelsoni* probe hybridized to plasmodia and prespores mainly in the digestive tubules (Fig. 3B and D). This specimen represented a terminal infection of *H. nelsoni*, when migration of plasmodia to digestive tubules had already occurred. Both probes outlined mature spores but did not completely penetrate them. Serial sections of an oyster with mixed infection of *H. costale* and *H. nelsoni* plasmodia stained with hematoxylin-eosin and ISH with the DNA probes are illustrated in Figure 4A, B, and C.

DISCUSSION

The presence of *H. costale* in Long Island Sound has been suggested in several earlier reports (Andrews 1984; Andrews 1988; Katkansky & Warner 1970; Newman 1971); however, in the absence of sporulating stages, positive identification has been impossible. Failure to detect sporulating stages is easily explained on the basis of the results of the present report: spores were detected in only 0.08% of the oysters studied. We can assume that *H. costale* has been enzootic to the area, but its presence was finally verified, not because of increased prevalence, but because of increased sampling effort and the use of species-specific diagnostic tools. Furthermore, absence of spores in 1997 and in any of the samples collected from the north shore of Long Island, New York, is most likely due to small sample sizes (510 and 600 oysters, respectively), which would give <0.5% probability to encounter a sporulating specimen. High occurrence of positive specimens detected in Branford-Clinton area (Table 1) was due to intensive sampling in that area.

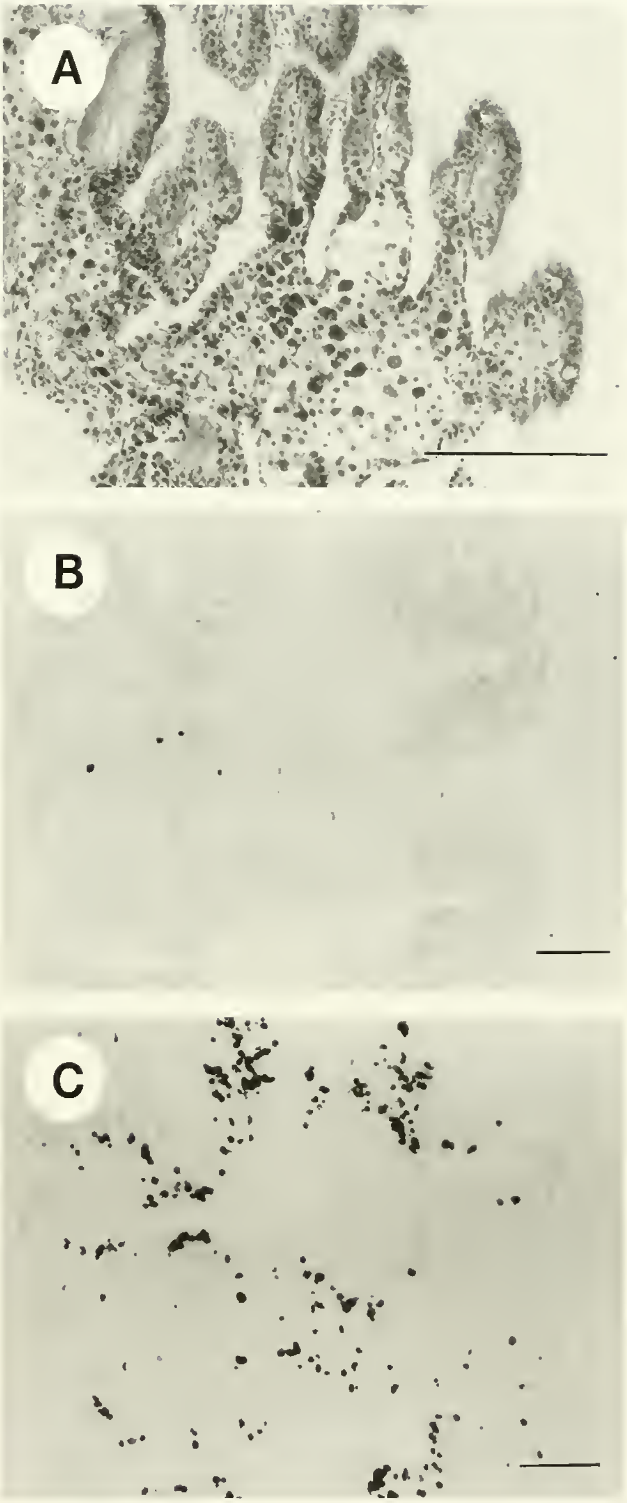

Figure 4. Serial sections of an oyster with mixed haplosporidian plasmodial infection. (A) Hematoxylin-eosin stain. (B) ISH with *H. costale* DNA probe detecting rare plasmodia in the gill. (C) ISH with *H. nelsoni* DNA probe of the same area shown in item (B). Scale bars 100 µm.

Detailed descriptions of sporulation and spore structures of *H. costale* or *H. nelsoni* are presented by Couch et al. (1966), Rosenfield et al. (1969), and Perkins (1969). Although morphological characteristics of *H. costale* spores or plasmodia in our material did not differ from those described in previous publications, *H. costale* in Long Island Sound appears to have a different pathogenesis than in the south. Gross signs as defined by Andrews (1988) (emaciation, failure of new shell growth in spring, high epizootic mortality mid-May–mid-June, and discoloration of gapers by spores) did not apply to our samples. In Virginia, infected oysters sporulate synchronously in May–July, presenting an obvious, easily diagnosed stage. All plasmodia develop into sporonts, and oysters die promptly after sporulation (Andrews 1984). *H. costale* is considered to be a well-adapted parasite that infects new oysters via spores that are released during mortality season (Andrews 1982). Spores are usually detected in moribund oysters and infection causes 20%–50% yearly mortalities (Andrews 1988). Cosporulation with *H. nelsoni* was reported previously by Couch (1967) in oysters collected from Chincoteague Bay, Virginia. The six cases with both spore types were dead and dying oysters collected in May and June during the characteristic *H. costale* sporulation time for oysters in Virginia (Couch 1967).

SSO disease in Long Island Sound differed from the above description in several ways. First, plasmodia and sporulation in the present material were found in October to December. During this time period, SSO disease in Virginia is subpatent and no plasmodia are detected before spring (Andrews 1988). However, in a recent paper describing *H. costale* probes also used in the present publication, Stokes and Burreson (2001) reported the presence of a positive ISH to *H. costale* plasmodia in an oyster sampled in October 1994 in Virginia. In the present paper, spores were detected in live specimens with no evidence of a synchronous mortality event. Spores were very rare, and infection always occurred as a coinfection with *H. nelsoni*. Because *H. costale* plasmodia were also detected in the samples, sporulation may occur infrequently. Rare sporulation such as presented in this report cannot sustain a widespread infection. Actual prevalence of *H. costale* in the area is not known based on the results of the present study, but the rare sporulation and the difficulty of finding positive specimens with ISH suggest a low prevalence.

Possible mortality associated with *H. costale* is impossible to estimate because of an *H. nelsoni* epizootic that occurred in Long Island Sound in 1997–1998 (Sunila et al. 1999). Oyster production in Connecticut decreased from more than 500,000 bushels in 1996 to 170,000 in 1999, reflecting high MSX-associated mortalities. According to Andrews (1984), *H. nelsoni* kills oysters much quicker than *H. costale*, and depresses manifestation of SSO disease during MSX epizootics.

H. costale is usually restricted to high-salinity bays with salinities >30‰. Its lower limit is 25‰ and salinities <20‰ appear to cause disease regression (Andrews 1979). It is possible that salinity in Long Island Sound's oyster beds is not high enough to sustain full epizootic *H. costale* activity. In Branford lots (Table 1), salinity varies between 26‰ and 27‰; in the Clinton Cedar Island Marina (in the mouth of Hammonassett River), salinity varies from 13‰ to 28‰. Oysters in the area are transplanted four times before they are marketed. This exposes them to even lower salinities, which may have provided a control for the disease.

Classically, the diagnosis of *H costale* relies on the presence of sporulating stages and the site of initial infection, which for *H. costale* is the epithelium of the digestive system and for *H. nelsoni* is the gill epithelium. In addition, history of the sampling area relating to past *H. costale* or *H. nelsoni* infections directs the diagnosis. In the present report, we were able to diagnose *H. costale* in a new geographic area with a deviating sporulation time by using DNA probes (Stokes & Burreson 1995; Stokes & Burreson 2001). Further research to study the infection cycle, prevalences, and possible association with mortalities is under way.

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INFECTION INTENSITY, PREVALENCE, AND HISTOPATHOLOGY OF *PERKINSUS* SP. IN THE MANILA CLAM, *RUDITAPES PHILIPPINARUM*, IN ISAHAYA BAY, JAPAN

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ABSTRACT Infection intensity, prevalence of infection, and pathologic features of *Perkinsus* parasitism among Manila clams inhabiting in Isahaya Bay, Japan, were investigated. Ray's fluid thioglycollate medium technique followed by Choi's 2 M NaOH digestion assay was applied to determine the infection intensity and prevalence. The infected tissues were also microscopically examined from histological preparations. The prevalence of infection was 57% in clams sampled in February 2001, and the mean infection intensity was 351,603 *Perkinsus* cells per clam or 225,701 *Perkinsus* cells/g of tissue. *Perkinsus* sp. was abundantly distributed in the gill and visceral mass, whereas it was rare in the adductor muscles and siphons. The total number of *Perkinsus* in the clams was linearly correlated with the number of *Perkinsus* cells in the gill tissues ($r^2 = 0.908$), suggesting that gill could be a target tissue for efficient diagnosis of *Perkinsus* infection. Heavily infected clams exhibited white nodules on the surface of the mantle as a consequence of inflammatory response to *Perkinsus* infection. Numerous trophozoites were observed in the connective tissue around the gonads and gill filaments, suggesting that heavy infection with *Perkinsus* may exert potential deleterious effects on growth and reproduction by interfering with the reproductive maturation and filtration activities of the clams.

KEY WORDS: *Perkinsus*, *Ruditapes philippinarum*, infection intensity, histopathology, Isahaya Bay, Japan

INTRODUCTION

The protozoan parasite *Perkinsus* sp. (Apicomplexa, Perkinsea) has been known to cause mass mortalities worldwide in commercially important shellfish, including oysters, scallops, clams, and abalones (Andrews & Hewatt 1957; Mackin 1962; Lester & Davis 1981; Navas et al. 1992; Blackbourn et al. 1998; Canestri-Trotti et al. 2000; Park & Choi 2001; Liang et al. 2001). In particular, *P. atlanticus* has been associated with mass mortalities of the venerid clams of the genus *Ruditapes* (i.e., *Tapes* or *Venerupis*) inhabiting the Mediterranean and Atlantic coasts (Da Ros & Canzonier 1985, Chagot et al. 1987, Sagrista et al. 1996). *Perkinsus* parasitism in the Manila clam ("short-necked clam"), *R. philippinarum*, was also recently confirmed in Japanese (Hamaguchi et al. 1998, Maeno et al. 1999) and Korean waters (Choi & Park 1997; Park & Choi 2001; Lee at al. 2001). *Perkinsus* sp. distributed in Korea is believed to be responsible for the mass mortality of Manila clams. This has occurred yearly, in late summer, since the early 1990s. Park and Choi (2001) postulated that the decline in Manila clam landings for the previous decade in Korea could be caused by *Perkinsus*-associated mortalities occurring in every late summer. As many other studies have reported, *Perkinsus* infection is associated with high salinity, temperature, and density of clam populations (Andrews & Hewatt 1957; Soniat 1996; Burreson & Ragone Calvo 1996; Cigarria et al. 1997).

Perkinsus-like microorganisms have been discovered in Manila clams distributed in Japan (Hamaguchi et al. 1998; Maeno et al. 1999) and China (Liang et al. 2001). Hamaguchi et al. (1998) reported on the occurrence of *Perkinsus* in the clams collected from Kumamoto and Hiroshima, in southern Japan. Comparison of the DNA sequence of *Perkinsus* with *P. atlanticus* and *P. olseni* indicated that the species of *Perkinsus* found in Kumamoto and Hiroshima, Japan, is taxonomically very close to *P. atlanticus* and *P. olseni* reported from Portugal and Australia (Hamaguchi et al. 1998). Manila clams are abundant in Isahaya Bay, southern Kyushu, Japan, where tidal flats are well developed and the clams are commercially cultured. Ishii et al. (2001) reported that the clam population in Ariake Sound has been declining significantly since 1987 due to overfishing, pollution, and predators. Because the presence of *Perkinsus* in clam populations inhabiting Ariake Sound was confirmed by Hamaguchi et al. (1998), detrimental effects of *Perkinsus* parasitism on clam growth, as well as on annual landings, cannot be ruled out in Isahaya Bay located on the west of Ariake Sound. However, infection intensity and prevalence of *Perkinsus* in the bay has not been reported previously.

Infection intensity and prevalence of *Perkinsus* parasitism on Manila clams was investigated from a clam population distributed in Isahaya Bay in the present study. This paper reports diagnosis, histopathologic features of the infected clams, infection intensity, and prevalence of *Perkinsus* parasitism in the clams collected in February 2001.

MATERIALS AND METHODS

A total of 191 clams were collected from Isahaya Bay, Kyushu, Japan, in February 2001 (Fig. 1). In the laboratory, shell length (SL), width (SW), thickness (ST), and tissue wet weight (TWT) of individual clams were recorded. Condition index (CI) was then calculated for evaluating fatness of the clams as

$$CI = [TWT/(ST \times SW \times SL)] \times 1{,}000$$

For evaluating *Perkinsus* infection, the clams were placed in two groups: one for histopathological examination and the other for measuring total body burden, which is the infection intensity of each clam as a total number of *Perkinsus* in a clam or number of *Perkinsus*/g tissue. For histopathology, a longitudinal section was made in the middle of the body, which included gills, digestive glands, gonads, mantle, and foot. A 5-μm thin section was cut for each clam after dehydration. The sections were then stained with Harris' hematoxylin and eosin Y. For measuring total body burden, the whole flesh of individual clams was immersed in fluid thioglycollate medium (FTM), fortified with nystatin and chloramphenicol to prevent bacterial activity (Ray 1966), and placed in

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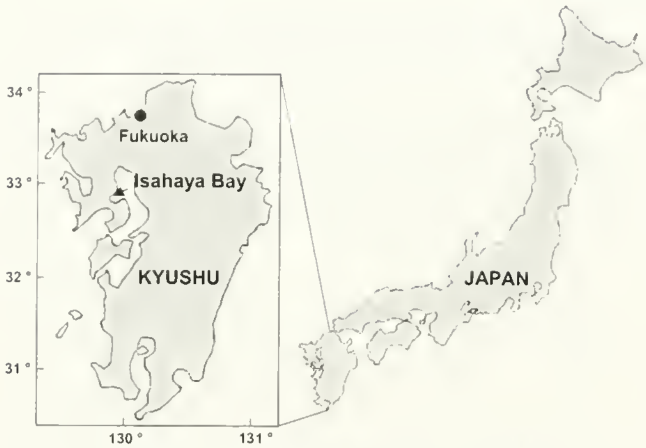

Figure 1. Location of the sampling site, Isahaya Bay, Kyushu, Japan.

a dark area for a week. After immersion, the tissues were digested in 2 M NaOH, and the number of *Perkinsus* cells was counted using a hemocytometer according to Choi et al. (1989). Total body burden was then standardized as the number of *Perkinsus*/g tissue. To determine the distribution pattern of *Perkinsus* per clam, the gills, mantle, adductor muscle, and body containing the visceral mass were excised separately from each clam and immersed independently in FTM. After 1 wk of immersion, the number of *Perkinsus* cells in the tissue was measured as described above (Choi et al. 1989). The infection intensity was standardized and expressed as the number of *Perkinsus* cells/g tissue.

RESULTS

Histopathological Observation of Perkinsus *sp.*

Numerous trophozoites were observed in the clams collected from Isahaya Bay. Eccentric vacuoles, nuclei, and nucleoli were observed from histological sections of the infected tissues (Fig. 2A). The diameter of the trophozoites, estimated microscopically, ranged from 7.73 to 15.80 μm, with a mean of 10.98 μm. Diameters of the nuclei varied from 4.20 to 6.59 μm, with a mean of 5.44 μm, whereas the diameters of the nucleoli varied from 1.90 to 2.67 μm, with a mean of 2.27 μm. Most trophozoites in the gills and mantle formed different sizes of clusters (Fig. 2A and B).

Perkinsus was predominantly found in gill filaments, mantles, and digestive tubules, although a few of the trophozoites were observed in the foot (Fig. 2B, C, and D). Heavy infection with *Perkinsus* in the gill lamellae resulted in swollen connective tissue with severe hemocyte infiltration (Fig. 2B). An inflammatory response to the parasite was also observed in heavily infected clams in the form of nodules on the mantle surface (Fig. 2C). Those nodules appeared as white spots on the mantle surface and could be observed even with the naked eye. Numerous *Perkinsus* trophozoites were also observed around the digestive glands, indicating that *Perkinsus* could inhibit the digestive activity of the clams in the visceral mass (Fig. 2D). Some clams exhibited mature eggs or sperm even in February because of the influence of the warm Kuroshio current in the bay. *Perkinsus* was also observed in the connective tissues of female as well as male gonads (Fig. 2E and F), indicating that *Perkinsus* infection in the Manila clam might disturb the reproductive processes. Sporocysts of a cercaria-like organism were also observed in the female gonad, although the prevalence was much lower than that of *Perkinsus* (Fig. 2H); only 2% of total clams investigated were infected.

Prevalence and Infection Intensity of Perkinsus *per Clam and in Different Types of Tissues*

The results of this *Perkinsus* infection survey conducted from a clam population in Isahaya Bay are summarized in Table 1. A total of 191 clams with a mean SL of 31.0 mm and a mean TWT of 1.566 g were analyzed in the study. Prevalence, the percentage of infection in the clams investigated, was 57.4%. Total body burden, in terms of the total number of *Perkinsus* cells in individual clams, varied from 0 to 2,609,375, with a mean of 351,603. Infection intensity, as number of *Perkinsus* cells/g tissue, varied from 0 to 1,817,196, with a mean of 225,701 (Table 1). No obvious correlation was observed between the infection intensity and size of the clams, as well as the CI.

Table 2 shows prevalence and infection intensity in various types of clam tissues. Among the four types of tissues examined, the gills showed the highest prevalence and infection intensity, with 85.71% and 1,019,817 cells/g gill tissue, respectively. Prevalence of infection in the visceral mass was as high as that observed in the gills, whereas the infection intensity as the number of *Perkinsus*/g tissue was much lower than the value observed in the gills. Infection intensity and prevalence was much lower in the adductor muscle and siphons compared to that in the gills and visceral mass. A positive correlation was observed between number of *Perkinsus* cells/g gill tissue and the total body burden (Fig. 3, $r^2 = 0.908$). The number of *Perkinsus* cells in the visceral mass was also highly correlated with the total body burden ($r^2 = 0.893$), suggesting that the gill and visceral mass are the main target tissues for *Perkinsus* infection in this species. Quantitative evaluation of *Perkinsus* infection among the various tissue types indicated that *Perkinsus* sp. is not evenly distributed in the clams; rather, it is concentrated in the gills and visceral mass.

DISCUSSION

Histopathological Features of Perkinsus

Although histology is not widely used in the diagnosis of *Perkinsus* infection, the technique provides valuable information on host-parasite cellular interactions (Hine & Thorne 2000; Diggles & Hine 2001; Lee et al. 2001). In the present study, pathogenicity of *Perkinsus* sp. was visually examined from histological preparations of the infected clams. Figure 2A shows typical *Perkinsus* trophozoites displaying a *Perkinsus*-specific "ring" structure (i.e., vacuole and nucleolus in a nucleus, Azevedo 1989; Azevedo et al. 1990; Auzoux-Bordenave et al. 1995; Perkins 1996; Park & Choi 2001). Trophozoite diameter measured in the present study was somewhat comparable to the size reported by Hamaguchi et al. (1998) and Maeno et al. (1999) in Japan. Trophozoite diameter measured in our study varied from 7.73 to 15.80 μm, with a mean of 10.98 μm. Hamaguchi et al. (1998) reported 5.3–32.5 μm, with a mean of 14.8 μm, as diameter of trophozoites in Manila clams, and Maeno et al. (1999) reported 5.7–11.4 μm. In contrast, trophozoites of *Perkinsus* sp. found in *R. philippinarum* on the northern coast of China varied from 2 to 10 μm (Liang et al. 2001). The size of trophozoites estimated in our study is also similar to the size of *P. olseni* [which is taxonomically very close to *Perkinsus* sp. found in Japan (Hamaguchi et al. 1998)], discovered in the Australian black-ribbed abalone, *Haliotis rubra* (Lester & Davis 1981).

Some heavily infected clams exhibited numerous clusters of trophozoites on their gill plicae and digestive tubules with severe hemocytic infiltration (Fig. 2B and D). Such a heavy infection in

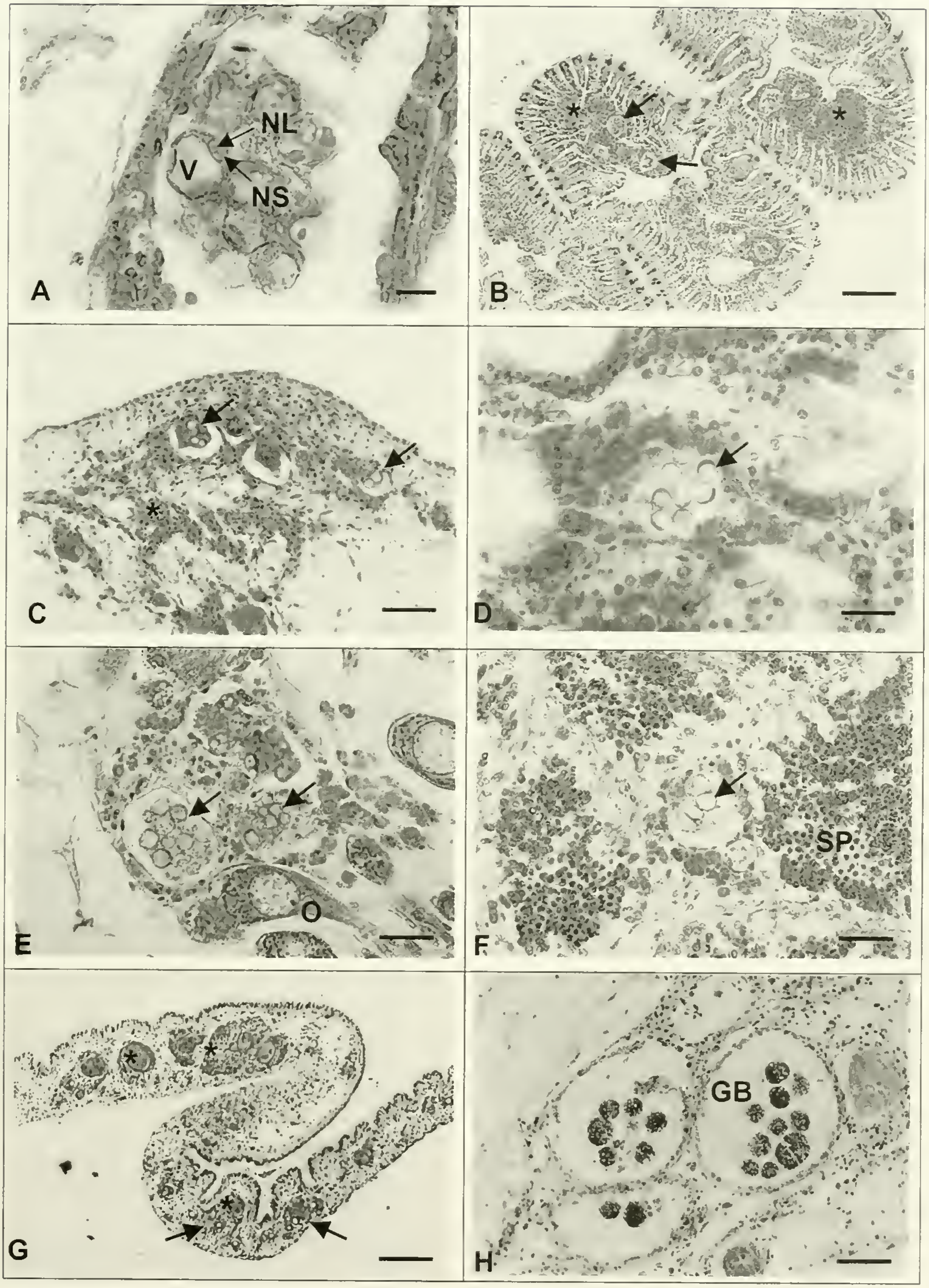

Figure 2. Histopathological features of *Perkinsus* infection. (A) Mature trophozoites containing vacuole (V). Nucleolus (NL) occurs in nucleus (NS). 1,000×, scale bar = 5 μm. (B) Severe inflammatory reaction (asterisk) occurs around trophozoites in gill tissues. 100×, scale bar = 100 μm. (C) Early stage of nodule formation. Infiltration of hemocytes (asterisks) around trophozoites (arrows) results in the swollen connective tissue of the foot. 200×, scale bar = 20 μm. (D) Trophozoites in connective tissues of digestive glands. 400×, scale bar = 40 μm. (E) Trophozoites in connective tissues of female gonad. Young oocytes (O) are seen around the capsulated trophozoites. 400×, scale bar = 500 μm. (F) Grouped trophozoites in connective tissues of male gonad. Concentrated hemocytes of the host enclose the trophozoites forming a capsule around spermatogonia (SP). 400×, scale bar = 20 μm. (G) Inflammation of host hemocytes (asterisk) in the mantle tissues. 100×, bar = 100 μm. (H) Sporocysts containing germ balls (GB). No inflammation observed. 200×, bar = 40 μm.

TABLE 1.

Survey results of *Perkinsus* infection in the clams distributed on Isahaya Bay.

	N	Average	SD	Min	Max
SH (mm)	191	21.4	2.1	15.6	25.9
SL (mm)	191	31.0	3.1	22.3	39.9
TWT (g)	191	1.566	0.521	0.537	2.769
CI	89	0.167	0.028	0.059	0.232
Total *Perkinsus* (cells/clam)	89	351,603	549,046	0	2,609,375
Unit *Perkinsus* (cells/g twt)	89	225,701	365,002	0	1,817,196
Prevalence	150	57%			

SH = shell height; SL = shell length; TWT = total tissue wet weight; CI = condition index; SD = standard deviation; Min = minimum; Max = maximum.

gill tissues may lower filtration efficiency and, in turn, cause retarded growth, although the effects of *Perkinsus* infection on filtration activity of the clams has not been experimentally proven. Infestation of *Perkinsus* in digestive tubules would cause digestive tubule atrophy and exert deleterious effects on the food digestion of the clams, as reported by Lee et al. (2001). Clams more heavily infected with *Perkinsus* exhibited white nodules on their mantle surfaces as well as gills, as was reported in other studies (Azevedo 1989; Navas et al. 1992; Montes et al. 1996; Almeida et al. 1999; Lee et al. 2001; Park & Choi 2001). A cross-section of the nodules revealed that they are the result of hemocytic encapsulations of trophozoites and massive hemocytic infiltration around the mantle tissues, evoking tissue inflammation (Fig. 2C). Several studies also have reported that *Perkinsus* secretes extracellular enzymes that perturb the host's immune system, which, in turn, allows the host animal to become more susceptible to other pathogens (Garreis et al. 1996; La Peyre et al. 1996; Faisal et al. 1999; Ordas et al. 2000).

Perkinsus was also observed among the connective tissues of female as well as male gonads (Fig. 2E and F), suggesting that *Perkinsus* infection also affects the reproduction of the clams in some way. Several studies have suggested that *Perkinsus* could (1) slow the gonad development process, or (2) reduce the reproductive output of the host animals by consuming net energy production needed for gamete production (White et al. 1988; Wilson et al. 1988; Choi et al. 1993; Choi et al. 1994). In *C. virginica*, no obvious correlation was observed between estimated fecundity and *P. marinus* infection (Choi et al. 1993). In contrast, the rate of egg protein synthesis was found to be slower in *C. virginica* heavily infected with *P. marinus*, whereas the rate was faster in oysters with relatively low infection (Choi et al. 1994). This suggests that the main effect of *Perkinsus* on host animal reproduction could be retardation of gonadal maturation rather than reduced gamete production. Reduced fecundity and impeded gonadal maturation were observed in Manila clams heavily infected with *Perkinsus* sp. in Korea (Park & Choi, in preparation).

Infection Intensity and Prevalence per Clam and in Various Types of Tissues

Numerous methods have been applied in the examination of *Perkinsus* parasitism since the first report on the occurrence of *P. marinus* in the American oyster, *Crassostrea virginica* (Mackin et al. 1950). *Perkinsus* infection has been diagnosed using histology (Perkins & Menzel 1966; Azevedo et al. 1990; Navas et al. 1992; Sagrista et al. 1995; Sagrista et al. 1996; Montes et al. 1996; Bower et al. 1998; Hine & Thorne 2000), FTM assay (Ray 1952; Ray 1966; Choi et al. 1989; Bushek et al. 1994; Rodriguez & Navas 1995; Fisher & Oliver 1996; Ford 1996; Almeida et al. 1999), immunological probes using *Perkinsus*-specific antibodies (Choi et al. 1991; Dungan & Roberson 1993; Maeno et al. 1999), and polymerase chain reaction (PCR) techniques (Marsh et al. 1995; Hamaguchi et al. 1998; Robledo et al. 1998; Figueras et al. 2000; Coss et al. 2001). Among these methods, FTM assay has been the most widely and frequently used in all types of *Perkinsus* diagnosis (Lester & Davis 1981; Azevedo 1989; Rodriguez & Navas 1995; Choi & Park 1997; Cigarria et al. 1997; Almeida et al. 1999; Liang et al. 2001), although FTM assay was initially designed for the detection of *P. marinus* (Ray 1953; Ray 1966). In the FTM assay, suspected tissues are immersed in 10 to 15 mL of FTM fortified with antibiotics for 1 or 2 wk. After immersion, hypnospores of *Perkinsus* developed in FTM and stained dark blue or brown with Lugol's iodine and were readily identifiable under a light microscope. The number of *Perkinsus* spores in FTM-assayed tissues can be accessed after digesting the tissues with 2 M NaOH, according to Choi et al. (1989). FTM assay combined with 2 M NaOH digestion has been successfully used in the quantification of *P. marinus* (Choi et al. 1989; Bushek et al. 1994; Fisher & Oliver 1996), as well as other *Perkinsus* species (Rodriguez & Navas 1995; Choi & Park 1997; Park 1999; Liang et al. 2001; Park & Choi 2001).

Infection intensity and prevalence of *Perkinsus* sp. in Manila clams in Isahaya Bay, Japan, is first reported in this study. Ray's FTM technique combined with Choi's NaOH digestion technique, which has been used in many other studies, was successfully applied in the quantification of *Perkinsus* in this study. Prevalence of infection and mean infection intensity of *Perkinsus* in the clams collected in February 2001 was 57% and 225,701 spores per gram tissue, respectively (Table 1). Hamaguchi et al. (1998) also reported the prevalence of *Perkinsus* in *R. philippinarum* collected from Kumamoto, east coast of Ariake Sound, and Hiroshima. The prevalence measured from Kumamoto was 87.5% in commercial clam beds and 56.3% in natural clam beds. The prevalence values reported by Hamaguchi et al. (1998) are similar to those estimated from Isahaya Bay. The prevalence observed in Hiroshima, 93.8% in commercial beds and 84.4% in natural habitats, was somewhat higher than the prevalence measured in Kumamoto and Isahaya Bay. No data on the infection intensity of *Perkinsus* in the Manila

TABLE 2.

Prevalence and average infection intensity of *Perkinsus* sp. among various tissues of *R. philippinarum* ($n = 35$).

Tissue Types	Prevalence (%)	Average Infection Intensity (*Perkinsus* Cells/g Tissue Wet Weight ρ SD)
Gills	85.71	1,019,817 ± 1,393,736
Siphons	45.71	66,579 ± 152,537
Adductor muscle	42.86	56,331 ± 120,086
Visceral mass	82.86	120,918 ± 164,124

SD = standard deviation.

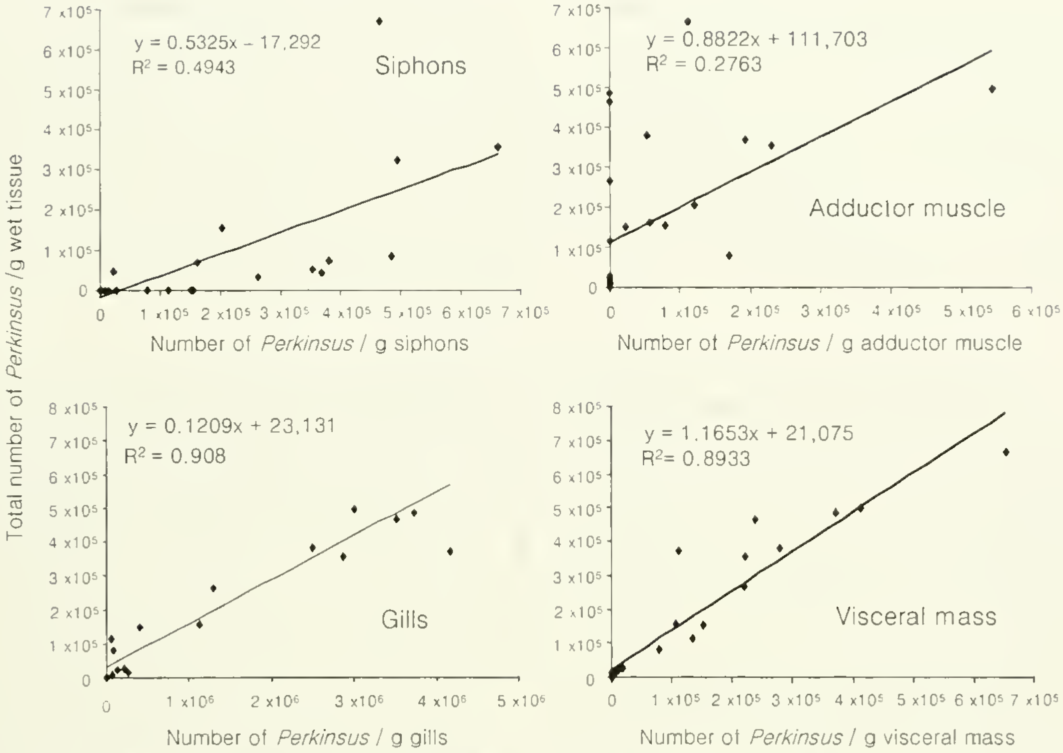

Figure 3. Correlation between the total number of *Perkinsus*/g tissue wet weight and the number *Perkinsus*/g of siphons, adductor muscle, gills, and visceral mass.

clam are available so far in Japan with which to compare the infection intensity measured in this study. The infection intensity and prevalence observed in the present study is somewhat lower than the values reported from neighboring countries. Prevalence of *Perkinsus* infection in a survey of *R. philippinarum* from Komsoe Bay, on the west coast of Korea, where tidal flats are well developed and used as a clam culture ground, was almost 100%, with a mean infection intensity of 709,028 spore/g tissue (Park & Choi 2001). *Perkinsus* infection reported from populations of *R. philippinarum* in the northern Yellow Sea (38°50′26″:39°27′09″) was also comparable to the values reported in the present study. The prevalence varied from 20% to 100%, with mean infection intensity of 2 to 1,670,615 spores/g tissue in the northern Yellow Sea (Liang et al. 2001).

No obvious correlation was observed between the infection intensity and size of the clams, as well as the condition index, in this study, although several studies have reported that *Perkinsus* infection is often positively correlated with oyster or clam size. Mackin (1951) and Ray (1953) found that *C. virginica* juveniles less than 1 y old have lower levels of infection compared to market-sized oysters. *Perkinsus* sp. found in *R. philippinarum* in Korea, as well as in China, also showed similar size-dependent infection. Clams smaller than 13 mm in shell length normally exhibit no infection while the larger clams appeared to be susceptible to *Perkinsus* (Choi & Park 1997; Liang et al. 2001; Park & Choi 2001). Absence of any correlation between the clam size and *Perkinsus* infection intensity in this study could be explained by the low prevalence of infection in the clams. In this study, 43% of the clams examined showed zero infection, which, in turn, resulted in a poor correlation coefficient. Several studies have indicated that low *Perkinsus* infection prevalence matches well with low infection intensity in terms of number of *Perkinsus* cells/g tissue (Choi & Park 1997; Liang et al. 2001; Park & Choi 2001). However, it is unlikely that the observed low prevalence is related to the size of clams, because no juvenile clams (i.e., less than 15 mm SL) were included in the analysis. The clams used in this study were 22.3–39.9 mm in shell length, with a mean of 31.0 mm, and all of them are considered to be over 2 y old. Relatively low infection prevalence in Isahaya Bay could be attributed to the density of clams in their habitat. The clam density in the bay was observed to be lower than the clam density reported from other commercial clam beds, although the clam density in the sampling location was not estimated. High infection intensity and prevalence of *Perkinsus* is common among clam beds where the clams are intensively cultured, resulting in high density (Da Ros & Canzonier 1985; Choi & Park 1997; Liang et al. 2001; Park & Choi 2001). In a high-density bed of clams, *Perkinsus* disease can be transmitted quickly because any life stage of *Perkinsus* would be infectious (Ray & Mackin 1954; Andrews & Ray 1988; Auzoux-Bordenave et al. 1995; Perkins 1996).

Infection intensity of *Perkinsus* among different types of tissues in *R. philippinarum* was compared in this study using Ray's FTM following the 2 M NaOH digestion (Ray 1966; Choi et al. 1989). Infection intensity of mantle, gills, siphon, and body containing visceral mass was separately determined in this study for diagnostic purposes. As shown in Table 2, the prevalence of infection was found to be highest in the gills, followed by the visceral mass. *Perkinsus* density in the gills in terms of the number of *Perkinsus* cells/g tissue was also the highest among various types of clam tissues analyzed (Table 2). A strong positive correlation was also observed between the numbers of *Perkinsus* spores/g gill tissue and the total number of *Perkinsus*/g in whole clams (Fig 3, r^2 = 0.908). The number of *Perkinsus* spores in the visceral mass was also strongly correlated with the number of *Perkinsus* spores in whole tissues (Fig. 3, $r^2 = 0.893$). Park (1999) and Rodriguez and Navas (1995) also observed strong positive correlations between infection intensity in gill tissues and infection intensity of whole clams in Korea and Spain. The high density of *Perkinsus* in the gill and visceral mass indicates that a favorable condition for *Perkinsus* growth and reproduction is provided in these tissues, where more energy is believed to be available for *Perkinsus* (Choi et al. 1989). Our data suggest that gill assay is an excellent alternative for whole clam assay (i.e., body burden assay, Rodriguez & Navas 1995; Park 1999).

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SEASONAL CHANGES IN THE HISTOLOGICAL AND BIOCHEMICAL PROFILE OF THE GONAD, DIGESTIVE GLAND, AND MUSCLE OF THE CALAFIA MOTHER-OF-PEARL OYSTER, *PINCTADA MAZATLANICA* (HANLEY, 1856) ASSOCIATED WITH GAMETOGENESIS

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ABSTRACT The relationship between the energy storage cycle and gametogenesis of the pearl oyster, *Pinctada mazatlanica* was studied over an annual cycle (January to December 1999). Histological analysis, combined with oocyte examination and measurements of carbohydrate, protein, total lipid, and triacylglycerides levels from gonadal tissue, digestive gland, and adductor muscle were performed. One-way ANOVA was used for assessing differences in the area of oocytes over time. Similarly, a two-way ANOVA was applied for differences in the biochemical composition of specimens over time and sex. The gametogenic cycle was affected by the presence of "La Niña" cold event during the first half of 1999. Gametogenesis commenced early in February and occurred synchronously throughout the annual cycle. There were two reproductive peaks, one in spring (March to May) and other in summer (July to September). A massive spawning was observed in September–October when water temperature was 29–29.5°C. Carbohydrates, either stored or obtained from ingested food, were used as an immediate fuel for the production of oocytes, which grew and increased their protein content during the first half of the year. Lipids and triacylglycerides also showed two important peaks in the female gonadal tissue and digestive gland, corresponding to the same peaks described histologically. Reserves stored in the muscle and digestive gland were actively used for gametogenesis. Muscle proteins were mobilized to the gonad during the first half of the year, while carbohydrates were used during the second half. The digestive gland acted as a short-term storage site of carbohydrates and lipids during gonad development. More studies on seasonal changes and energy storage and mobilization in pearl oysters are required, especially involving the participation of the mantle tissue.

KEY WORDS: Bahía de La Paz, gametogenesis, metabolism, pearl oysters, temperature, *Pinctada mazatlanica*

INTRODUCTION

Marine bivalves show cycles of energy storage and utilization that are closely related to gametogenic cycles (Gabbott 1975; Bayne 1976; Barber & Blake 1981). Most species are capable of storing nutrient reserves in their body tissues during periods of high food supply, which are subsequently mobilized during times of food shortage, decreased rates of feeding, and/or high energy demand (Ansell 1974; Gabbott 1975; Bayne 1976; Sastry 1979; Barber & Blake 1981, 1991; Epp et al. 1988). Gametogenesis represents a period of particularly high-energy demand, when both maintenance costs and the cost of gamete synthesis must be met by the food supply, stored reserves or a combination of both. The reproductive and biochemical cycles are thus closely coupled in most species of bivalves but vary in relation to environmental factors, between species, and between populations of the same species (Epp et al. 1988).

In addition, many marine bivalves inhabiting subtropical and temperate areas follow an annual reproductive cycle with precise periods of gonad maturation and spawning. Reproduction usually demands considerable consumption of energy and the weight loss during spawning can reach between 30 to 60% (Mathieu & Lubet 1993). In general, energy is stored prior to gametogenesis when food is abundant in the form of lipid, glycogen, and protein substrates, and subsequently is mobilized and utilized in the production of gametes when metabolic demand is high (Bayne 1976; Barber & Blake 1981, 1983, 1991).

Pearl oysters are marine bivalves having a considerable economic and commercial value because of their natural capacity to produce pearls of high quality (Monteforte 1990, 1996). In spite of this, the factors which affect reproductive success in these species are poorly studied and understood, although their basic reproductive biology has been well documented histologically (Tranter 1958a; Tranter 1958b; Wada 1959; Sevilla 1969; Chellam 1987; Rose et al. 1991; Hernández-Díaz 1993; Arizmendi-Castillo 1996; García-Domínguez et al. 1996; Saucedo & Monteforte 1997; Behzadi 1997).

Presently, the growing importance and value of pearl oysters has promoted the use of hatchery-produced stock, and as such, the need for knowledge relating to broodstock conditioning and larval rearing. However, in order to produce oocytes and spermatozoa of the best quality, which will develop into strong and viable larvae, it has been essential to understand the seasonal behavior of oysters from wild population. This information will be used further to determine the basic needs of broodstock in the laboratory. As an initial step toward a better understanding of reproductive success in the Calafia mother-of-pearl oyster *Pinctada mazatlanica* (Hanley), the relationship between the build-up and utilization of energy reserves and the annual cycle of gametogenesis and spawning has to be investigated. The objective of this work was to study the seasonal cycles of energy storage and depletion in the gonadal tissue, digestive gland, and adductor muscle of this species.

MATERIALS AND METHODS

Specimen Collection and Dissection

From January to December 1999, twenty oysters were collected monthly from the Submarine Experimental Farm of CIBNOR, located in Caleta El Merito, in Bahía de La Paz, México (24°16′N and 110°19′W). Only adult specimens ranging 140 (±8 mm SD)

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shell height were collected to assure the presence of both sexes in the sample (Saucedo & Monteforte 1997). Water temperature was recorded at the collecting site. Specimens were taken to the laboratory to be dissected. The gonadal tissue (which develops gradually within the visceral mass at the expense of the interconnective tissue matrix and the digestive gland, as gametogenesis proceeds) and adductor muscle were excised from each specimen. The former tissue component was preserved in Davidson's solution for 48 h and used for histological analyses. The muscle was preserved at –80°C for biochemical analyses.

Histological Analysis

Preserved samples of gonadal tissues were dehydrated, embedded in paraplast, sectioned at 5 μm along an anteroposterior plane, and stained with hematoxylin-eosin (Howard & Smith 1983). Gametogenic state was characterized as either early, mid, or late developing, ripe, partially spawned, and spent, based on a larger version of Tranter's original scheme for pearl oysters (Tranter, 1958a, b). In this larger version, early development corresponds to small acini showing only few layers of spermatogonia and spermatocytes (in the testis) or oogonia connected to the follicular wall (in the ovary). In mid development, acini tend to shrink and the lumen becomes restricted, while oocytes and/or spermatids and spermatozoa become increasingly common. Late development is characterized by mature or maturing spermatozoa and oocytes strongly packed within the follicular lumen, although immature stages are still present.

The advancement of the oogenesis process was also evaluated by counting the frequency of oocytes and measuring their size variation over time (Grant & Tyler 1983). As a previous step, oocytes were classified accordingly to their vitellogenic stage as previtellogenic, vitellogenic, and postvitellogenic, following the criterion of Gaulejac (1995) for the marine bivalve *Pinna nobilis*. Aside from Gaulejac's work, additional descriptions about the cytological characteristics of each type of oocyte may be found in Saucedo et al. (2001a).

The frequency was calculated by counting the total number of each type of oocyte appearing in three randomly selected areas of the ovary. The size of oocytes was also assessed measuring thirty oocytes of any type appearing in three random areas of the ovary. The diameter was not used for the analysis because this dimension usually provides data that vary considerably as a consequence of the plane on which thin sectioning is made with the microtome. Instead, the area is not affected that much by the sectioning process and thus provides more homogeneous and reliable data about the size of oocytes. Images were captured from the computer with a digital Cole-Snap camera (I-media Cybernetics) and processed with Sigma Scan Pro (vers. 5.0) designed for digital image analysis.

Biochemical Analysis

For the biochemical analyses, preserved samples of gonadal tissue and digestive gland were weighed and homogenized in 3 mL of cold saline solution (NaCl 35%) to obtain a crude extract. Similarly, muscle samples were weighed and homogenized in 5 mL of 10% trichloracetic acid (TCA) for carbohydrate analysis. Homogenized samples were then centrifuged at 3600 rpm at –5°C for 15 min, and the supernatant stored at –20°C for 24 h for further analyses.

The TCA supernatant was directly used for carbohydrate analysis in muscle. For other tissues, the saline crude extract was diluted with 20% TCA. After centrifugation, 0.1 mL of the supernatant was mixed with 1 mL of anthrone reagent (0.1% dissolved in 76% sulfuric acid), incubated for about 2 min at 90°C and immediately cooled to 4°C to stop further reaction (Van Handel 1965). Absorbance was read at 620 nm against a reagent blank and carbohydrate was quantified using dextrose solution as standard.

For protein determination, the saline crude extract was diluted 1:5 in 0.5 N NaOH for soluble protein determination (Bradford 1976), using commercial chromogen reagent (Sigma) and bovine serum albumin (Sigma) as standard solution. Absorbance was read at 595 nm. For muscle protein determination, 0.01 g of tissue was digested in 3 mL of 0.5 N NaOH for 24 h before following the above methodology.

Commercial kits from Merck were used to determine the composition of total lipids (Merck num. 3321) and triacylglycerides (GPO-PAP, Merck num. 1.1434.001). These methods were adapted to a microplate using 20 μL of supernatant and 200 μL of enzyme chromogen reagent (Racotta et al. 1998; Palacios et al. 2000). Absorbance was recorded on a microplate reader (Labsystems, Uniskan II) at 560 nm for total lipids and 495 nm for triacylglycerides.

Statistical Treatment

For studying the histological and biochemical variations related to the ongoing gametogenic cycle, one-way ANOVA was used to determine significant differences in the area of oocytes (factor T with 12 levels or months). A two-way ANOVA was applied for differences in the biochemical composition of gonadal tissue, digestive gland, and adductor muscle over time and sex of specimens (factor S with two levels). In addition, correlation analyses were performed to assess the grade of relation between some histological and biochemical parameters of the above tissue components. For all analyses, the significance level was set at $P < 0.05$ (Sokal & Rohlf 1981).

RESULTS

Variation of Temperature

Part of 1997 and 1998 were warm years because of the presence of "El Niño" phenomenon in waters of Bahía de La Paz. As a consequence, the second half of 1998 and first half of 1999 were "Niña" years characterized by abnormal cold-water temperatures (Lluch-Belda et al. 1999). The chronology and duration of the reproductive stages defined in this study over the annual cycle January to December 1999 are based in this abnormal cold event. Figure 1 presents the historical register of mean temperature variation recorded at Bahía de La Paz from 1990 to 1999. Values of water temperature in the present study showed a relatively high variation of ±1–2.5°C from the summer and winter average values registered during such period of time.

Histological Analysis

Reproductive Cycle

The annual gametogenic cycle is depicted in Figure 2. Inactive gonads (unable to be sexed) were found only in January and February. Gonadal development started early in February and proceeded continuous and synchronously throughout the annual cycle in both male and female gonads, showing two peaks of maximum

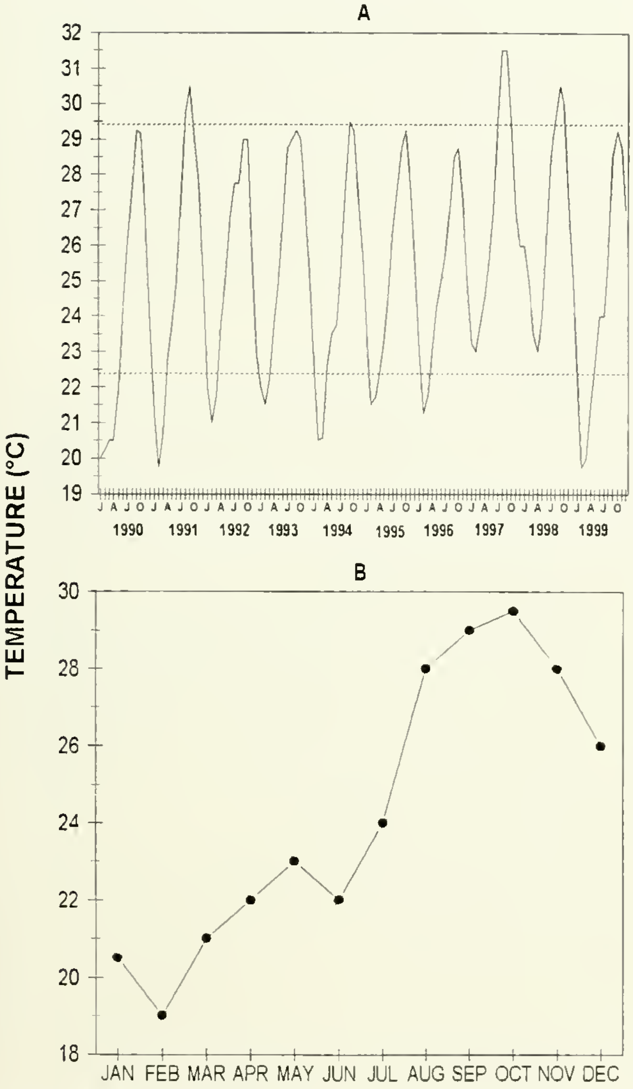

Figure 1. Mean range of temperature variation at different sites of Bahía de La Paz. A) Historical record from 1990 to 1999. Dotted lines at the top and bottom of the graph are the summer and winter average values, respectively; B) Values obtained from the present study during January to December 1999.

reproductive activity (defined in terms of the high incidence of specimens in late-development and ripe stages). The first peak was observed in spring (March to May), when water temperature was 21–23°C. However, no trace of spawning was detected during this season. The second peak was seen in summer (July to August), when water temperature rose to 25–26°C. In this case, a massive spawning occurred in the following month, when temperature reached its highest record (29–30°C). Organisms in spent stage were observed from October to November, although some other specimens were still spawning or starting a new gametogenic process.

Sex Ratio

The analysis for the whole gametogenic cycle revealed a higher frequency of males (49%) than of females (38%). Therefore, the female/male sex ratio was 0.78:1. The sample was also conformed by indeterminate specimens (7%), hermaphrodites (5%), and a small percentage of animals behaving as functional hermaphrodites (1%). Particularly, from January to May males completely outnumbered females and reached a mean percentage of 63% vs. 24% of females. From June to August this trend changed to 54% of males and 44% of females. Finally, from October on, the population of males decreased considerably (25%) while females strongly increased (51%).

Oocyte Examination

Figure 3 shows the variation of previtellogenic, vitellogenic, and postvitellogenic oocytes over time. All types of oocytes were observed throughout the annual cycle, although their frequencies of appearance were variable each month. Vitellogenic oocytes showed a relatively constant presence over time. An increase of previtellogenic oocytes was clear in November (31%) and December (58%) after the main spawning was achieved. In comparison, postvitellogenic oocytes showed higher incidences during March (51%), May (49%), and August (48%), corresponding to the same peaks of maximum reproductive activity detected histologically.

Temporal variations in the size (area) of oocytes are showed in Figure 4. Both decreases and increases in the volume of oocytes observed over the annual cycle are probably associated with changes in the temperature of water. Thus, small oocytes were observed in February (early development: 19°C), June (resorption process between both reproductive peaks: temperature dropped to 24°C), and October, November, and especially December (spawning and spent stage: 29.5, 28, and 26°C respectively). Large oocytes were observed in March, April, and May and August during the two reproductive peaks histologically detected. The

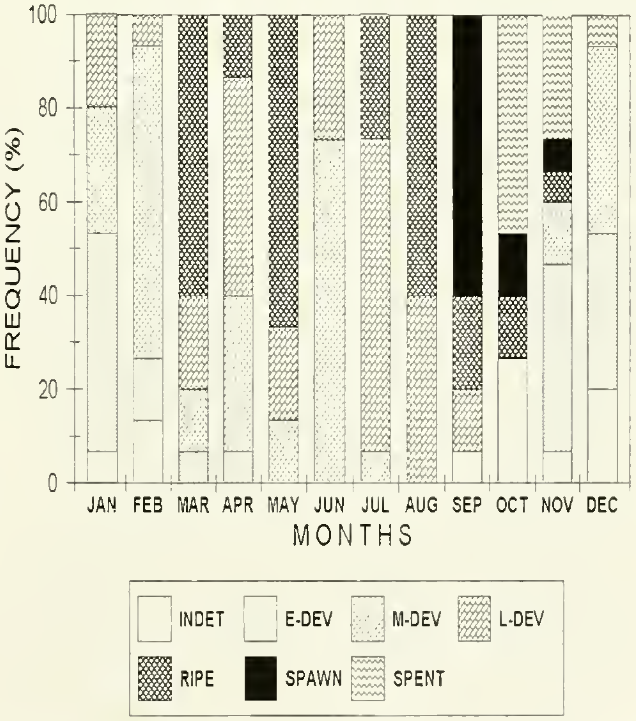

Figure 2. Sexual gametogenic stages in *Pinctada mazatlanica* over an annual cycle. Indet = Indeterminate stage; E-Dev = early-developmen stage; M-Dev = mid development; L-Dev = late-development; spaw spawning.

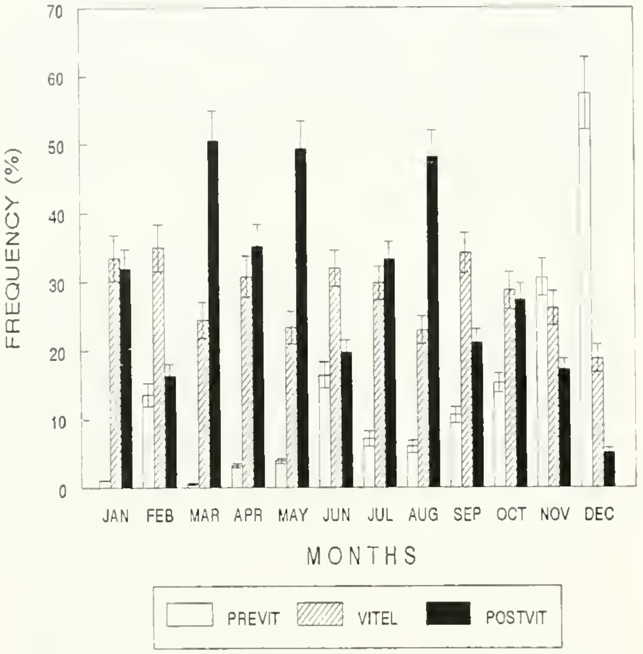

Figure 3. Temporal variations in the mean frequency of occurrence of different types of oocytes in *Pinctada mazatlanica* over an annual gametogenic cycle. Previt = previtellogenic oocytes; Vitel = vitellogenic oocytes; Postvit = postvitellogenic oocytes. Bars denote standard deviation.

ANOVA detected highly significant differences in the mean area of oocytes over time ($F = 102.97$; $P < 0.001$).

Biochemical Analysis

Gonadal Tissue

Preliminary tests made to measure the amount of carbohydrates in the gonadal tissue, digestive gland, and muscle indicated that over 80% was glycogen. Thus, glycogen is reported in this study as total carbohydrates (CHO).

When water temperature rose from 20 to 25°C, CHO were actively depleted during the first half of the year and decreased from 12 mg/g in January to 4 mg/g in July (Fig. 5A). Afterwards, CHO were not used for the production of gametes, yielding a slight increase in their concentration between 5 and 6 mg/g. The variation in the concentration of this component showed significant differences over time ($F = 35.83$; $P < 0.001$), but not according to the sex of specimens ($F = 0.414$; $P > 0.05$). The decrease in the levels of CHO in the gonadal tissue was not significantly correlated with the increase in the area of postvitellogenic oocytes ($r = 0.102$; $P > 0.05$).

Proteins (PRO) accumulated mainly in the female gonadal tissue during the first half of the reproductive cycle, resulting in a sharp rise in their concentration from 52 mg/g in January to 100 mg/g in May (Fig. 5B). After a drop in June to July, the concentration varied between 58 and 65 mg/g for both sexes during the second half of the year. There were not significant differences in the PRO content over time ($F = 0.954$; $P > 0.05$), but differences were significant in relation to the sex of specimens ($F = 8.16$; $P < 0.05$).

There were two peaks depicting the temporal variations in the concentration of total lipids (LIP; Fig. 5C) and triacylglycerides (TG; Fig. 5D) in the female gonadal tissue. The first peak was seen in April for LIP (20.4 mg/g) and in May for TG (13.5 mg/g), while the second peak was observed in August to September for both components (13.6 mg/g for LIP and 9.4 mg/g for TG). There were significant differences in the temporal variations of LIP ($F = 13.23$; $P < 0.001$) and TG ($F = 2.84$; $P < 0.05$) and also according to sex ($F = 14.24$; $P < 0.001$ for LIP; $F = 43.58$; $P < 0.001$ for TG). There was a positive and significant correlation between the variations of both elements over time ($r = 0.973$; $P < 0.001$). Similarly, variations in the concentration of LIP were significantly correlated to the increase in the area of postvitellogenic oocytes ($r = 0.64$; $P < 0.005$).

Digestive Gland

There was a continuous accumulation of CHO in this tissue from 23.4 mg/g in March to over 32 mg/g until November (Fig. 6A). After the spawning, the concentration of CHO decreased to 22 mg/g in December. There were significant differences in the concentration of this component over time ($F = 27.8$; $P < 0.001$). However, differences were not significant in relation to the sex of oysters ($F = 0.434$; $P > 0.05$).

PRO showed a gradual decrease from January (156 mg/g) to October (96 mg/g), especially in males (Fig. 6B). In females, this trend showed two minimum peaks in May and October. After the spawning in late September, a shift in the concentration of PRO was recorded again. The variations in the concentration of PRO denoted significant differences over time ($F = 24.86$; $P < 0.001$), while differences were not significant according to the sex of specimens ($F = 0.093$; $P > 0.05$).

LIP and TG in this tissue presented two peaks of concentration throughout time (Figs. 6C and D). The first peak occurred in April (25 mg/g for LIP and 15 mg/g for TG). The second peak was observed in August, with values of 17 mg/g (LIP) and 10 mg/g (TG). Unlike gonadal tissue, the concentration of both components during winter (spent stages) was the same or higher than that of the summer. There were significant differences in the variation of the LIP and TG content over time ($F = 56.28$; $P < 0.001$ for LIP; $F = 26.82$; $P < 0.001$ for TG), but differences were not significant

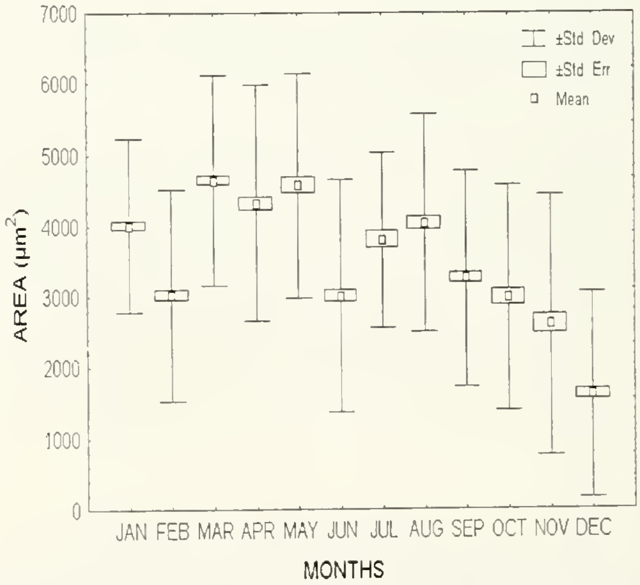

Figure 4. Box and Whisker for temporal variations in the mean area of oocytes in *Pinctada mazatlanica* over an annual gametogenic cycle.

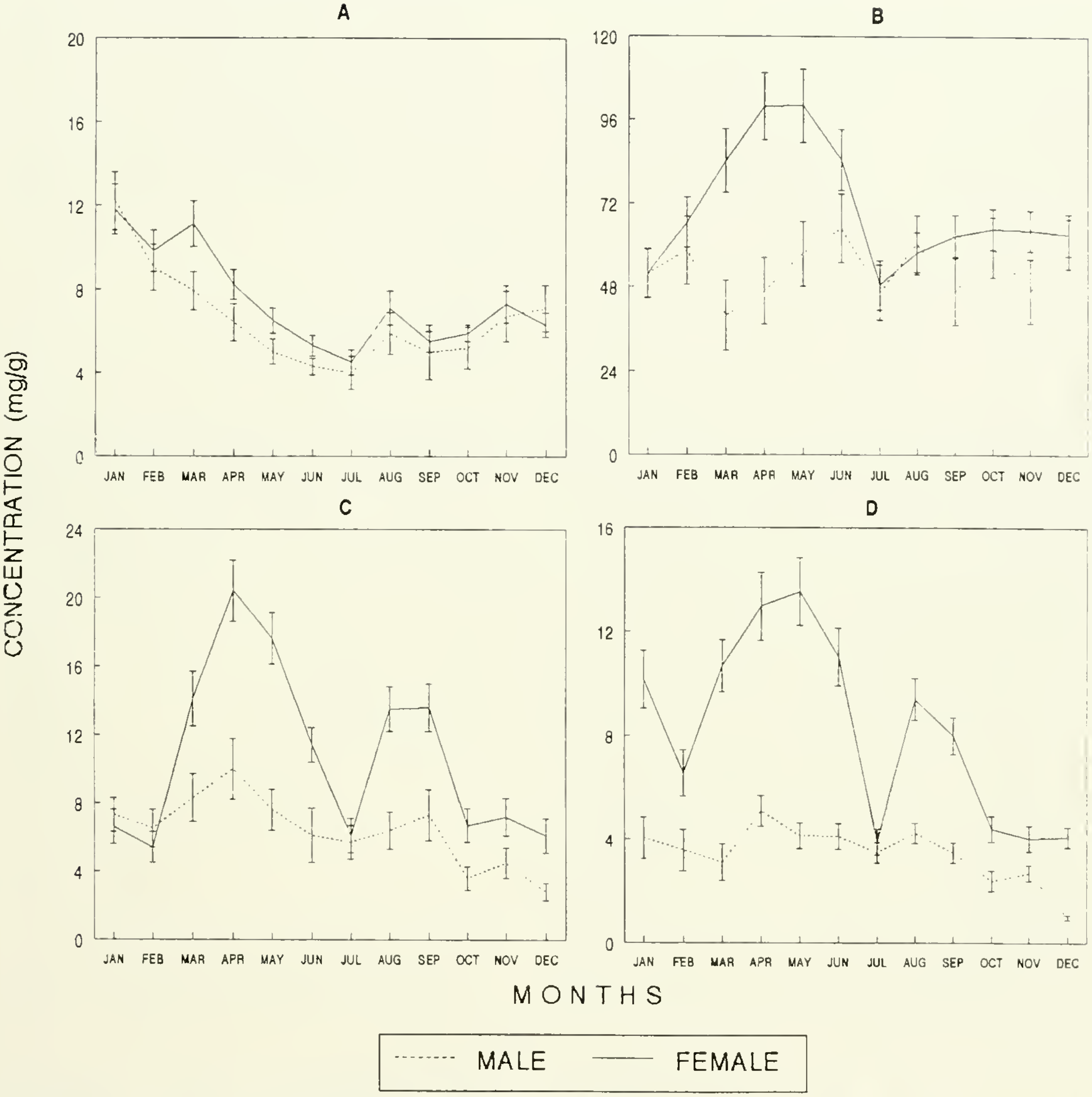

Figure 5. Temporal and sexual variations in the mean levels of total carbohydrates (A), proteins (B), total lipids (C), and triacylglycerides (D) in the gonadal tissue of *Pinctada mazatlanica* over an annual gametogenic cycle. Bars denote standard deviation.

between sexes ($F = 0.849$; $P > 0.05$ for LIP; $F = 0.267$; $P > 0.05$ for TG). The temporal variations of both components were significantly correlated ($r = 0.963$; $P < 0.001$).

Adductor Muscle

CHO in the muscle showed high concentrations during the first half of the gametogenic cycle, varying between 95 mg/g (January) and 115 mg/g (June) (Fig. 7A). Afterwards, a sharp drop to 50 mg/g in October was observed mainly during the mid and late-developing and ripe stages. After the spawning, a recovery in the content of CHO was noticed. The ANOVA found significant differences in the concentration of this component on time ($F = 47.95$; $P < 0.001$). Neither sex showed significant differences in their CHO content over time ($F = 1.39$; $P > 0.05$).

During the first reproductive peak, the level of PRO in the muscle decreased from 235 mg/g in February to 128 mg/g in May. After a short period of accumulation in June, such levels gradually rose and reached 227 mg/g in December (Fig. 7B). The variations in the concentration of PRO denoted highly significant differences over time ($F = 103.61$; $P < 0.001$), but differences were not significant between sexes ($F = 0.138$; $P > 0.05$). The decrease in the PRO content of the muscle was significantly correlated to the increase in the area of postvitellogenic oocytes ($r = 0.632$; $P < 0.05$).

DISCUSSION

Seasonal changes in energy storage and depletion in relati gametogenesis have been well documented during the las

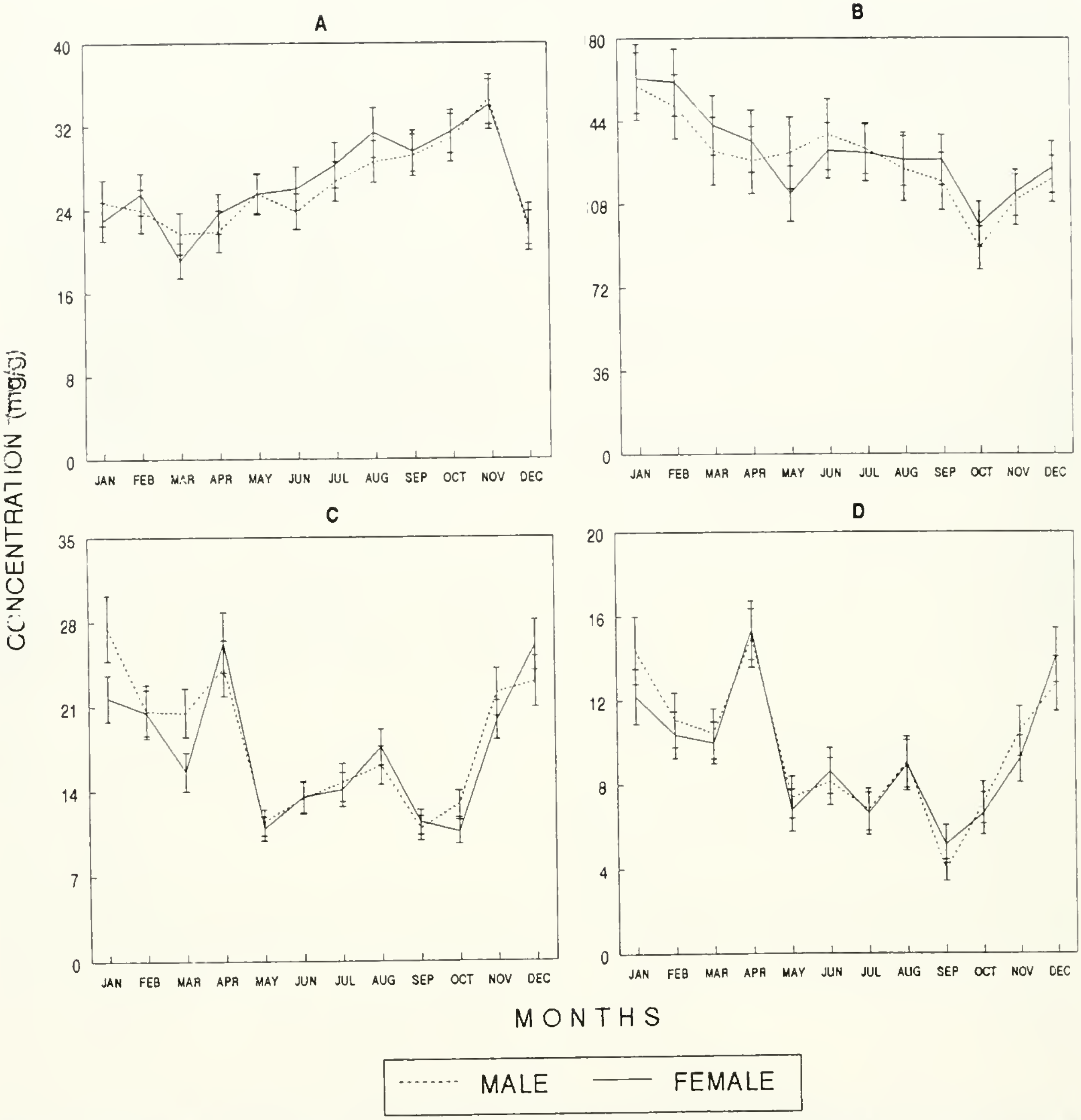

Figure 6. Temporal and sexual variations in the mean levels of total carbohydrates (A), proteins (B), total lipids (C), and triacylglycerides (D) in the digestive gland of *Pinctada mazatlanica* over an annual gametogenic cycle. Bars denote standard deviation.

decades for some commercial-edible species of marine bivalves, such as scallops, mussels, and oysters (Giese 1969; Sastry & Blake 1971; Ansell 1974; Comely 1974; Gabbott 1975, 1976, 1983, Bayne 1976; Taylor & Venn 1979; Zandee et al. 1980; Barber & Blake 1981, 1983, 1991; Robinson et al. 1981; Bayne et al. 1982; Epp et al. 1988; Couturier & Newkirk 1991; Martínez 1991; Pazos et al. 1997; Racotta et al. 1998). Nevertheless, most of the aspects concerning the storage capacity and mobilization of nutrients to satisfy metabolic needs related to gametogenesis seem to be species-specific (Barber & Blake 1981, 1991) and some important interspecific differences have been reported (Pazos et al. 1997). The present work couples for the first time both gametogenic and biochemical cycles for a species of pearl oyster.

Both histological and biochemical analyses showed the existence of two peaks of intense reproductive activity over the annual cycle; one in spring (March to May) when water temperature was rising from 21 to 23°C, and other in summer (August to October) when water temperature was about to reach a maximum value of 28–29°C. The first reproductive peak was clearly defined in terms of its high incidence of ripe specimens, high frequency of postvitellogenic oocytes, and high levels of protein, lipids, and triacylglycerides within the gonadal tissue. However, the histological analysis did not identify any spawning activity in the sample. This was confirmed by very low spatfall recorded in parallel field monitoring (CIBNOR-Pearl Oyster Research Program database). Such result was probably ascribed to the low and abnormal water tem-

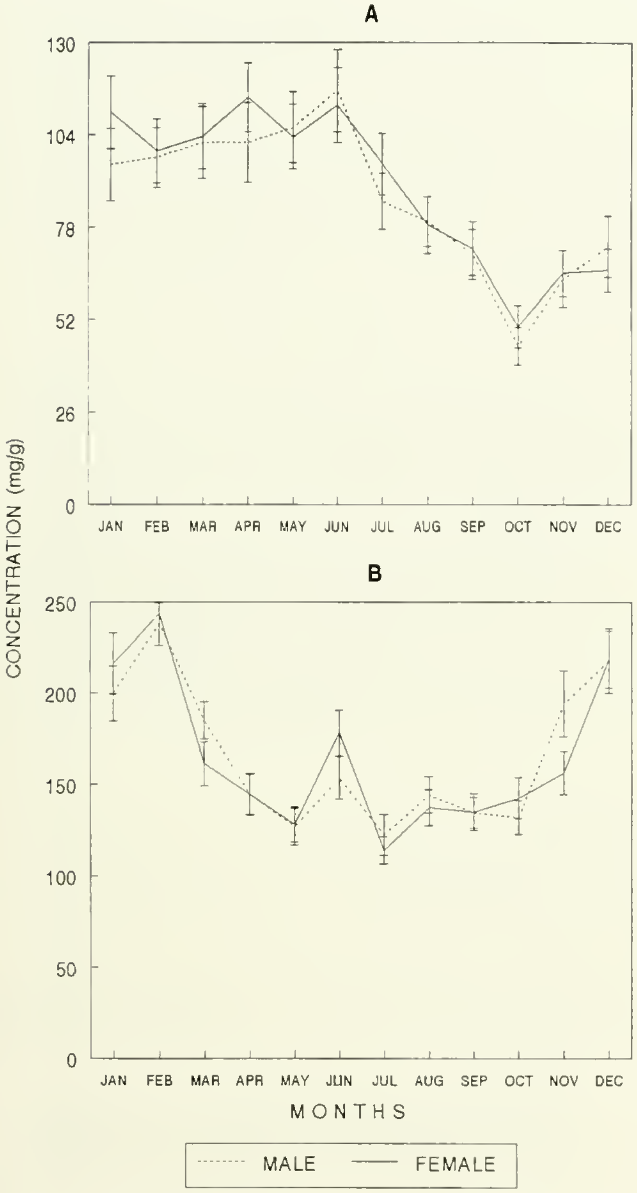

Figure 7. Temporal and sexual variations in the mean levels of total carbohydrates (A) and proteins (B) in the adductor muscle of *Pinctada mazatlanica* over an annual gametogenic cycle. Bars denote standard deviation.

perature registered during March to May (21–23°C) and also to the drop-out in the same parameter detected in June (22°C) as a consequence of the presence of "La Niña" phenomenon in waters of Bahía de La Paz during the first half of 1999, which caused gonads to undergo a partial lysis and resorption. Similar processes have been reported to occur in the scallops *Argopecten irradians concentricus* (Sastry 1966; Barber & Blake 1981, 1991; Epp et al. 1988) and *Placopecten magellanicus* (Robinson et al. 1981) under low temperatures conditions or during times of food shortage.

In this study, the three major tissues related to gametogenesis (gonad, digestive gland, and adductor muscle) were utilized differently over time, but showed a clear seasonal cycle of gonadal development, spawning, energy storage, and mobilization of nutrients. Gametogenesis started early in February, when food is commonly abundant from the phytoplankton (Signoret & Santoyo 1980; Lechuga-Devéze 1997), and wild specimens had stored enough energy reserves in the adductor muscle (mainly) and digestive gland (secondarily). When gametogenesis had already reached the early-development stage in March, the first previtellogenic and vitellogenic oocytes started to differentiate within acini. Because proteins in the muscle and digestive gland showed a downward trend from January to May, it seems evident that gonad growth took place at their expense during the first reproductive peak. Therefore, a substantial growth in the size of oocytes, an increase in the weight of gonadal tissue, and their levels of protein, lipids, and triacylglycerides was recorded. This was particularly evident in the female gonad, where the content of the last three elements doubled that of males, at least during the first reproductive peak, and partially during the second reproductive peak. On the contrary, as gametogenesis proceeded into the mid and late-developing and ripe stages (June to October), carbohydrates (glycogen) from the muscle were the only substrate mobilized and used for the build-up of gametes. Following the spawning in October–November, when gonadal tissue decreased in weight and their basic levels of lipids and triacylglycerides fell sharply, the condition of the digestive gland and muscle improved again.

Carbohydrates obtained from ingested food were used as an energy-rich fuel for the build-up of gametes, via their conversion into lipids and triacylglycerides reserves (lipogenesis). This process has been reported to occur in some bivalves to satisfy the metabolic demands derived from vitellogenesis (Gabbott 1975, 1976, 1983), especially because lipids and triacylglycerides are the basic energetic reserves for sustaining embryonic and larval development of most species of marine bivalves (Holland 1978; Fraser 1989). However, there was a lack of correlation between carbohydrates of the gonadal tissue and the total area of postvitellogenic oocytes. On the contrary, the correlation was significant between the area of oocytes and the content of lipids in this tissue. Several authors have discussed the relative contribution of food intake vs. energy reserves to satisfy the metabolic demands of growth and gonadal production in marine bivalves (Bayne 1976; Gabbott 1976; Barber & Blake 1981, 1983; Robinson et al. 1981; Epp et al. 1988; Racotta et al. 1998). However, because these processes are highly dependent on several exogenous and endogenous factors, no pattern has yet been established. For example, in *Pecten maximus* (Comely, 1974), *Chlamys opercularis* (Taylor & Venn, 1979), and *Mytilus edulis* (Gabbott, 1975), energy reserves are required for both the initiation of gametogenesis and subsequent gonadal growth, while in others such as *A. irradians concentricus*, food intake is necessary to sustain gonadal growth since reserves from the muscle, digestive gland, and mantle are inadequate.

Parallel to their utilization for gametogenesis, ingested nutrients were also incorporated into the adductor muscle and digestive gland for storage. The muscle stored high levels of protein both at the beginning and end of the annual cycle to sustain the start of gametogenesis. No other study with bivalves has reported protein values as high as those presented here for the adductor muscle of *P. mazatlanica*, neither for scallops whose adductor muscle is associated with locomotion. Because the decrease in protein content of the muscle was significantly related to the increase in the size of oocytes during vitellogenesis, the role of this organ as the most important site of energy storage is evident. In fact, a significant loss of weight in the muscle to less than half its maximum value was detected as both protein and carbohydrate reserves [illegible] rapidly consumed in benefit of gametogenesis. The contribution of the muscle has also been emphasized for other bivalves, such as *Chlamys* sp (Ansell 1974; Taylor & Venn 1979), *M. edulis* (Gabbott 1975; Bayne 1976), *A. irradians concentricus* (Barber & Blake 1981, 1991; Epp et al. 1988), *P. maximus* (Faveris & Lubet 1991), *A. purpuratus* (Martínez 1991), and *A. ventricosus* (Racotta et al. 1998).

Unlike the muscle, the digestive gland appears to have a secondary role in gonadal development of *P. mazatlanica*. This seems evident since protein was the only fuel transferred to the gonadal tissue during the first half of the year. On the contrary, carbohydrates were progressively stored from March to November, indicating that despite the advancement of gametogenesis, these nutrients are not used for the cycle in course and rather accumulated for further energy needs. This result is in agreement with the finding of Barber and Blake (1981, 1991) that the digestive gland acts as a short-term storage and transfer site of carbohydrates to meet the reproductive events in *M. edulis*. Regarding the storage function, Saucedo et al. (2001b) found specialized vesicular connective tissue (VCT) cells—filled mainly with carbohydrates—surrounding excretory conduits among the digestive gland and gonadal tissue. We believe that VCT cells from both tissues are probably communicated via the interconnective tissue matrix, which may be the vehicle for the transport of stored carbohydrates between the digestive gland and the gonadal tissue, and vice versa. Lipids and triacylglycerides reserves were stored and rapidly mobilized after each of the reproductive peaks detected. Therefore, *P. mazatlanica* may be considered a species that uses a strategy of storing large quantities of energy for starting and sustaining gametogenesis. However, our knowledge about the metabolic control of reproduction in pearl oysters is still limited. More studies on seasonal cycles of energy storage and mobilization are required, especially involving the participation of the mantle tissue, which has been proposed to be an important site of storage of glycogen and lipids in some bivalves. It would also be interesting to obtain data similar to that from this study for tropical species of *Pinctada*.

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HYBRIDIZATION OF TETRAPLOID AND DIPLOID *CRASSOSTREA GIGAS* (THUNBERG) WITH DIPLOID *C. ARIAKENSIS* (FUJITA)

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ABSTRACT Three replicates of hybrid crosses of tetraploid and diploid *C. gigas* (Thunberg) with diploid *C. ariakensis* (Fujita) were produced with controls. Larval survival and growth were documented. Cytological events were also monitored in oocytes from hybrid crosses following insemination. Among the four types of hybrid crosses, diploid *C. gigas* (female) × diploid *C. ariakensis* (male) (GA) was the most successful. Survival of GA was about the same as that of controls in two of three replications, although its growth rate was 25–30% lower. Crosses of tetraploid *C. gigas* (female) and diploid *C. ariakensis* (male) (GGA) had poor yield at day 2 post-fertilization (0.05%), but grew nearly as well as controls subsequently. The other two types of hybrids (i.e., diploid *C. ariakensis* [female] and tetraploid *C. gigas* [male] [AGG], diploid *C. ariakensis* [female] and diploid *C. gigas* [male] [AG]) suffered very low yield at day 2 (0.01% and 0.003%) and grew very slowly. Spat were obtained from all replicates of GA crosses and one of three replicates of GGA, and proved to be hybrids by polymerase chain reaction/restriction fragment length polymorphism (PCR/RFLP) diagnosis. GGA hybrids were confirmed to be triploid by flow cytometry. No larvae survived to eyed stage in AGG or AG crosses. Cytological examination revealed that the vast majority (>99%) of oocytes from hybrid crosses had a prolonged meiotic prophase I or metaphase I at least through 180 min post-insemination.

KEY WORDS: *Crassostrea gigas, Crassostrea ariakensis,* diploid, hybrid, tetraploid, oyster, breeding, polyploidy

INTRODUCTION

There are numerous reports of attempted interspecific hybridization in the genus *Crassostrea* (Gaffney & Allen 1993). However, most should be viewed with caution because these reports were unaccompanied by genetic confirmation of putative hybrids. Even a modest amount of contamination may account for the majority or all of surviving progeny in hybrid crosses in which fertilization rate and viability are normally low or nil (Allen & Gaffney 1993). One case seems clear: Pacific oyster *C. gigas* (Thunberg) and Suminoe oyster *C. ariakensis* (Fujita—formerly *C. rivularis* Gould) can be crossed to produce viable hybrids (Allen & Gaffney 1993).

The production of hybrids is interesting because they may possess qualities that improve commercial traits. Hybrids also could be back-crossed to introgress certain traits into either of the parental species; for example, disease resistance. Introgression of disease resistance into *C. virginica* (the Eastern oyster) from *C. gigas* was the rationale for the extensive hybrid trials undertaken by Allen et al. (1993). Later, many more hybridization trials were attempted, using bridging crosses between races of *C. virginica,* using (only slightly) fertile *C. gigas* × *C. ariakensis* hybrids (GA), and using polyploidy (Lyu 1996). However, under no circumstances tested in the lab did *C. virginica* hybridize with *C. gigas* or *C. ariakensis.*

Although they failed as a bridging cross to *C. virginica,* GA hybrids are still of interest for several other reasons. First, no work has been done on the qualities of diploid GA hybrids as an aquaculture product, although this work might more appropriately be carried out where there is on-going commercial culture of these two Asian species. For the East coast, they are nonnative. Second, because diploid hybrids are possible, production of polyploid hybrids should also be possible. Polyploid hybrids are potentially useful for improvement of commercial traits (Longwell 1986). Virtually no work on polyploid hybrids of shellfish has been done. Third, and most apropos to research on the East Coast, is the issue of testing nonnative species as an alternative to the native Eastern oyster because of the decline in the fisheries there.

Trials of nonnatives were begun in Delaware Bay several years ago (Allen 1993) and have been conducted for *C. gigas* in the Chesapeake Bay (Calvo et al. 2000). Trials with *C. ariakensis* have been (Calvo et al. 2001) and continue to be conducted. *C. gigas* seems more suitable for higher salinity environments and *C. ariakensis* seems suitable for more estuarine conditions. In all field trials up to this point, triploids have been used to effect population control because of their sterility (Allen & Downing 1990, Gaffney & Allen 1992, Guo & Allen 1994a).

Triploid hybrids then are of interest because they are expected to be sterile, more so than diploids because of the added burden of gametogenesis in hybrids (Thorgaard & Allen 1986, Thorgaard & Allen 1992). Triploid hybrids may also have characteristics intermediate to the two parental species, for example, salinity preference. The genotypes that might be available for culture in an estuary as varied as the Chesapeake Bay, for example, could range from triploid *C. gigas* (GGG) through two types of triploid hybrids—either tetraploid *C. gigas* × diploid *C. ariakensis* (GGA) or diploid *C. gigas* × tetraploid *C. ariakensis* (GAA)—to triploid *C. ariakensis* (AAA), with phenotypes potentially encompassing the full range of estuarine and marine conditions.

To date, all hybrid crosses between *Crassostrea* species have been made between diploids. Diploids are also used in the production of triploid hybrids using ploidy induction techniques (Allen et al. 1989). For example, triploid hybrids were attempted between *C. virginica* and *C. gigas* by inhibiting polar body 2 with cytochalasin B treatment (Allen et al. 1993). However, this hybrid seems to be inviable in any form. Triploid hybrids were also attempted (S. K. Allen, Jr., unpublished data) between *C. gigas* and *C. ariakensis.* These, too, were unsuccessful for another rea fertilization in this cross is protracted, taking more than 3 h.

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consequence, polar body 2 formation is asynchronous and treatments to inhibit polar body 2 are useless. The availability of tetraploid *C. gigas* (Guo & Allen 1994b), tetraploid *C. ariakensis*, or both, provides a new opportunity to produce triploid hybrids by crossing (Guo et al. 1996), rather than induction and to further investigate the fitness of them. As a first step, we examined the feasibility of hybridizing tetraploid *C. gigas* and diploid *C. ariakensis*. At the same time, we repeated crosses of diploid *C. gigas* with diploid *C. ariakensis* as controls and also examined early development of eggs cytogenetically.

MATERIALS AND METHODS

Oyster and Gametes

Sexually mature oysters used in this study were 2 years old and obtained from stocks held at the Cape Shore Laboratory, Haskin Shellfish Research Lab. Ploidy of tetraploid Pacific oysters was confirmed in all individuals by flow cytometry prior to spawning. Gametes were obtained by strip spawning. All surfaces and instruments contacting the oysters were cleaned with dilute bleach and rinsed with fresh water between handling and opening of different individuals. Sex was determined by gonad biopsy under a light microscope. Once the sex was determined, the animals from different sexes were removed to separate containers. Gametes from each oyster were dissected into individual beakers. Eggs were passed through a 60-μm Nytex screen to remove the large tissue debris and rinsed on a 25-μm screen, then suspended in filtered (2 μm) seawater at 23–25°C for at least 30 min to confirm that the eggs were not self-fertilized. Sperm were separated from debris by passing the suspension through a 15-μm screen.

Experimental Design

Abbreviations for gamete contributions of the two oyster species are as follows: G = diploid *C. gigas;* GG = tetraploid *C. gigas;* and A = diploid *C. ariakensis*, with female listed first. Eight types of crosses were conducted (Table 1) overall, although not all crosses were possible in all three replicates. For each replication, an individual female and male were used. After spawning, parents were frozen at −80°C for subsequent genetic confirmation of the progeny.

TABLE 1.

Experimental Design for Crosses Among 4n *C. gigas*, 2n *C. gigas*, and 2n *C. ariakensis*.

		♂		
	G	**GG**	**A**	
♀				
G	GG		GA	
GG			GGA	Rep 1
A	AG	AGG	AA	
♀				
G	GG	G/GG	GA	
GG	GG/G		GGA	Rep 2
A	AG	AGG	AA	
♀				
G	GG	G/GG	GA	
GG			GGA	Rep 3
A	AG	AGG	AA	

Individual females and males were used for each replication, and three replicates were made. Gamete contribution is represented by GG, G, or A, respectively, with female listed first.

Embryonic and Larval Development

Insemination was conducted at 23–25°C and for hybrids, high densities of sperm were used (Lyu & Allen 1999). Fertilization rate was assessed by directly examining at least 100 oocytes under the light microscope at 60–90 min post-insemination for controls and up to 180 min post-insemination for hybrid crosses. After determining fertilization rate, oocytes were transferred to culture vessels whether fertilization was observed or not. Fertilization was considered successful if the oocyte was at or beyond polar body 1 formation.

Yield at 48 h post-insemination was estimated by directly counting straight-hinge larvae with normal appearance. Yield was calculated as

(no. of straight-hinge × 100)/no. of eggs incubated

Temperature and salinity for larval cultures of crosses of GG, GA, GGA, G/GG, and GG/G, where *C. gigas* was the egg source, were 25°C, 22–23 ppt (Breese & Malouf 1975). For crosses of AA, AG, and AGG, where *C. ariakensis* was the egg source, temperature and salinity were 26°C and 20 ppt (Breese & Malouf, 1977). Seawater in the larval cultures was renewed every 2 days. In all hybrid cultures, densities of larvae were sufficiently low to prevent density-related growth effects; densities in parental culture were within those used in standard larval culture, beginning at 10/mL and winnowing out to 1–2 /mL. During water changes, numbers of remaining larvae were estimated and shell length was measured for 20 individuals for each cross. When larvae reached eyed stage, eyed larvae were collected and treated with a solution of 10^{-4} M epinephrine for 16 h (Coon et al. 1986). Following treatment, metamorphosed larvae were held in a downweller system until they reached a shell length of approximately 1 mm, when they were transferred to an upweller silo. We took great care to eliminate all sources of contamination throughout the culture process.

For cytological observations, eggs from each hybrid cross were sampled and fixed with Carnoy's solution (1:3 glacial acetic acid and absolute methanol) at 90, 120, 150, and 180 min post-insemination. Fixatives were changed twice following light centrifugation. Chromosomes were observed by acetic orcein stain (Guo et al. 1992).

Genetic Confirmation

We randomly sampled 28 spat from each replicate of GA crosses and all GGA spat. In progeny, the whole body was prepared for DNA extraction, whereas mantle tissue (2–8 mg) from corresponding parental species was prepared using a commercial kit (PureGene, Gentra, Minneapolis, MN). An additional gill tissue sample from GGA spat was taken and stored in DAPI/DMSO (Sigma, St. Louis, MO) solution at −80°C for flow cytometric analysis.

An approximately 550-bp region of the nuclear rDNA genome was amplified via polymerase chain reaction (PCR) using primer ITS-1. (The primer pair was designed by Dr. Patrick M. Gaffney, University of Delaware [Hedgecock et al. 1999]). Reaction volume of 25 μL contained 50 MM $MgCl_2$, 0.2 mM of each dNTP, 0.2 μM of each primer, 5 U/μL polymerase (Taq DNA polymerase, Sigma) and 1 μL DNA extraction. DNA amplifications were performed in a programmable thermal cycler (PTC-100, M.J. Research, Inc., Walthram, MA) using a 2-min initial denaturation at

94°C and then 34 cycles of 45-sec denaturation at 94°C, 1-min annealing at 52°C, 1-min extension at 72°C, and finally a 5-min extension at 72°C.

Restriction enzyme digestion of PCR products was made with restriction endonuclease Hinf I (Sigma). Digestion volume of 20 μL contained 1 × buffer (supplied by New England Biolab Inc., Beverly, MA), 5 units Hinf I, and 8 μL PCR product. Digestion mix was incubated at 37°C for 3–4 h, followed by 5 μL 10× loading dye to stop the digestion.

All PCR products and restriction digest fragments were electrophoresed in a 3% agarose (Sigma) gel in 1× TBE (0.089 M Tris-borate, pH 8.3, 0.002 M ethylenediamine tetraacetic acid [EDTA]) buffer. A molecular weight marker (pUC 18, digested with Hae III, Sigma) was loaded along with the product of interest. The gel was run at 60–90 V, stained with ethidium bromide (0.2 mg/mL) for 10–15 min, and visualized by transillumination.

Statistical Analyses

All data were analyzed with the computer program SYSTAT (Wilkinson 1990). Fertilization rates and yield data were arcsine transformed prior to statistical analysis (Sokal & Rohlf 1981). To compare the performance of hybrids to their controls, a two-way ANOVA was used. Paired *t*-tests were conducted to compare certain crosses to their reciprocals.

RESULTS

Fertilization Rate and 48-h Yield

Mean fertilization rates in the parental (nonhybrid) crosses were 94% (GG), 77% (AA), 88% (G/GG), and 85% (GG/G) (Table 2), with no statistically significant difference among them ($F = 3.118$, $P = 0.132$) by ANOVA. In hybrids, signs of fertilization did not appear until 180 min after insemination, precluding estimates of fertilization rate.

Yield at 48 h post-insemination varied significantly among crosses (Table 2) ($F = 3.964$, $P = 0.018$). Yield in GG was significantly greater than that in AA ($t = 4.162$, DF = 4, $P = 0.014$) but there was no difference between other parental crosses. Yields were similar in GGA and AGG ($t = 1.010$, DF = 4, $P = 0.369$), whereas GA had higher yields than AG ($t = 5.364$, DF = 4, $P = 0.006$). Yields of GA and AA crosses were about the same. GGA and AGG crosses produced many fewer larvae than controls. AG crosses suffered extremely low yield (0.003%).

TABLE 2.

Mean fertilization rates and yields ± SD (*n*) at 48-h in parental and hybrid crosses combined from three replicates.

Cross	Fertilization Rate (%)[a]	Yield (%)
GG	94 ± 4.9 (3)	21 ± 7.5 (3)
GA	ND	4 ± 1.2 (3)
GGA	ND	0.05 ± 0.01 (2)
AA	77 ± 13.9 (3)	3 ± 2.5 (3)
GG/G	85 (1)	4 (1)
G/GG	88 ± 5.0 (2)	18 ± 24.4 (2)
AGG	ND	0.01 ± 0.02 (3)
AG	ND	0.003 ± 0.006 (3)

ND = no data.

[a] Fertilization rate was observed at 60–90 min post-insemination for pure crosses; 180 min post-insemination for hybrid crosses.

Larval Survival and Growth

After 48 h post-insemination, survival of GA crosses was about equal to controls in two of three replicates (Fig. 1). Larvae of GGA crosses had high survival, although the number of eyed larvae was small (of 450 larvae on day two, 280 survived to eyed stage). For AGG and AG crosses, mortality was severe and steady for 13 days, with no survival to eyed stage. In GG crosses, mortality was generally density related, moderating at lower densities at about day 6–10. For AA crosses, survival was generally poor, making this cross a poor control. In general, survival ranked GG, GA, and AA, in descending order. Survival of G/GG was variable: better than GG in replicate 2 but worse than AA in replicate 3. Survival of

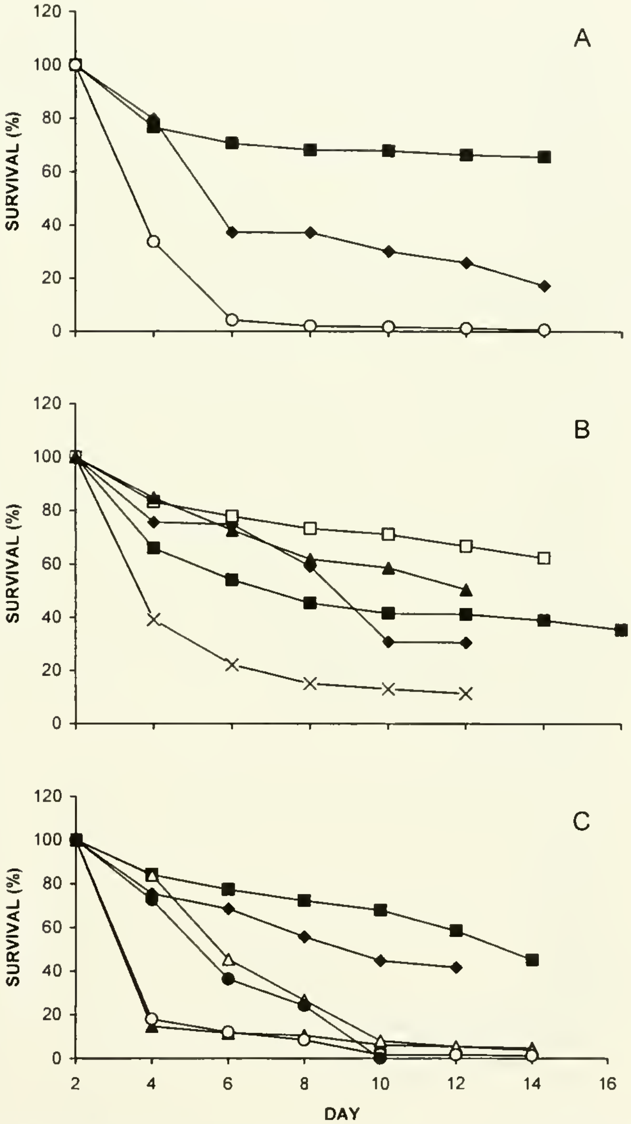

Figure 1. Mean survival of hybrid larvae and their respective controls from day 2 up to day 16 in crosses of diploid and tetraploid *C. gigas* with diploid *C. ariakensis*. (A) Six matings were made in replicate 1. No larvae survived to day 2 in AG, AGG, and GGA. (B) Eight matings were made in replicate 2. No larvae survived to day 2 in AG, AGG, and AA. (C) Seven matings were made in replicate 3. No larvae survived to day 2 in GGA. Counts were terminated when harvesting of eyed larvae was begun. GG (◆), GA (■), G/GG (▲), GG/G (×), GGA (□), AGG (△), AA (○), AG (●).

G/GG crosses was better than its reciprocal, GG/G. Larvae survived to setting in all replicates of GG and GA, 2 of 3 replicates of G/GG and 1 of 3 replicates of GGA. Spat were obtained from all of these.

Larvae of GG, G/GG, GG/G, and GGA crosses grew at similar rates. Crosses of GA grew slower than GG controls but faster than AA (Fig. 2). GA larvae were generally smaller than GG larvae. Both AG and AGG crosses grew very slowly (AG larvae died at day 10). AA crosses grew slowest of all controls. Mean size of eyed larvae was 350 μm for GG, 336 μm for GA, 360 μm for GGA, 361 μm for G/GG, and 361 μm for G/GG. ANOVA showed no significant difference in eyed larvae size among crosses ($F = 1.712$, $P = 0.199$). After 90 days post-setting, spat from GGA reached 12.0–17.5 mm in shell size compared with 2.87–8.0 mm in the corresponding GA cross ($t = 8.49$, DF = 6, $P < 0.001$).

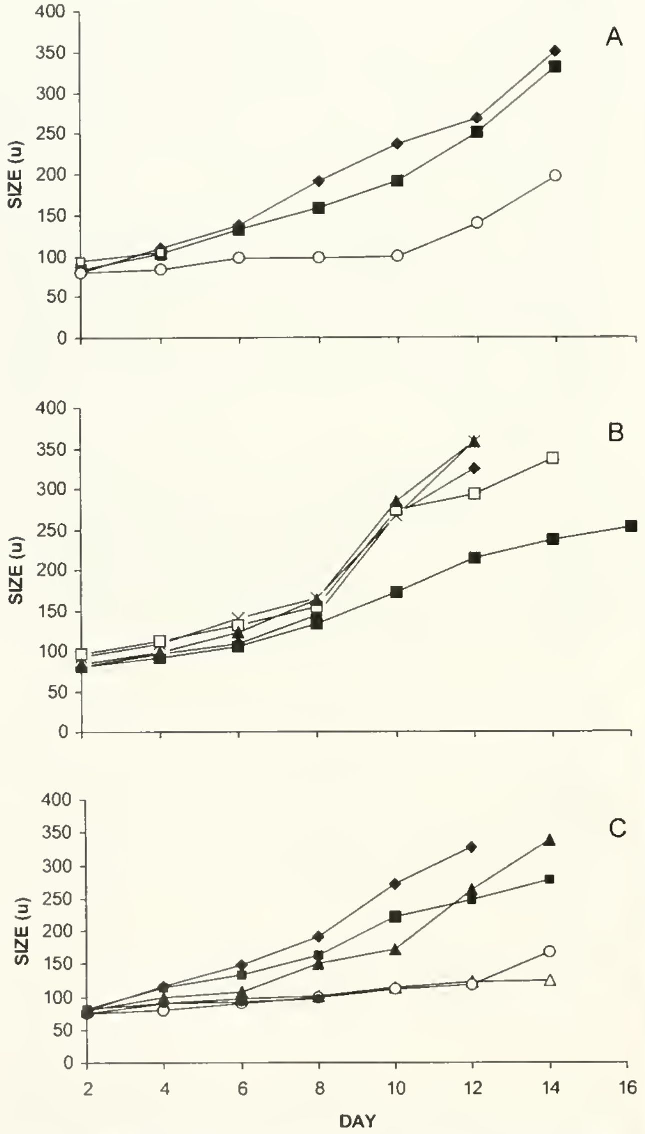

Figure 2. Mean size (μm) of hybrid and control larvae from day 2–16 in crosses of diploid and tetraploid *C. gigas* with diploid *C. ariakensis*. (A) Larvae from GG, GA, GGA, and AA in replicate 1; (B) larvae from GG, GA, GG/G, and G/GG in replicate 2; and (C) larvae from GG, GA, G/GG, AGG, AA, and AG in replicate 3. Measurement of surviving larvae was made until eyed larvae appeared. GG (◆), GA (■), G/GG (▲), GG/G (×), GGA (□), AGG (△), AA (○), AG (●).

Cytological Observation of Eggs from Hybrid Crosses

The vast majority of eggs from hybrid crosses were delayed at prophase I or metaphase I at least through 180 min post-insemination (Fig. 3A–E). In fact, of all eggs examined at 180 min post-insemination (≥150 observations from each of GA, AG, GGA, and AGG), only 2% of eggs from GGA had entered anaphase I (Fig. 3F). In GA, AG, and AGG, 10 bivalents were still observed at this time. Chromosome aggregation was much more complicated in eggs from GGA crosses. In general, eggs contained an average of 10 quadrivalents, although other types of synaptic chromosomes were also present, i.e., univalents, bivalents, and trivalents (Fig. 3D, E).

Genetic Confirmation

Agarose gel electrophoresis of the PCR products consistently revealed two bands, one at around 587 base pairs (bp) and the other at around 527 bp (Fig. 4, top). Restriction digest fragments resolved distinct bands in both parental species. Two bands were resolved, one at around 434 bp and the other around 138 bp in *C. gigas* females, both tetraploid and diploid. In contrast, two bands were resolved at around 267/257 bp and 174 bp in *C. ariakensis* males (Fig. 4, bottom). The hybrids (GA and GGA) expressed all four bands corresponding to their parental species. An additional band was detected at around 458 bp in hybrids. Identical band resolution was observed in replicates 1 and 2 of GA crosses, whereas an extra band was resolved at 587 bp consistently in both parental species and hybrids in replicate 3 (Fig. 4, bottom, last four lanes). All progeny samples (28 individuals from each replicate of GA and a total of 4 individuals from GGA) were hybrids. Three of four GGA hybrids were triploid as confirmed by flow cytometry; the other hybrid was diploid.

DISCUSSION

In hybrid crosses, fertilization was not apparent even as late as 180 min post-insemination. These same observations were reported previously (Miyazaki 1939, Imai & Sakai 1961), both

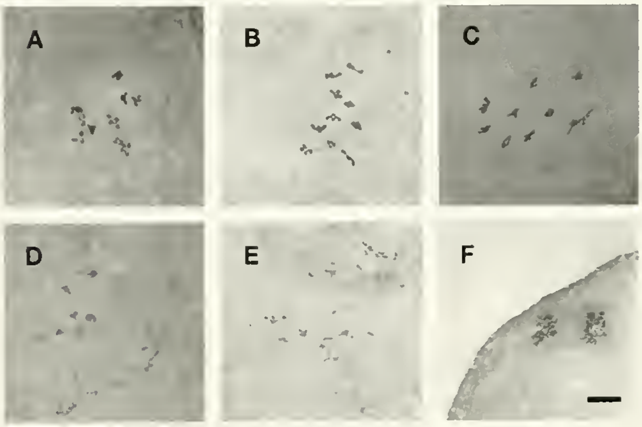

Figure 3. Chromosome observations in crosses of diploid and tetraploid *C. gigas* with diploid *C. ariakensis* at 180 min post-insemination. (A) Prophase I in oocytes from GA; (B) prophase I in oocytes from AG; (C) prophase I in oocytes from AGG; (D) and (E) prophase I in oocytes from GGA; and (F) anaphase I in oocytes from GGA. Scale bar on (D): 10 μm.

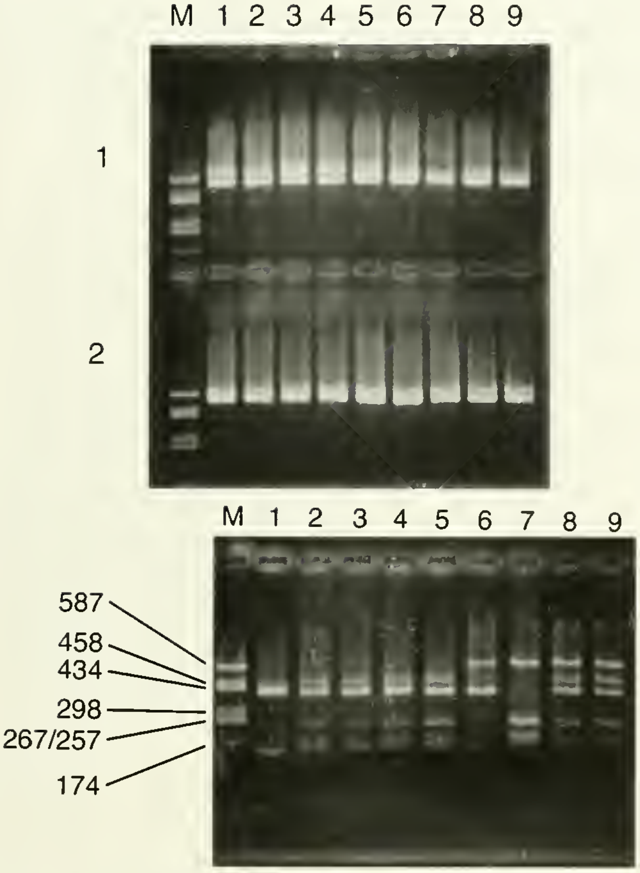

Figure 4. Top: Electrophoretic separation of undigested DNA products resulting from PCR amplification of genomic DNA, using primer of ITS-1. rDNA originated from parental species and their hybrid progeny. Row 1: lane 1 = diploid *C. gigas* (♀); lane 2 = diploid *C. ariakensis* (♂); lanes 3–4 = G × A progeny; lane 5 = diploid *C. gigas* (♀), lane 6 = diploid *C. ariakensis* (♂), lanes 7–9 = G × A progeny. Row 2: lane 1 = tetraploid *C. gigas* (♀), lanes 2–5 = GG × A progeny; lane 6 = diploid *C. gigas* (♀), lane 7 = diploid *C. ariakensis* (♂), lanes 8–9 = G × A progeny. Lane M = molecular weight marker. Bottom: Electrophoretic separation of PCR amplified rDNA products from GA and GGA crosses digested with restriction endonuclease Hinf I. M = molecular weight marker; lane 1 = tetraploid *C. gigas* (♀; lanes 2–5 = GG × A progeny, lane 6 = diploid *C. gigas* (♀), lane 7 = diploid *C. ariakensis* (♂), lanes 8–9 = G × A progeny. The molecular weights of bands resolved from the marker are, from largest (slowest migrating) to smallest: 587, 458, 434, 298, 267/257, 174, and 102 base pairs, respectively.

of whom found little or no fertilization between *C. gigas* and *C. ariakensis* in their studies of Japanese oysters. However, we observed 8%–9% fertilization rate in GA crosses in other experiments (data not shown). In a previous study, a mean fertilization rate of 12% was reported for GA crosses (Allen & Gaffney 1993). Zhou et al. (1982) revealed that fertilization rates in *C. gigas* (♀) × *C. ariakensis* (♂) were 0–52.6% and its reciprocal, 2.3%–18.8%. These data indicate that the fertilization rate in these hybrids varies widely. Success of hybridization should not be assessed solely on the fertilization rate. In contrast, fertilization between other species of *Crassostrea* occurred readily, and often showed relatively high rates, but larvae survived for only a short time before complete mortality (Menzel 1986; Allen et al. 1993).

Despite the apparent lack of fertilization (observed up to 180 min post-insemination), replicates of GA yielded viable spat, which reconfirms the compatibility of the gametes from *C. gigas* (♀) and *C. ariakensis* (♂). Buroker et al. (1979) reports a relatively high genetic similarity between these two species. Other studies also demonstrate the feasibility of hybridization between these two species (Allen & Gaffney 1993; Downing 1988; Downing 1991; Zhou et al. 1982), although only Allen & Gaffney (1993) confirmed hybrids genetically. In contrast to the success of GA, the reciprocal AG failed to produce any spat, although no morphological deformities were observed in the larvae. AG larvae were previously shown to be much less viable compared with the reciprocal although a few spat were obtained (Allen & Gaffney, 1993). The diploid control AA (three replicates) consistently survived poorly and grew slowly in this study. This may partly account for the failure to obtain any spat from the AG cross. According to published accounts (Breese & Malouf 1977; Langdon & Robinson 1996), the survival and growth of *C. ariakensis* are similar to *C. gigas* under appropriate culture conditions. It is not clear why larvae of *C. ariakensis* performed so poorly in this study, because we have routinely cultured *C. ariakensis* on other occasions.

Only one of three replicates of GGA yielded spat. The failure of the other two replicates might be attributable to low fecundity of tetraploid *C. gigas* used here (data not shown), although generally tetraploids have shown high fecundity (Guo et al. 1996; B. Eudeline, Taylor United, Inc. and S.K. Allen, Jr., unpublished data). In all three replicates, yield at day 2 was low. We suggest that the major barrier for GGA production on a pilot- or production-scale is low yield at day 2. Afterward, larvae of GGA crosses survived well (virtually no mortality). While it is clear that more GGA progeny could be obtained by using more parents, the real challenge is to find factors that lead to high levels of fertilization for the gametes that are available. We also suggest that there is a difference in growth rate between triploid (GGA) and diploid (GA) hybrids. First, GGA eyed larvae appeared 5–7 days earlier than GA and right after those of controls GG, G/GG, and GG/G. Second, the size of spat from GGA was greater than the size of GA at 90 days post-insemination, although the number of GGA spat was small.

All 28 progeny sampled from each of three replicates of GA were hybrids. With respect to GGA progeny, some eyed larvae attached to the culture containers, leading to loss of eyed larvae. Consequently, only four cultchless spat were obtained. However, the fact that three of the spat were triploid hybrids demonstrates that hybridization between tetraploid *C. gigas* (♀) and diploid *C. ariakensis* (♂) was successful. Triploid hybrids of *C. gigas* and *C. ariakensis* cannot be obtained in any other way. In particular, the use of cytochalasin B (or other polar body inhibitor) is precluded in GA crosses because of the prolonged period leading to syngamy and polar body formation. It is not possible to create triploids without some level of predictability and synchrony among developing eggs (Allen et al. 1989).

PCR restriction fragment length polymorphism (RFLP) diagnosis was an effective means to verify putative hybrid progeny. ITS-1 amplification/Hinf I digestion successfully distinguished among *C. gigas*, *C. ariakensis*, and hybrids, which show bands present from both parental species. Application of this method is based on availability of an appropriate primer: ITS-1 in this study Other methods have been used to confirm hybrid status (Allen & Gaffney 1993; Allen et al. 1993; Nakamura et al. 1990; Jiang et 1988). Karyotype analysis has been useful for hybrids among pearl oyster genus *Pinctada* (Jiang et al. 1988). For species within the genus *Crassostrea,* the great similarity in their karyotype renders this type of analysis useless in hybrid documentation until more discriminating markers are designed. Flow cytometry was equivocal in discriminating between hybrid and pure crosses using dissociated cells of pooled larvae (Allen et al. 1993).

Cytological examination of newly fertilized eggs from hybrid crosses revealed that either fertilization was severely delayed or early development of oocytes was extremely slow. The duration of meiotic maturation in hybrid crosses was at least triple that of controls. The delay of meiosis was probably not due to the quality of gametes because eggs in controls were normal and reached first cleavage at around 60 min post-insemination consistently (data not shown). Another hybridization attempt between *C. gigas* and *C. ariakensis* indicated there was no apparent difference in the time of development from eggs to the straight-hinge larvae (Zhou et al. 1982). Apparently, fertilization was also highly successful in the study by Zhou et al. leading us to suspect contamination. An alternative explanation is that Zhou et al. used a different population of *C. ariakensis.* The *C. ariakensis* used here are from a population derived from Ariake Bay in Japan. Those of Zhou et al. are likely from mainland China. In this study, despite the delay in fertilization, healthy larvae were obtained and cultured through metamorphosis in all replicates of GA and one replicate of GGA. Furthermore, hybrid spat were obtained. Success in obtaining larvae suggests that meiosis in GA and GGA crosses must have resumed at some time, despite being delayed for an uncommonly long time. A similar observation was made in *C. angulata* oocytes fertilized with sperm from *C. virginica,* where 47% of them exhibited metaphase I nearly 4 h after insemination. However, no parental crosses were made for comparison (Stiles 1973). Oocytes in hybrid crosses of *C. gigas* and *C. ariakensis* with *C. virginica* proceed through meiosis and early mitosis in fairly normal fashion (Scarpa & Allen 1992). The timing of meiotic and mitotic landmarks was the same for all crosses, parental and hybrid. However, hybrids of *C. gigas* and *C. ariakensis* with *C. virginica* were inviable (Allen et al. 1993) after a short larval period despite normal meiotic and early mitotic behavior. *C. gigas* × *C. ariakensis* crosses were not included in Scarpa and Allen's work.

Overall, this study reveals a new potential application for oyster breeding: triploid *C. gigas* × *C. ariakensis* hybrids. We were limited to relatively few tetraploid brood stock for this study, but since that time, tetraploids have become commercialized on the West coast of the United Staes. We were also limited to making the triploid hybrid in one direction; that is, using only *C. gigas* as the tetraploid, enabling experiments on GGA (4n♀) or AGG (4n♂). We were unable to explore GAA (4n♂ *C. ariakensis*) or AAG (4n♀ *C. ariakensis*). Presumably the former would behave like GA and GGA cultures, and the latter would behave like AG and AGG cultures. These experiments will have to wait for the tetraploid *C. ariakensis* that are currently under development. At present, GGA hybrids can be made only at a high cost of gametes from the parental species. Even with high fecundity, the losses are so severe that commercial production would be a problem. Studies on factors that promote higher fertilization rate, syngamy, development rates, or all of these would contribute to efforts to produce pilot-scale quantities of GGA for future testing.

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MICROSCOPIC ANATOMY OF GONADAL TISSUE AND SPECIALIZED STORAGE CELLS ASSOCIATED WITH OOGENESIS AND SPERMATOGENESIS IN THE CALAFIA MOTHER-OF-PEARL OYSTER, *PINCTADA MAZATLANICA* (BIVALVIA: PTERIIDAE)

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ABSTRACT As part of a major experiment of the reproductive biology of wild *Pinctada mazatlanica*, this study was aimed to characterize the microscopic anatomy of the gonadal tissue and storage cellular elements involved in the seasonal advancement of oogenesis and spermatogenesis. Tissue samples were collected every fifteen days over an annual cycle and processed with histological and histochemical techniques. The hematoxylin-eosin (cytology description), Blue Alcian-PAS (PAS, for carbohydrates), and Black Sudan (BBS) and Oil Red (OR, for lipids) techniques were employed. Temporal variations of the sex ratio and sexuality of specimens were also analyzed. Gonadal tissue developed synchronously over time at the expense of a matrix of interconnective tissue and reserves stored in the adductor muscle and digestive gland. The interconnective tissue communicates the gonadal tissue with the digestive gland and also serves as substrate for the differentiation of vesicular connective tissue cells and auxiliary cells. In fact, gametes (particularly oocytes) were exogenously nourished by both kinds of cells as gametogenesis advanced. The vesicular connective tissue cells, very abundant among acini, adenomeres, and pearl sac, were observed surrounding excretory conduits in the first two tissues, and were identified as highly PAS++ and moderately BBS+ and OR+. The auxiliary cells were found attached to previtellogenic and vitellogenic oocytes. The endogenous synthesis of lipids during vitellogenesis was associated to the Balbiani body, a storage compartment of the oocyte ooplasm not previously described for any species of pearl oyster. The average female/male ratio is 0.35:1 when specimens were obtained from cultured conditions, but females outnumbered males when collected from the wild. Several cases of protoginic specimens and a few functional hermaphrodite oysters were seen.

KEY WORDS: pearl oysters, *Pinctada mazatlanica*, histology, oogenesis, spermatogenesis, storage cells

INTRODUCTION

When dealing with endangered or protected species, like the majority of members of the genus *Pinctada*, the success of aquaculture requires a proper knowledge of the biology and ecology of broodstock, and in particular, a clear understanding of their reproductive biology and physiology (Southgate & Beer 1997). In experiments focusing on gonadal conditioning, larval rearing, and hatchery spat production under controlled conditions, such knowledge is especially important for two reasons: (1) to assure the formation of spermatozoa and oocytes of the best quality, that will develop into viable and competitive larvae and, later, into settled spat; and (2) to maintain the continuity of the spat production process throughout the year, avoiding the geographic limitations that prevail in subtropical environments like Bahía de La Paz. At such localities, the main spawning season for most species of marine bivalves is restricted to a short period of one to two months of the annual cycle (Saucedo et al. 2001a; Saucedo et al. 2001b; Saucedo et al. 2001c).

Pearl oysters are marine bivalves having considerable economic and commercial value because of their natural capacity to produce pearls of high quality (Monteforte 1990, 1996). Despite this, the factors affecting reproductive success in these species are poorly studied and understood, although their basic reproductive biology has been well documented histologically (Tranter 1958a; Tranter 1958b; Tranter 1958c; Wada 1959; Sevilla 1969; Chellam 1987; Rose et al. 1991; Wada et al. 1995; García-Domínguez et al. 1996; Saucedo & Monteforte 1997; Behzadi et al. 1997; Saucedo et al. 2001a; Saucedo et al. 2001b; Saucedo et al. 2001c). These studies reveal that most aspects of the gametogenic cycle are common to all members of the genus *Pinctada*, with differences ascribed basically to the geographic location of each species. The gonad is absent as an anatomically discrete organ, and instead, gonadal tissue gradually develops as part of the visceral mass, intermingled with the digestive gland and interstitial connective tissue. Gametogenesis occurs continuously and synchronously throughout the annual cycle, but shows a variable number of spawning peaks. Adults behave as protandrous hermaphrodites, maturing as males and changing to females at a certain size/age. A female/male sex ratio of 1:1 is reached with increasing age.

In México, there have been some studies of the reproductive biology of the Calafia mother-of-pearl oyster, *Pinctada mazatlanica* (Hanley 1856) (Sevilla 1969; García-Domínguez et al. 1996; Saucedo & Monteforte 1997; Saucedo et al. 2001a; Saucedo et al. 2001b; Saucedo et al. 2001c). Little is known, however, about the step-by-step advancement of the gametogenic cycle and the role of accessory tissues and cells upon the seasonal build-up of gametes. This study describes the microscopic anatomy of gonadal tissue and specialized storage cellular elements involved in the annual cycle of oogenesis and spermatogenesis in *P. mazatlanica*. This experiment was conducted as part of a major study of the reproductive biology of the species.

MATERIALS AND METHODS

Twenty adult specimens averaging 139 (±9.7 mm *SD*) shell height were collected every fifteen days over a year (January to December 1999) from Caleta El Merito, Bahía de La Paz, Baja California Sur, México (24°16′N, 110°19′W). Cultured specimens coming from the Experimental Marine Farm of the Centro de Investigaciones Biológicas del Noroeste (CIBNOR) were collected from January to August. Wild specimens were collected from September through December because of the scarcity of cultured specimens remaining in the farm during the last third of the year.

After dissecting each specimen, a sample of the visceral mass (where gonadal tissue develops concomitantly with the digest

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gland) was excised and preserved in Davidson's solution for 48 h. Samples were dehydrated in ascending ethanol series, embedded in Paraplast at 56°C, and sectioned with a rotatory microtome at 4–5 μm along the anteroposterior axis. To characterize the general morphology of tissues and the advancement of gametogenesis, thin sections were stained with the conventional hematoxylin-eosin technique (Howard & Smith 1983). To identify the presence of carbohydrates (CHO) and lipids (LIP) in storage tissular and cellular elements, additional sections were stained with blue Alcian-PAS (referred to as PAS for CHO) and black Sudan (BBS) and oil red (OR), both for LIP (Prophet et al. 1992). Finished slides were then examined with an Olympus BX-41 compound microscope. The resulting images were stored in the computer using a digital Cole-Snap camera (I-media Cybernetics) and processed with Sigma Scan Pro software (vers. 5.5).

To study the seasonal changes of oogenesis and spermatogenesis, a modified version of Tranter's original scheme (Tranter 1958a; Tranter 1958b; Tranter 1958c) for pearl oysters was used. Gametogenesis was characterized by eight stages: (a) inactive or resting); (b) sexual activation; (c) early development; (d) mid development; (e) late development; (f) ripeness; (g) partially spawned; and (h) spent. For oogenesis in particular, gametes were classified according to their vitellogenic stage as oogonias (corresponding in time to the sexual-activation stage), previtellogenic oocytes (for the early-developing stage), vitellogenic oocytes (mid-and late-developing stages during yolk formation), and postvitellogenic oocytes (ripe stage), adopting the criterion defined by de Gaulejac et al. (1995) for the bivalve *Pinna nobilis*. Apart from de Gaulejac's work, detailed descriptions of the cytological characteristics of each type of oocyte may be found in Saucedo et al. (2001a, b).

In addition, total area variation was recorded for thirty oocytes in each developmental stage measured at three randomly-selected areas of the ovary (Grant & Tyler 1983). The sex ratio and sexual condition of specimens were also recorded. One-way ANOVA was applied for differences in the mean area of oocytes over time.

RESULTS

Gonadal Tissue

Pearl oysters lack a true gonad organ. Instead, gonadal tissue develops gradually as part of the visceral mass by means of seasonal soma-germline interactions. Macroscopically, this tissue is formed by two symmetric lobules (a lobule corresponding to each valve) that grow asymmetrically toward the dorsal region of the visceral mass as gametogenesis proceeds, thus constituting an unpaired gland when specimens are ripe. When observed under the light microscope in a transversal, anteroposterior section (Fig. 1A), gonadal tissue appears delimited from the central region by the digestive gland, and from the pallial cavity (along the periphery) by a layer of neutral-acid mucopolysaccharides and collagen fibers. A thick matrix of interstitial connective tissue—appreciated in the form of a complex circulatory system running among acini—gives support, communication, and substrate for the differentiation of gonadal tissue and one kind of somatic nutritive-storage cells, named as vesicular connective tissue (VCT) cells. The size and grade of diffusion of the interconnective tissue matrix shows an inverse relation to the advancement of gametogenesis. The mucopolysaccharides layer is formed by eosinophilic highly PAS++ cells, with both fractions clearly contrasted, the neutral-inactive (with a pink-magenta color) from the acid-active (with an intense blue color) (Fig. 1A). This tissue is also BBS+ and OR+, although its low reaction to both colorants suggests the presence of relatively few lipids, which are present only in the form of esters of cholesterol.

The gonadal tissue is initially formed as a series of small and compact granular bags that latter enlarge and transform into a complex network of branched tubules as gametogenesis advances (Fig. 1A). These bags, representing the structural units of the gonadal tissue, are called acini or follicles, indistinct for the male testis or the female ovary. The sex cells, either oocytes or spermatozoa, develop within these units by permanent mitosis of oogonial or spermatogonial stem cells producing cloned-daughter cells by centripetal movements.

Digestive Gland

This tissue is formed by a large number of blind-end granular tubules that are connected to the stomach by branched conduits. When observed microscopically in transversal section (Fig. 1B), tubules are composed of structural units called adenomeres, each one having a typically round shape that conforms the blind-end of the tubule. Adenomeres are formed from an external layer of epithelial cells that have a round basophilic nucleus resting upon a thin, acidophilic basal membrane. A thin layer of interconnective tissue surrounds and supports the blind tubules. The digestive gland is involved in a double function; the first one, as tissue, is related to the digestion process of the oyster, while the second, as endocrine gland, is for storage and release of nutrients. Intracellular digestion takes place in the cells located in the basal membrane, via ciliated vesicles acting as carriers of the ingested food and their pinocytosis to enter the cell (processes observed only with an electron microscope). With light microscopy, the diameter of the blind tubules varies with regard to the light beam that can pass, indicating which digestive phase (assimilation or digestion) is being observed. The glandular function of the digestive gland is described in the section below.

Vesicular Connective Tissue (VCT) Cells

The glandular function of the digestive gland, which is closely related to that of gonadal tissue, is associated directly with the presence of a dense matrix of small, round or oval pleomorphic somatic VCT cells. As an integral part of the connective tissue network in which development and morphogenesis of the gonadal tissue takes place, these eosinophilic cells are widely distributed among adenomeres and acini (particularly in the former tissue) surrounding wide excretory conduits (Fig. 1C and D). These conduits are probably communicating with each other via the vascular interconnective tissue matrix, and may be the vehicle for transporting stored nutrients and energy reserves from the gonadal tissue to the digestive gland, or vice versa. When specimens reach the late-developing or ripe stage, this tissue network can grow and invade the intestinal loop (known as the pearl sac for pearl culture purposes), constituting an important storage site for nutrients. VCT cells are strongly PAS++ (Fig. 1C and D) and moderately BBS+ and OR+ (Fig. 1E), confirming a high proportion of carbohydrates (mainly glycogen), but also lipid droplets in a lower and variable percentage. Transverse sections through the gonad indicate that the proportion of VCT cells versus germ cells appears to be approximately the same in ovaries and testis.

Auxiliary Cells (AC)

These specialized intragonadal cells are observed only in female acini, always in intimate relation with developing oocytes, to

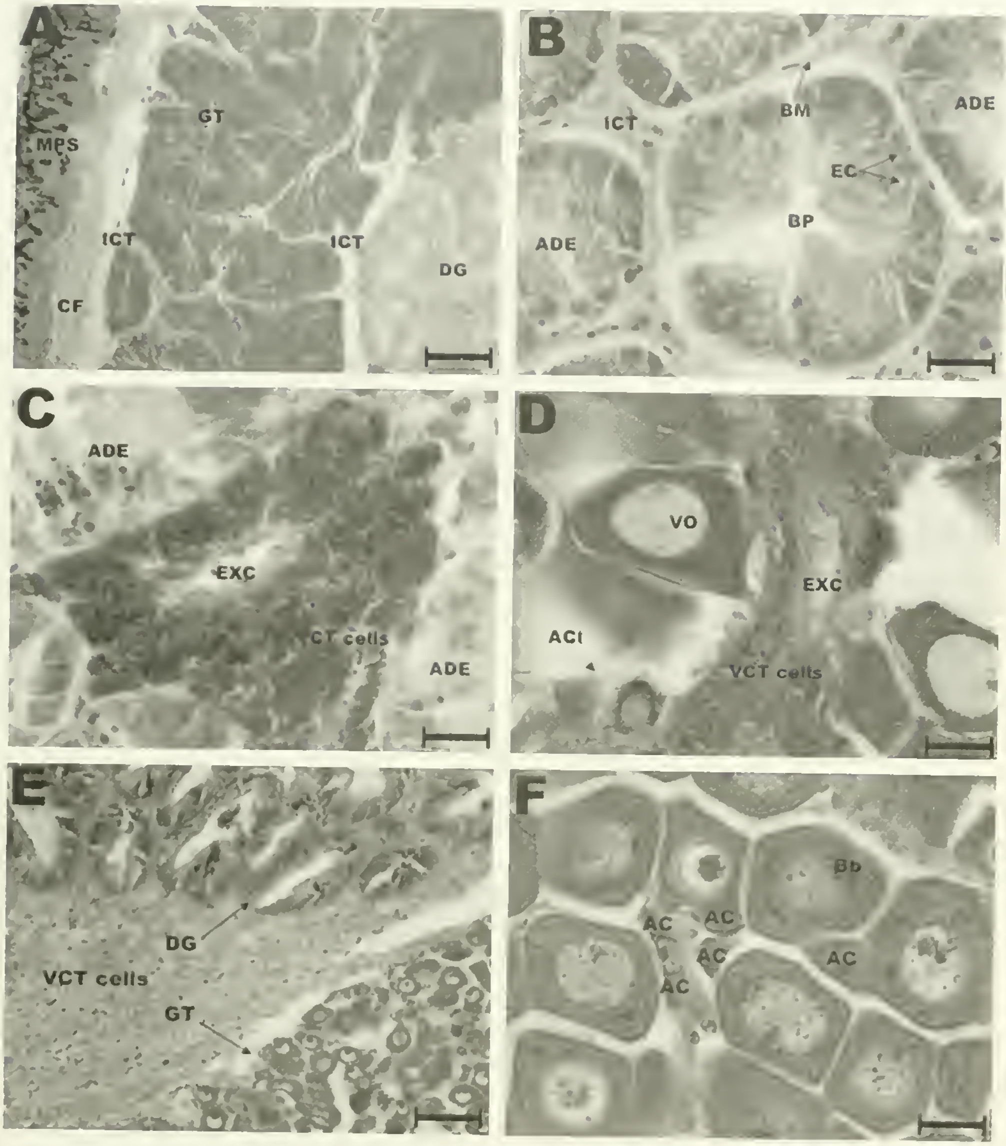

Figure 1. Color photomicrographs of accessory tissues and cells associated with oogenesis and spermatogenesis in *Pinctada mazatlanica*. A) Transversal section of the visceral mass at 10x, showing, from the periphery to center, the neutral (magenta) and acid (blue) highly PAS++ mucopolysaccharides layer (MPS), collagen fibers (CF), and interconnective tissue (ICT) supporting and communicating the male gonadal tissue (GT) with the digestive gland (DG); B) Microscopic anatomy of the DG at 40x, in which adenomeres (ADE) show several layers of eosinophilic cells (EC) resting upon a basal membrane (BM) and the blind part (BP) in the center; C) and D) Highly PAS++ vesicular connective tissue (VCT) cells at 40x, observed surrounding excretory conduits (EXC) among ADE and acini (ACI), respectively; E) Moderately BBS+ VCT cells at 10x, found in the boundaries between female GT and DG; F) Basophilic auxiliary cells (AC) at 40x, observed in different positions in relation to developing oocytes, and the Balbiani body (Bb). Scale bar = 50μm.

which they are attached by desmosome-like gap junctions (observed only with the electron microscope). AC range in size from 20–30 μm, and because of their basophilic character and strong affinity to hematoxylin, are easily distinguishable from oocytes (except those previtetellogenic that are also basophilic) by their dark purple color. AC exhibit an enormous plasticity to change their morphology according to the stage of gametogenesis and to their exact location with respect to growing oocytes. This plasticity is most likely due to the disposition and arrangement of the microtubule skeleton (cytoarchitecture) of these cells. Thus, AC may be observed basally (near the stalk region), laterally, or eccentrically (Fig. 1F). AC serve a nutritive function during oocyte development, especially during the construction of the lipid fraction of the yolk in the early-, mid-, and late-developing stages. In ripe specimens, these cells gradually disappear or, if still appearing, are detached from the oocytes.

Oogenesis

The step-by-step advancement of the annual oogenic cycle in *P. mazatlanica* is depicted in Figure 2. Here, the stages are described.

Inactive or Resting

There is no evidence of gonadal development and specimens are undifferentiated by sex. Acini are observed collapsed and empty, with some granulocytes and phagocytes remaining from th gamete resorption process. On the contrary, the vascular inter

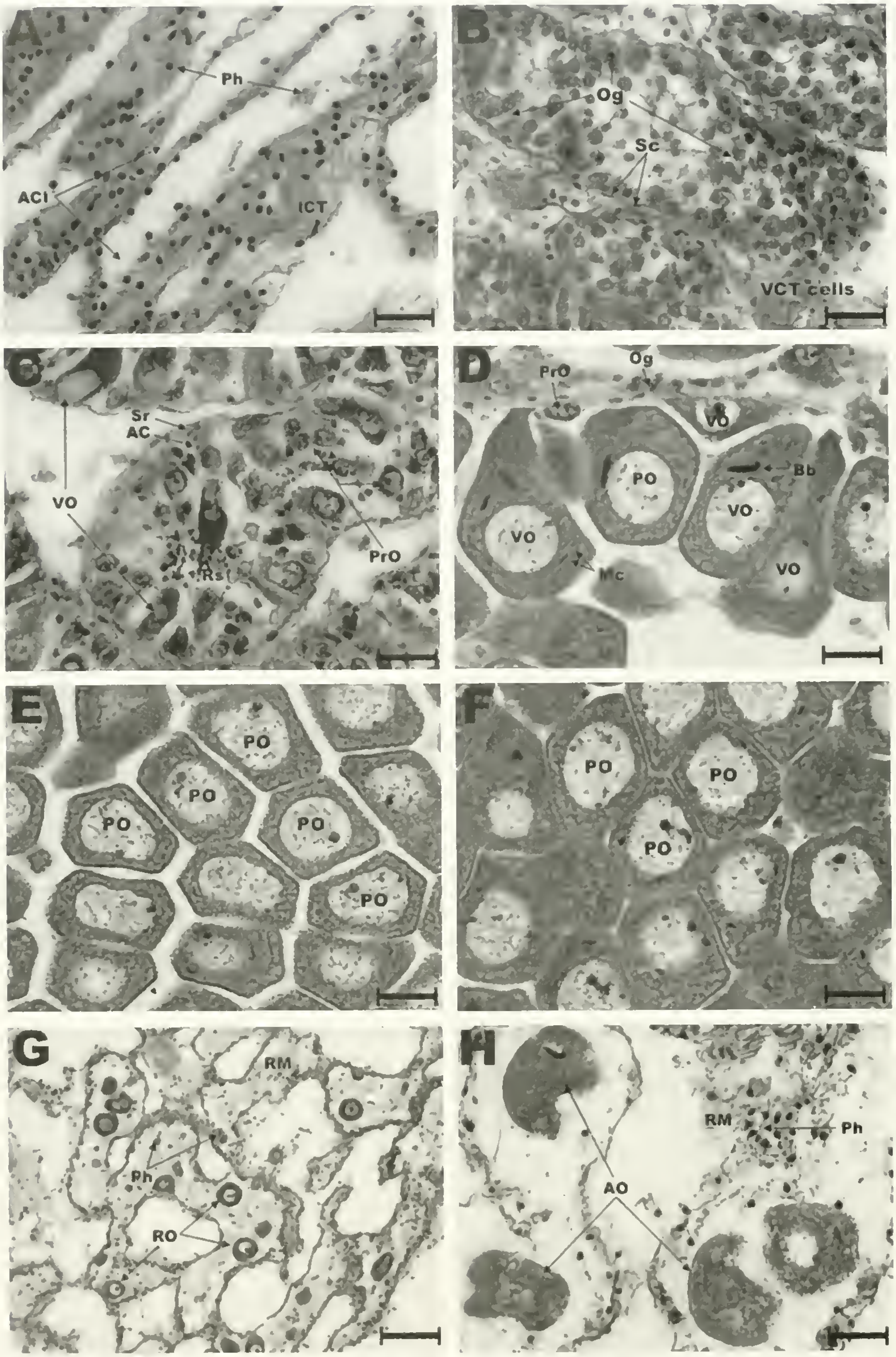

Figure 2. Photomicrographs of female gonadal tissue (40x), showing the sexual stages of oogenesis in *Pinctada mazatlanica.* A) Inactive stage, with empty and collapsed ACI containing some phagocytes (Ph) and abundant ICT; B) Activation stage, in which only stem cells (Sc) and oogonia (Og) are attached to ACI walls, and, among them, large amounts of VCT; C) Early development, dominated by previtellogenic oocytes (PrO) still attached to the acinus wall by the stalk region (Sr) and accompanied by AC and a few vitellogenic oocytes (VO); D) Mid-developing stage, with only a few Og and PrO present, while more VO and postvitellogenic oocytes (PO) start dominating the ACI. The Bb and the mitochondrial clusters (Mc) are common during this stage; E) Late development, showing free PO filling the ACI lumen, but still leaving a wide interoocyte space; F) Ripe stage, with large, free PO and the interoocyte space reduced to its minimum; G) Partial spawning, presenting many scattered residual oocytes (RO), resorptive material (RM), and a few Ph; H) Spent stage, showing empty ACI with atresic oocytes (AO), RM, and more signs of Ph. Scale bar = 50 μm.

nective tissue grows and appears distended and greatly diffused among acini (Fig. 2A).

Sexual Activation

Acini look small, round or oval, and easily distinguished from other acini. They are supported by interconnective tissue and nourished by VCT cells. Each acinus is lined up exclusively with small (3–4 μm diameter) spherical stem cells and larger oogonias (5–6 μm), either attached in clusters to the acinus wall or progressively expanding toward the lumen (Fig. 2B). Apart from these cells no other developmental stage has yet appeared.

Early Development

Acini begin to grow and enlarge, but their individuality is still clear. Oogonias rapidly proliferate by mitosis and give rise, by centripetal movements, to the first previtellogenic oocytes (Fig. 2C). These oocytes are easily distinguished from other types of oocytes by the dark blue-purple color of their ooplasma. They enlarge to a diameter of 14–23 μm in diameter, lack yolk, and are still connected to the acinus wall by the stalk region, in which some AC are commonly observed. At the end of this stage, a few vitellogenic oocytes appear in the acinus, but the interoocyte space is still wide.

Mid Development

Acini continue to grow, adopting a more complex anastomosed shape. The proportion of interconnective tissue and VCT cells decreases, but AC are still observed. All developmental stages are present within acini, but vitellogenic oocytes—characterized by the growth of the yolk ooplasm and increase in diameter to 30 to 55 μm—are the dominant stage (Fig. 2D). This oocyte is still connected to the acini wall, presents a peduncle or pear shape, and shows a large nucleus and one or two dense, blue-stained nucleoli. Two different basophilic structures are observed in the ooplasm for the first time: the Balbiani body (seen as a black spot of variable size and shape) and many smaller, irregularly scattered black spots that probably correspond to mitochondrial clusters (Figs. 1F and 2D).

Late Development

Acini continue to grow and stratify and interconnective tissue and VCT cells to decrease. AC tend to disappear or, if still occurring, they appear separated from oocytes. The interoocyte space reduces as previtellogenic oocytes diminish and vitellogenic and postvitellogenic oocytes progressively develop (Fig. 2E). Postvitellogenic oocytes are seen free in the lumen, have a polyhedral or polygonal shape, and increase in diameter to 60–70 μm. A dense amorphous mass (probably chromatin) is observed in the nucleoplasm. The nucleolus is dense, compact, and usually in a marginal position. The Balbiani body and mitochondrial clusters may still be present.

Ripeness

This stage marks the end of vitellogenesis. Acini are strongly anastomosed and packed almost entirely with mature, free postvitellogenic oocytes that attain a maximum diameter of 70–75 μm (Fig. 2F). VCT cells in gonadal tissue almost disappear and interoocyte space is reduced to the minimum. The nucleus now occupies a large area in the middle of the oocyte. Many oocytes begin to enter the germinal vesicle stage, in which a gap between the nuclear envelope and the ooplasm appears, indicating a readiness for spawning.

Partially Spawned

After the germinal vesicle breakdown, oocytes are released outside the acini, which look distended and partially empty (Fig. 2G). Some types of phagocytes and granulocytes appear in the space between the free residual oocytes, which now look rounded or pear-shaped. Large amounts of resorptive material are noticeable. The nucleolus and chromatin disaggregate, but if still present, they may be associated with a new developmental process. This may be a strategy to recycle nutrients (proteins and lipids of the yolk) destined for the new oocyte.

Spent

Acini are collapsed and empty, with clear signs of phagocytic activity, oocyte degeneration, and much resorptive material. Depending on the duration of this phase, residual oocytes, especially those of small diameter whose nucleus is not polylobed, undergo atresia (Fig. 2H). This process represents deterioration and disintegration of most of the major cellular constituents and thereby the breakdown of the oocyte.

Spermatogenesis

The step-by-step advancement of the annual spermatogenic cycle in *P. mazatlanica* is depicted in Figure 3. The stages are described as follows:

Sexual Activation

Acini initially are small, round or oval, and supported by vascular interconnective tissue and VCT cells. The germinal epithelium is formed exclusively by a single layer of small (3–4 μm diameter) spherical stem cells that are strongly attached to the acinus wall and by some spermatogonias starting to differentiate. Development into spermatocytes, spermatides, or spermatozoa has not yet appeared (Fig. 3A).

Early Development

Acini grow and look enlarged and slightly branched, but their individuality is still recognizable. Spermatogonias rapidly proliferate in a centripetal direction, giving rise to several layers of small (2–2.5 μm diameter) primary and secondary spermatocytes expanding toward the lumen, which are the dominant stage present (Fig. 3B). A few isolated pockets of spermatids and spermatozoa can also be observed. The proportion of interconnective tissue and VCT cells decreases.

Mid Development

Acini begin to show stratification, causing a reduction of the interconnective tissue and storage cells. Starting from the outer layers to the center, all developmental stages are present: spermatogonia, abundant spermatocytes, spermatids, and ripe spermatozoa (showing their acidophilic tails as pink lines radiating from the center of the lumen) (Fig. 3C). Cell diameter decreases to approximately 1–0.5 μm.

Late Development

Acini continue their growth and stratification, appearing highly anastomosed. As a consequence, the matrix, previously occupied by interconnective tissue and VCT cells, has almost disappeared. Spermatogonia and spermatocytes have been reduced in thickness to a few layers of cells located at the periphery. Acini are pac[illegible]

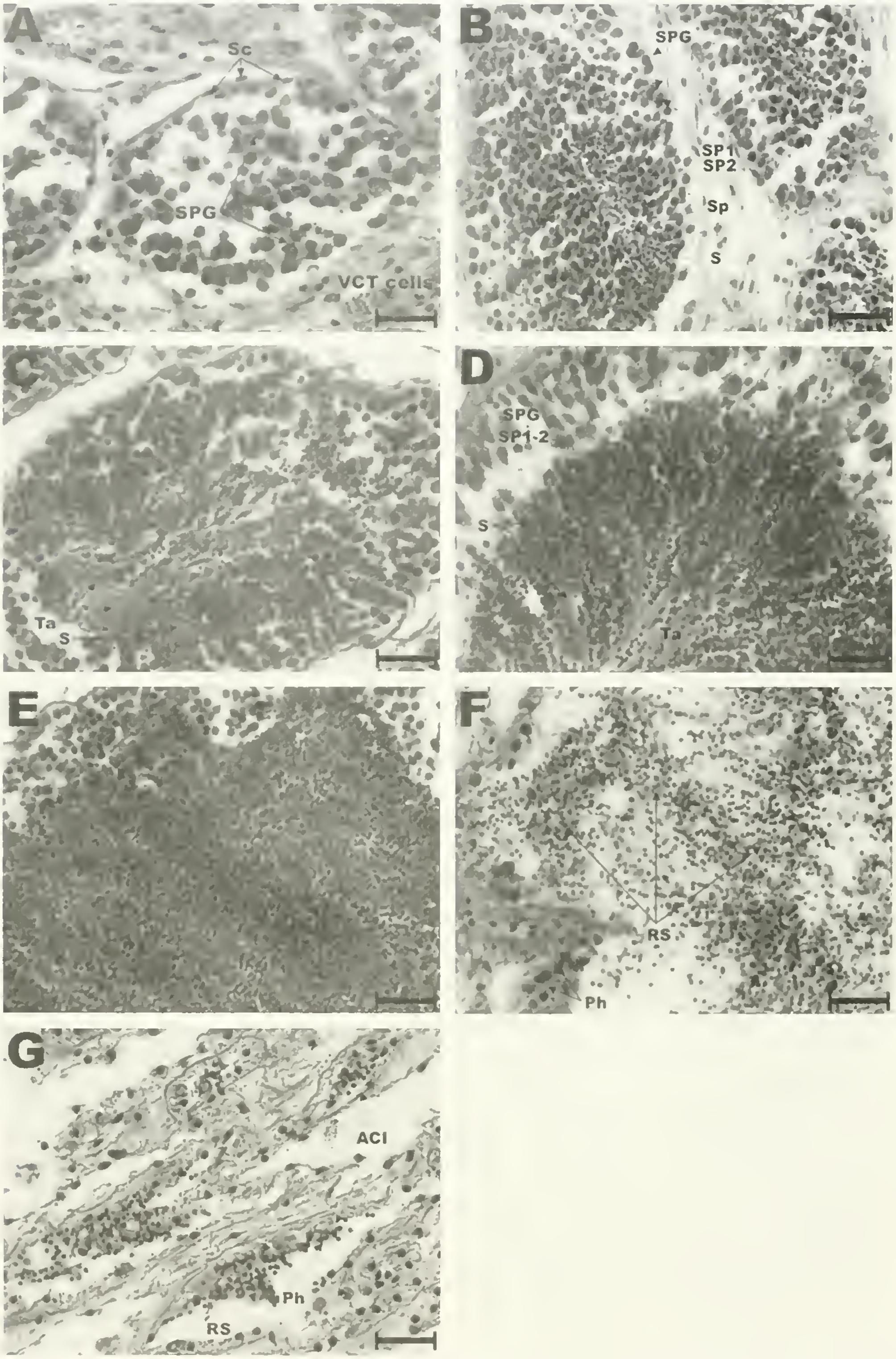

Figure 3. Photomicrographs of male gonadal tissue (40x), showing the sexual stages of spermatogenesis in *Pinctada mazatlanica*. A) Activation stage, showing ACI filled only with stem cells (Sc) and spermatogonias (SPG) proliferating toward the lumen. Abundant ICT with VCT cells can be observed; B) Early development, with many SPG and primary and secondary spermatocytes (SP1 and SP2) rapidly differentiating into the first pockets of spermatides (Sp) and spermatozoa (S); C) Mid development, characterized by the presence of all type of cellular stages, but more mature S showing their acidophilic tails (T) radiating from the center; D) Late-developing stage, in which SPG decrease in number and instead S sharply increase their frequency; E) Ripe stage, evidencing a dense volume of ripe S packing the acini; F) Partial spawning, showing distended but empty acini, residual spermatozoa (RS) and some Ph starting to appear; G) Spent stage, with collapsed ACI and evident signs of RS undergoing cytolysis. Scale bar = 50 µm.

with a dense, dark-blue band of ripe spermatozoa several cells deep (Fig. 3D).

Ripeness

Acini look like a complex network of branched tubules that make distinguishing the boundaries between them difficult. The dominant stage is now the spermatozoa, which strongly pack the acini. Stem cells and spermatogonia are latent and restricted to a thin layer at the periphery of the acini (Fig. 3E). Only a small amount of interstitial connective tissue is evident at this stage.

Partially Spawned

After spermatozoa are expelled into the surrounding environment, acini walls look broken but still distended (Fig. 3F). Many residual spermatozoa are observed scattered, with the first signals of phagocytic activity. Much residual material is also noticeable. A gap between acinus walls and the mass of residual spermatozoa appears. In some cases, redevelopment or sex reversal processes occur.

Spent

Because spawning is never complete, the spent stage may be skipped when new gonadal replenishment occurs off the main reproductive cycle. However, when gamete resorption starts, acini look collapsed and empty, with no evidence of active spermatogenesis taking place. This phase is characterized by the rapid proliferation of different kinds of phagocytes, granulocytes, and amoebocytes surrounding and destroying residual spermatozoa (Fig. 3G). The matrix of interconnective tissue starts to grow and develop again.

Bisexuality (Hermaphroditism)

This condition was observed in 8.3% of the sample (40 of 480 specimens). Bisexuality was classified as either consecutive sexuality or functional hermaphroditism.

Consecutive Sexuality

The individual functions as one sex when young and later changes to the opposite sex. As for bisexuality, two manifestations of this condition were observed: (1) Protandrous hermaphroditism: when sex reversal occurs, this is the normal condition reported for many bivalves and pearl oysters. In this situation both germinal lineages overlap in the same acinus, but the male gonadal tissue, which developed initially, undergoes regression and lysis. Only a few residual spermatozoa accompany oocyte development (Fig. 4A). This condition, which corresponds to the relation between stage 7 of spermatogenesis and stages 1, 2, or 3 of oogenesis, was observed in 5.8% of the sample (28 of 480 specimens). (2) Protoginic hermaphroditism: observed in only 1.7% of the total sample (8 of 480 specimens). Again, both germinal lineages overlap in the same acinus, but now the male gonadal tissue proliferates as female gametes gradually disappear. Therefore, only atresic oocytes are found in the acini together with developing male gametes (Fig. 4B). This condition corresponds to the relation of stage 7 of oogenesis and stages 1, 2, or 3 of spermatogenesis.

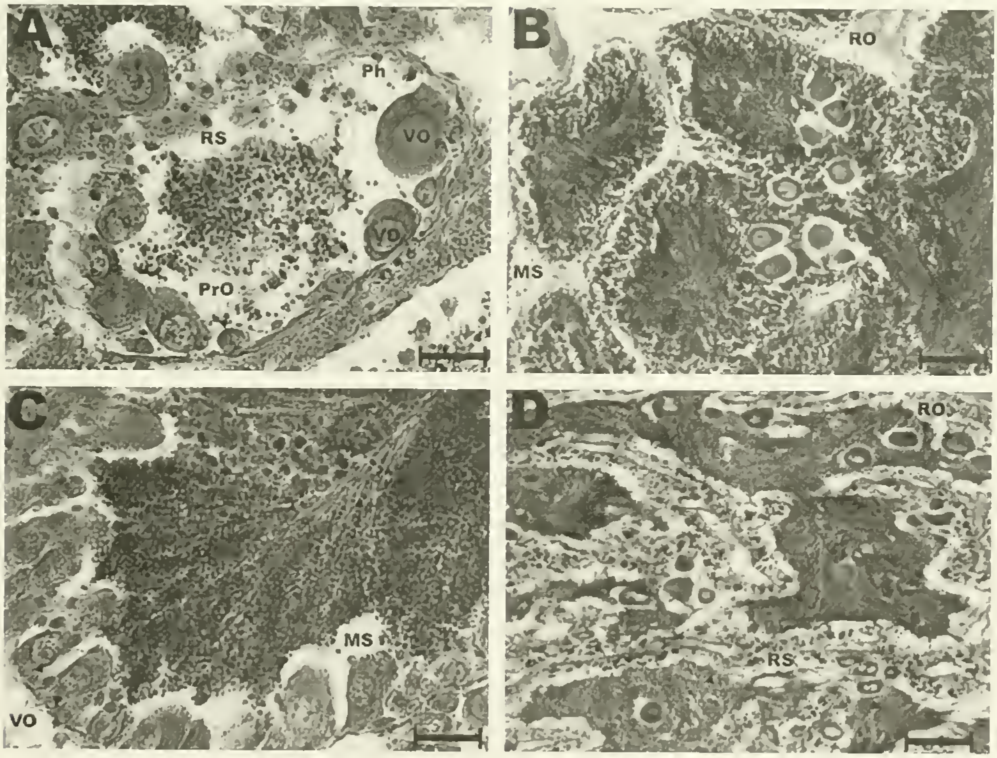

Figure 4. Photomicrographs of hermaphrodite specimens of *Pinctada mazatlanica stained with hematoxylin-eosin.* A) Protandrous hermaphrodite, with active Ph surrounding and destroying RS, while PrO and VO present active development; B) Protoginic hermaphrodite, showing a few RO undergoing regression among mature spermatozoa (MS) filling the ACI; C) Functional hermaphrodite, presenting MS with tails and growing oocytes (PrO and VO) in equal proportion and apparent functionality; D) Functional hermaphrodite, in which both male and female lineages spawned at the same time, and only RS and RO can be seen within ACI. Scale bar = 50 μm.

Functional Hermaphroditism

This condition was detected in 0.8% of the sample (4 oysters). Both sexes are present concomitantly within the same acinus in apparently equal proportion and display no sign of gonadal regression in either gamete (Fig. 4C). Both lineages may be observed spawning and undergoing regression at the same time (Fig. 4D).

Temporal Variation of Gametogenesis, Oocyte Area, and Sex Ratio

Temporal variations of the main developmental stages of gametogenesis, oocyte area, and sex ratio of *Pinctada mazatlanica* are shown in Table 1. Gonadal development started early in February and proceeded synchronously throughout the annual cycle, showing two reproductive peaks, one in March to May (in which no trace of spawning activity was detected) and other in July to August (concluding with a massive spawning in September and October). Small oocytes were observed in February, June, and October to December. Large oocytes were observed in March, May, and August, corresponding to the two reproductive peaks detected histologically. There were significant differences in the mean area of oocytes over time ($F = 102.97$; $P < 0.001$). During most of the annual cycle (January to August), when cultured specimens were collected, males outnumbered females and led to an average female/male ratio of 0.6:1. From September until December, when wild animals were collected, females doubled in number relative to males, yielding an average female/male ratio of 2:1.

DISCUSSION

In this study of *P. mazatlanica*, oogenesis and spermatogenesis were analyzed using a scheme of eight stages that adequately describe developmental variations in the microscopic anatomy of the gonadal tissues and storage cells. A 15-day sampling interval was selected as the best procedure for obtaining detailed documentation of the step-by-step advancement of gametogenesis. The timing of sampling also avoided the major problem of missing partial spawns or sex reversal phenomena, and allowed us to observe many unreported phenomena of the reproductive process of *P. mazatlanica*. We introduced, for the first time, the activation stage to classify acini in which no other developmental stage rather than oogonias or spermatogonias can be distinguished. This stage — not previously described for any marine bivalve—clearly marks the moment of sexual differentiation of germ cells and the very incipient commencement of gametogenesis. Both events occurred early in February to March, when phytoplankton is usually abundant in Bahía de La Paz (Lechuga-Devéze 1997) and wild oysters had stored sufficient energy reserves in the adductor muscle and digestive gland for starting gametogenesis (Saucedo et al. 2001b).

TABLE 1.

Temporal variations in the main developmental stages, mean oocyte area (± standard deviation), and sexual condition of *Pinctada mazatlanica* during an annual gametogenic cycle.

Shell Height (mm ± sd)	ORI	Dev Stage	TIM	Oocyte Area (*) (μm ± sd)	Sexual Condition M (%)	F (%)	I (%)	B (%)	F/M SR
142 ± 7.4	Cult	Inactive	Jan	4004 ± 928	60	25	15	0	0.4:1
151 ± 11.8	Cult	Activ	Jan		50	25	5	20	0.5:1
140 ± 9.9	Cult	E-Dev	Feb	3020 ± 649	80	15	0	5	0.2:1
139 ± 9.6	Cult	M-Dev	Feb		50	35	10	5	0.7:1
146 ± 6.5	Cult	M-Dev	Mar	4644 ± 868	50	25	5	20	0.5:1
142 ± 6.2	Cult	L-Dev	Mar		45	40	0	15	0.9:1
150 ± 10.7	Cult	L-Dev	Apr	4318 ± 959	60	25	0	15	0.4:1
143 ± 9.5	Cult	ML-Dev	Apr		65	25	0	10	0.4:1
130 ± 7.6	Cult	ML-Dev	May	4566 ± 1085	50	40	0	10	0.8:1
139 ± 8.5	Cult	L-Dev	May		65	25	0	10	0.4:1
141 ± 8.1	Cult	L-Dev	Jun	3009 ± 644	70	30	0	0	0.4:1
149 ± 9.3	Cult	M-Dev	Jun		45	55	0	0	1.2:1
123 ± 9.1	Cult	L-Dev	Jul	3806 ± 931	75	25	0	0	0.3:1
148 ± 10.6	Cult	L-Dev	Jul		45	45	0	10	1:1
139 ± 9.8	Cult	Ripe	Aug	4037 ± 1132	50	40	0	10	0.8:1
140 ± 10.1	Cult	Ripe	Aug		50	40	0	10	0.8:1
134 ± 12.2	Wild	Spawn	Sep	3259 ± 786	40	50	0	10	1.3:1
148 ± 10.3	Wild	Spawn	Sep		25	70	0	5	2.8:1
144 ± 11.0	Wild	Spawn Spent	Oct	2981 ± 724	25	60	5	10	2.4:1
127 ± 10.9	Wild	Spent	Oct		20	55	20	5	2.8:1
133 ± 10.2	Wild	Spent	Nov	2602 ± 587	25	40	25	10	1.6:1
137 ± 9.6	Wild	Spent	Nov		35	50	10	5	1.4:1
129 ± 12.3	Wild	E-Dev	Dec	1608 ± 825	25	40	20	15	1.6:1
125 ± 11.6	Wild	E-Dev	Dec		30	50	20	0	1.7:1

* Oocyte area was evaluated on a monthly basis only.

ORI = origin of specimens; DEV. STAGE = developmental stage; TIM = timing correspondence (Activ = activation stage; E-Dev = early development; M-Dev = mid development; L-Dev = late development; M = male; F = female; I = inactive or resting; B = bisexual or hermaphrodite; F/M SR = female/male sex ratio).

The developing stage was divided into three subcategories (early, mid, and late) as a strategy to identify, especially in previtellogenic, vitellogenic, and postvitellogenic oocytes, the presence of specific cellular structures whose expression is short lived. The Balbiani body and the mitochondrial clusters observed in the oocyte ooplasm are examples of these structures. The Balbiani body was initially described by Sastry (1968) in *Aequipecten irradians concentricus*, and thereafter, it has been rarely cited (Guraya 1979; Dohem 1983; as cited in de Gaulejac et al. 1995 for the bivalve *Pin. nobilis*). This structure, not previously described for any species of pearl oyster, was mostly seen in vitellogenic oocytes and seldom in postvitellogenic oocytes of *P. mazatlanica*. According to Pipe (1987a, 1987b) and de Gaulejac et al. (1995), this body is probably filled with lipid droplets arising from the degeneration and transformation of mitochondria and other membranous organelles, such as the Golgi complex. However, the mechanisms for the endogenous supply of lipids and other materials needed by the growing oocyte are poorly understood, although pinocytosis was proposed (Pipe 1987a; Pipe 1987b; de Gaulejac et al. 1995). The mitochondrial clusters (whose presence cannot be confirmed using light microscopy, but suggested because of their strong basophilic character), appeared initially near the stalk region and later scattered in the vicinity of the nucleus during the previtellogenic and vitellogenic stages of oogenesis. Certainly, their expression is ascribed to the intense respiratory rate of early and mid-developing oocytes and the active synthesis of energy related to the formation of the yolk molecule.

Several bisexual specimens were detected during the annual cycle. Although sex reversal in pearl oysters is basically protandrous (Gervis & Sims 1992), many protoginic and functional hermaphrodite specimens were found in this study, especially from September through December, when wild oysters were collected. This alteration in the sexual behavior of specimens was previously considered by Coe (1945) an accidental or abnormal mode of embryonic development, resulting from the failure of the hereditary sex-differentiating mechanism to function normally. However, environmental influence is presently recognized as one of the most important factors controlling sexual differentiation of germ cells in the direction of maleness or femaleness. We believe that females appear more frequently in the wild population, while males predominate under culture conditions. Thus, oyster's age (as a biotic factor) and the density in which individuals are kept within the culture baskets (abiotic factor) may be important factors regulating sex reversal from female to male in *P. mazatlanica*. Taylor (1999) reported a similar expression of sexuality in cultured *P. margaritifera*.

In subtropical areas, such as in Bahía de La Paz, the range of temperature variation over an annual cycle is 11–12°C (Pearl Oyster Project database). Therefore, the energy to fuel gametogenesis, parallel to that arising from food intake, have to come from specialized somatic cells involved in the storage of nutrients (Lubet 1959). According to Mathieu and Lubet (1993), there are three types of cellular elements commonly recognized as participating in these processes in bivalves: specific storage cells (adipogranular cells and VCT cells), intragonadal cells (follicular cells, Sertoli cells, and AC), and muscular cells. We identified the presence of two of them in *P. mazatlanica*: VCT cells (constituting an integral part of the interconnective tissue matrix, observed in the gonadal tissue, digestive gland, and pearl sac) and AC (within acini, always attached to developing oocytes). The muscular cells were not considered in this study, since their active role upon gametogenesis has been clearly demonstrated for several bivalves (Ansell 1974; Gabbott 1975; Bayne 1976; Taylor & Venn 1979; Barber & Blake 1981, 1991; Epp et al. 1988; Faveris & Lubet 1991; Martínez 1991; Racotta et al. 1998), including the pearl oyster, *P. mazatlanica* (Saucedo et al. 2001b). Follicular and Sertoli cells were not observed with light microscopy. Storage cells are expressed differently between families. For example, Mytilidae possess both types, VCT and ADG cells (Lubet 1959; Bayne et al. 1982; Pipe 1987a; Pipe 1987b; Peek & Gabbott 1989a; Peek & Gabbott 1989b), Glycymeridae only one type corresponding to ADG cells (Mathieu & Lubet 1993), and Ostreidae only VCT cells (Swift et al. 1988), but with an intermediate structure between ADG and VCT cells as described for Mytilidae. In contrast, Pectinidae, Veneridae, Cardidae, and Pinnidae possess none of them (Yongqiang & Xiang 1988; Dorange & Le Pennec 1989; Dorange et al. 1989; Rodríguez-Jaramillo et al. 2001) since they rely mostly on energy stored in the adductor muscle for the synthesis of gametes.

Storage cells also show important differences in their biochemical composition and ways of releasing nutrients. In this study, VCT cells were strongly PAS++ and moderately BBS+ and OR+, demonstrating that although specialized in the storage of carbohydrates (glycogen), they also contain lipids. In *M. edulis*, nutrients are released by a progressive autophagic mechanism that involves the sequestration of small membrane-bound vacuoles from the large, stored glycogen vesicle, which causes a reduction in cellular volume (Bayne et al. 1982; Mathieu & Lubet 1993). As proposed by Pipe (1987a, 1987b), these vacuoles fuse with the cell membrane and release their glycogen content by eccrine secretion. VCT cells were commonly observed surrounding excretory conduits in the gonadal tissue and digestive gland, indicating that both tissues must be communicating by the interconnective tissue matrix, which probably serves as the vehicle to transport the stored carbohydrates from the gonadal tissue to the digestive gland, or vice versa. Although it is difficult to determine the timing when carbohydrate reserves flow from one tissue to another, we believe that carbohydrates obtained from ingested food are initially used as an energy-rich fuel for the immediate build-up of gametes, and later incorporated into the digestive gland for storage. This proposal is in agreement with results of Saucedo et al. (2001b), who observed progressive accumulation of carbohydrates in this tissue, despite the advancement of gametogenesis in the same species (at least during the developing and ripe stages and later during the spawning occurring in October to November). This trend indicates that carbohydrates from the digestive gland are not transferred to the gonadal tissue for usage during the reproductive cycle in course, but instead stored and used to cover further energy demands. This argument also coincides with the finding of Barber and Blake (1981, 1991) that the digestive gland acts as a short-term storage and transfer site of nutrients to meet the reproductive events in *M. edulis*.

In this study, another cellular storage component associated with the growing oocyte was the auxiliary cells. Although their presence has been associated mainly with the nutrition of previtellogenic and vitellogenic oocytes, an alternate function related to the resorption of residual oocytes was hypothesized by de Gaulejac et al. (1995). The first function was attributed to the intimate relation of these cells with oocytes by means of desmosome-like junctions and to their rich content of glycogen granules and lipid droplets. In contrast, the presence of an active Golgi complex and large autophagic vacuoles suggests these cells may also have the ability to phagocytize, and as such, auxiliary cells might be implicated in the growth of oocytes by recycling nutrients originating by phagocytosis.

With the results previously reported in Saucedo et al. (200

2001c) and those presented in this study, we believe that successful trials for the continuous production of *P. mazatlanica* spat can be conducted if nutrients with the correct biochemical composition, energy profile, and quantity are supplied to broodstock during their gonadal conditioning. This is especially important to allow the vital energy storage process, either extracellular (in VCT cells and auxiliary cells) and intracellular (in the Balbiani body) to be accomplished. More studies on seasonal cycles of expression, mobilization, and depletion of VCT cells and AC are needed to confirm these findings, especially for the role of mantle tissue, which is an important site of storage of glycogen and lipids associated with gametogenesis in bivalves. Ultrastructure studies of gametogenesis are also required.

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GROWTH AND GAMETOGENIC CYCLE OF THE BLOOD ARK, *ANADARA OVALIS* (BRUGUIÉRE, 1789) IN COASTAL GEORGIA

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ABSTRACT We collected two cohorts (1994, 1995) of blood arks, *Anadara ovalis* (Bruguiére 1789) from sets that had occurred on surf clam cages in Wassaw Sound, Georgia. Subsequent to transferal into pearl nets, the arks were suspended from the main dock at the Skidaway Institute of Oceanography on the Skidaway River, Georgia, between January 1995 and April 1997. To determine the reproductive cycle and growth rate, we collected arks monthly, measured each individual for shell length, and took a gonadal sample for histological analysis. Arks from the 1994 cohort grew from a mean shell length of 17.8 mm to 39.9 mm in 15 months, a rate of 1.47 mm per month. Arks from the 1995 cohort grew from a mean shell length of 9.9 mm to 42.6 mm in 16 months, a rate of 2.04 mm per month. Growth was most rapid during the spring and summer months. Of the 747 arks sectioned for histological examination, males dominated the population (66%) and 7.2% were sexually indeterminate. A minor spawning event occurred in the winter months of the first year for each cohort (0+ years) followed by a major spawning commencing in April and continuing through the summer months. In Georgia, it appears that blood arks recruit in summer-early fall, and that juveniles grow rapidly to reach sexual maturity at an early size (10–12 mm) and age (~8 months).

KEY WORDS: *Anadara ovalis*, arks, gametogenesis, growth, sex ratio, spawning

INTRODUCTION

A member of the ark shell family (Arcidae), the blood ark, *Anadara ovalis* (Bruguière 1789) is reported to inhabit estuarine waters from Cape Cod, Massachusetts, to the West Indies and Brazil, at depths ranging from the low-tide line to >3 m (Abbott 1974; Anderson et al. 1984; Rehder 1981; Walker & Gates 2001). This bivalve is found over a variety of substrate types, but is most commonly found in sandy deposits (Alexander 1993). The blood ark inhabits Chesapeake Bay waters in area where salinity is above 15 ppt (Chanley & Andrews 1971). Blood arks are equivalved, and somewhat oval in shape, ranging in size (shell length) from 28 to 76 mm (Rehder 1981). Shell height is slightly less than the shell length, with a reported length/height ratio of 1.16 (Alexander 1993); shell depth reaches approximately 70% of height (McGraw et al. 1996). The blood ark is a short-lived species, which has up to 80% mortality in the third year of life, and a maximum life span of five years (McGraw et al. 1996; Walker 1998).

Several ark shell species form the basis of economically significant molluscan fisheries and extensive culture operations throughout the world (Baqueiro et al. 1982; Broom 1985; Baqueiro 1989; Manzi & Castagna 1989; Nie 1990; Umezawa 1992). The 1997 worldwide harvest of arks (*Scapharca*, *Arca*, and *Anadara* spp.) was 97,296 metric tons, landed in Cuba, Fiji, Indonesia, Korea, Japan, Mexico, Philippines, and Venezuela (FAO 1999). In the United States, ark resources have been largely ignored by the fishing industry until recently. Some interest was expressed in South Carolina in the 1980s, however, there were problems identifying viable markets (Anderson et al. 1984; Anderson and Eversole 1985). Since then a small fishery, primarily for the blood ark, *Anadara ovalis* (Bruguiére 1789), and the ponderous ark, *Noetia ponderosa* (Say 1822), has developed in Virginia. Meats are sold primarily as an ethnic food in Chicago, New York, Los Angeles, and Washington D.C. or exported to Mexico (McGraw & Castagna 1994; McGraw et al. 1996; McGraw et al. 1998).

The demand for arks has recently outpaced the numbers that can be supplied by the Virginia fishery. Since blood arks have been reported to grow up to twice as rapidly as ponderous arks in the first 2 years after settlement in Virginia waters, it has become the principal aquacultural species of interest (McGraw et al. 1996). Growth rates of Georgia stocks have been reported to almost double those observed in these more northern waters, reaching a marketable size in a 1-year period (Walker 1998). Consequently, the great potential of *Anadara ovalis* as a new commercial resource in Georgia has been recognized, and an aquacultural fishery is currently being investigated (Power & Walker 2001). Unfortunately, little life-history information other than from Virginia (McGraw & Castagna 1994; McGraw et al. 1996; McGraw et al. 1998) exists for this species in the United States. This study describes the growth and gametogenic cycle of the blood ark from the coastal waters of Georgia.

MATERIALS AND METHODS

We collected blood ark cohorts in January 1995 (1994 cohort) and December 1995 (1995 cohort) at the mouth of House Creek, Little Tybee Island, Wassaw Sound, Georgia. The clams had attached themselves to surfclam (*Spisula solidissima similis*, Say 1822) grow-out cages (6 mm vinyl coated, 1 × 1 × 0.6 m) that were partially buried (0.3 m) at the spring-low-water mark on a sand flat. Cages had been deployed in September of both years and when we stocked with surfclam seed in October, we noted that ark wild spat had settled and attached with byssal threads to the wire mesh sides of the cages. In the laboratory on Skidaway Island, we measured for shell length (longest possible measurement, i.e., anterior-posterior) using Vernier calipers. Arks from the 1994 and 1995 cohorts had a mean shell length of 17.8 ± 1.52 (SE) mm (range: 11.9–28.3 mm) and 9.9 ± 0.19 mm (range: 4.3–17.7 mm), respectively. Each cohort was placed in a separate 3 mm mesh pearl net, and suspended from a floating dock on the Skidaway River. The 1994 recruitment was a small set (approximately 20–30 arks per cage), while the 1995 recruitment was a massive set with over 500 arks collected per cage.

Between January 1995 and April 1996 we randomly collected arks (N ≈ 10, 1994 cohort) from the pearl nets. We repeated this procedure for the 1995 cohort collecting arks (N ≈ 30) monthly between December 1995 and April 1997. We measured these for shell length, and dissected a mid-lateral gonadal sample (ca. 1 cm^2) from each animal. Smaller specimens with meats <1 cm^2 were preserved and sectioned whole. We also noted the coloration of gonads during the dissections. Gonadal tissue was fixed in Davidson's solution, refrigerated for 48 h, washed with 50% ethanol, and preserved in 70% ethanol until processing. We processed tissues according to procedures outlined in Howard and Smith (1983). The examination of prepared gonadal slides was conducted with a Zeiss Standard 20 microscope (20X). Each section was sexed, and assigned to a developmental stage as described by Walker and Heffernan (1994) and Spruck et al. (1994). A staging criteria of 0 to 5 was employed for Early Active (EA = 3), Late Active (LA = 4), Ripe (R = 5), Partially Spawned (PS = 2), Spent (SP = 1), and Inactive (IA = 0). The determination of monthly gonadal index (G.I.) values was obtained by averaging the number of specimens ascribed to each category score. We tested sex ratios against a 1:1 ratio with Chi-square statistics (Elliott 1977).

Surface water temperature and salinity data were taken daily from the dock of the Marine Extension Service, adjacent to the grow-out site at 0800 h (Monday–Friday) from January 1995 to April 1997.

RESULTS

Monthly mean water temperature and salinity data for the Skidaway River are given in Figure 1. Water temperatures were coolest during the months of January and February 1995, 1996, and 1997 ranging from a mean low of 9.34°C to a high of 13.32°C. Water temperatures reached their peak during the months of July and August 1995, and 1996 ranging from 28.61°C to 30.46°C. Mean salinity values were less cyclical but typically reached their lowest in the spring months each year (14.63 ppt in March 1995). The highest salinity recorded over the study period was 28.00 ppt in December 1996.

Both cohorts exhibited similar growth trends (Fig. 2), increasing in size rapidly during the spring-summer months with growth rates diminishing in the fall of the first culture year (i.e., 1+ aged individuals). Arks from the 1994 cohort grew from a mean shell length of 17.8 mm to 39.9 mm in 15 months, a rate of 1.47 mm per month. Arks from the 1995 cohort grew from a mean shell length of 9.9 mm to 42.6 mm in 16 months, a rate of 2.04 mm per month.

Of the 747 arks examined, 54 (7.2%) were sexually indeterminate, 493 (66%) were male, and 200 (26.7%) were females. The Chi-square test revealed that the overall male/female ratio of 2.44 was significantly different from parity (χ^2 = 123.9; $P < 0.001$). Males dominated in both cohorts (1994: 1.00:0.36; and 1995: 1.00: 0.42) and in every monthly sample. Histological examination and visual observations of the gonads revealed that all orange-red colored gonads were late active or ripe females, while those showing white coloration were typically ripe males.

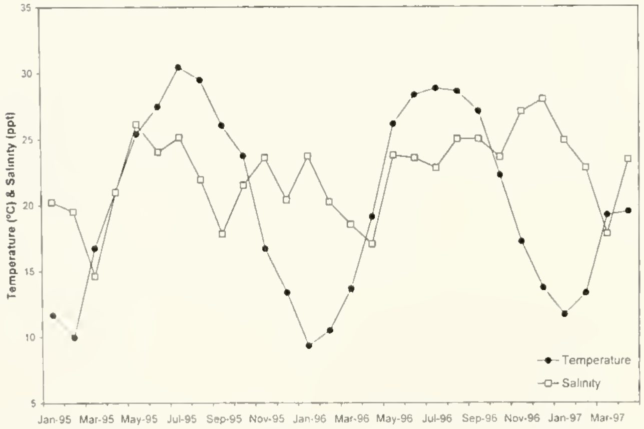

Figure 1. The mean monthly water temperature and salinity of the Skidaway River, Georgia from January 1995 to April 1997.

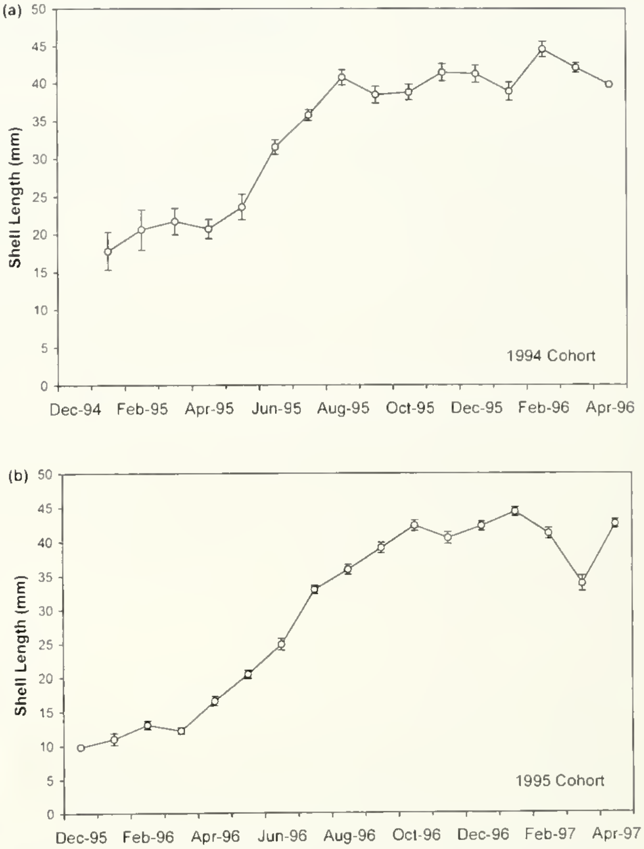

Figure 2. (a) The mean monthly shell length (mm ± standard error) of the 1994 cohort of blood arks, *Anadara ovalis*, grown in pearl nets suspended in the Skidaway River, Georgia from January 1995 to April 1996. (b) The mean monthly shell length (mm ± standard error) of the 1995 cohort of blood arks, *Anadara ovalis*, grown in pearl nets suspended in the Skidaway River, Georgia from December 1995 to April 1997.

Blood arks reached sexual maturity at an early age, exhibiting ripe specimens in the winter months of the first year in both cohorts with major spawning commencing after April–May and continuing to September–December (Figs. 3–5). A comparison of the gonadal stages for the two cohorts shows a striking difference for the cohorts in December 1995 (1994 cohort age 1+ years, 1995 cohort age 0+ years) indicating that the 0+ year arks develop gametes more easily during the winter months of their first year. For the 1994 cohort, 22% were ripe, 22% were partially spawned and 56% were spent in January 1995. In February and March, arks were either spent or in the early active stage. By April, late active (37.5%), ripe stages (25%) and spent stages (25%) were present. The gonadal index increased between March (1.9) and April (3.1)-May (3.2) as more animals became reproductively active, with ripe individuals dominating in May (60%) and June (42.9%). The gonadal index decreased to 1.4 by June, reflecting the occurrence of partially spawned individuals. Partial spawning dominated from

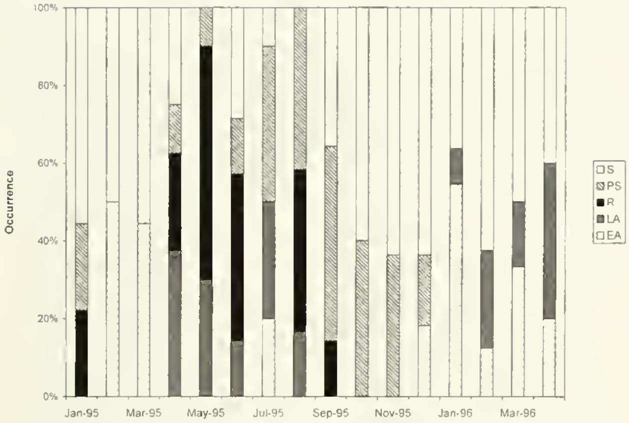

Figure 3. The relative frequency (percentage) of each gonadal developmental phase (EA = early active, LA = late active, R = ripe, PS = partially spawned, and S = spent) for the 1994 *Anadara ovalis* cohort from January 1995 to April 1996.

July (40%) through September (50%), with spent individuals occurring most frequently through the latter part of the year. This resulted in low gonadal index values until the initiation of gametogenesis again in January (G.I. = 1.5 in December to 2.36 in January).

For the 1995 cohort, 22% of arks were ripe in December 1995, with 28% in the early active and 50% in the late active stage (G.I. = 3.93). Most arks were in the early active stage (50%) with 41.7% exhibiting the spent stage by January (G.I. = 2.25), indicating a minor spawning event having taken place. Ripe individuals occurred again in March (9.4%) with most (76.7%) being ripe in April (G.I. = 4.53). Ripe individuals were present through August, but partially spawned and spent individuals dominated until December 1996. As in previous years, early active and late active individuals began occurring in December 1996 with ripe individuals again dominating by April (69.2%) (G.I. = 4.82).

For the 1995 set, 225 arks in December 1995 were histologically processed to determine the size at sexual maturity for the blood ark. Of the 225 arks examined, 26 (11.6%) were sexually indeterminate, 156 (69.6%) were males and 43 (19.2%) were females. The sex ratio was 1.00:0.28 (M:F), which significantly differed from parity ($\chi^2 = 64.17$; $P < 0.001$). Males averaged 9.9 ± 0.26 mm and ranged from 4.3 mm to 16.2 mm in shell length. Females average 11.5 ± 0.37 mm and ranged from 7.4 mm to 17.7 mm in shell length. Indeterminate animals averaged 6.5 ± 0.29 mm and ranged from 4.5 mm to 8.9 mm. Of the 199 arks that had started gametogenesis, most were in the late active stage for males (54.5%, 10.2 ± 0.27 mm, range: 6.3–16.2 mm) and the ripe stage for females (44.2%, 11.8 ± 0.50 mm, range: 8.2–16.6 mm). At this time, males also had 30.1% in the early active stage (7.3 ± 0.25 mm, range: 4.3 mm–13.4 mm), while females had 20.9% (9.6 ± 0.68, range: 7.4–14.2 mm).

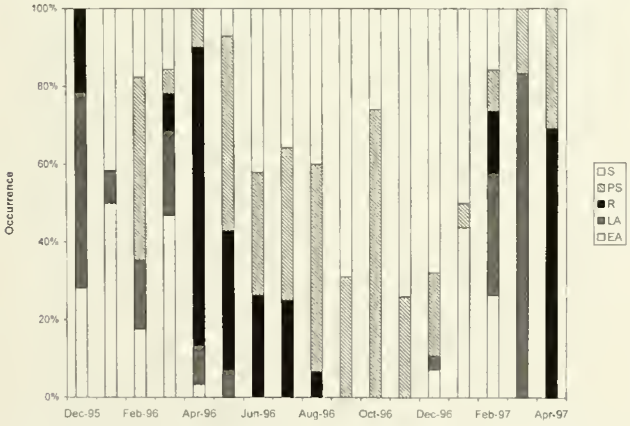

Figure 4. The relative frequency (percentage) of each gonadal developmental phase (EA = early active, LA = late active, R = ripe, PS = partially spawned, and S = spent) for the 1995 *Anadara ovalis* cohort from December 1995 to April 1997.

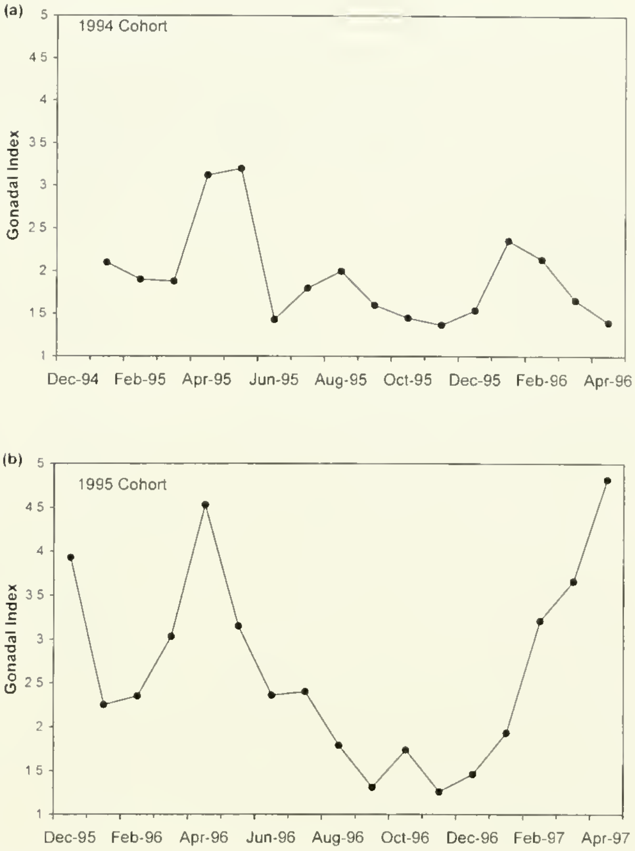

Figure 5. (a) The monthly gonadal index values of the 1994 cohort of blood arks, *Anadara ovalis*, from the coastal waters of Georgia between January 1995 and April 1996. Monthly gonadal index (G.I.) values were determined by averaging the number of specimens ascribed to each category score (EA = 3, LA = 4, R = 5, PS = 2, SP = 1). (b) The monthly gonadal index values of the 1995 cohort of blood arks, *Anadara ovalis*, from the coastal waters of Georgia between December 1995 and April 1997. Monthly gonadal index (G.I.) values were determined by averaging the number of specimens ascribed to each category score (EA = 3, LA = 4, R = 5, PS = 2, SP = 1).

DISCUSSION

In Georgia, bloods arks recruit during the summer and early fall; the spat grow rapidly and reach sexual maturity by the end of the year. A minor spawning event occurs at this point, followed by the major spawning period in the subsequent summer months. As observed with one-year-old arks from the 1995 cohort, the minor winter spawning event is not repeated in the subsequent year.

Rapid growth rates recorded for arks (<2-years-old) in

TABLE 1.

Size at initial gametogenesis and sexual maturity for various marine bivalves species from the Family Arcidae.

Species	Shell Length at Initial Gametogenesis (mm) Male	Female	Not Stated	Shell Length at Sexual Maturity (mm) Male	Female	Not Stated	Source
Anadara cornea			20				Broom, 1985
Anadara granosa						21	Narasimham, 1968
Anadara granosa			17.5			24–25	Broom, 1983
Anadara ovalis	4	7		10	12		This study
Anadara rhombea	19	22		21–25	21–25		Natarajan & John, 1983
Anadara scapha			22			30	Baron, 1992
Anadara subcrenata			15				Ting et al., 1972
Anadara transversa			10				Walker & Power (in press)
Anadara tuberculosa				32	36		Broom, 1985
Senilia senilis	5–9	19	10–17				Yoloye, 1974

present study (1.47 and 2.04 mm/mo) were comparable to earlier reported growth rates for natural populations in Virginian waters, and cultured arks in the coastal waters of Georgia. In Georgia, arks that were similarly grown in pearl nets for 36 months exhibited rapid growth in the first year (<10 mm size class: 2.58 mm/mo; 10–20 mm: 2.01 mm/mo) with decreased annual growth for years two (0.83 and 0.89 mm/mo) and three (0.19 and 0.18 mm/mo) (Walker 1998). In Virginia, blood arks grew from an initial size of 14 mm to 30 mm in 11 months, a lower rate of 1.45 mm/mo (McGraw & Castagna 1994). Arks were noted in the present study to grow faster in the spring and summer months, therefore the slower growth rates in Virginia may result from colder water temperatures.

Sexual maturity was attained at an approximate mean size of 10 mm in shell length for males and 12 mm for females (age 7 to 8 months) however; gametogenesis was noted to occur at minimum sizes of 4 mm and 7 mm, for males and females, respectively. Animals from the 1995 cohort were known to be at least three months old when gathered in December, since the cages were deployed in September. At this time, 20% of the 225 collected were ripe and 44% were in the late active stage. Because major spawning for blood arks starts in April–May, the maximum age of the new recruits on the cages is about 8 months. We believe that blood arks had already recruited to the sand flat prior to the placement of the field grow-out cages. Once the cages were in place, we postulate that the arks migrated up the sides and attached themselves with byssal threads. Blood ark juveniles are commonly found attached to the upper ends of *Dioptera* tubes in the estuary (Walker, personal observations). An upward migration of juvenile arks has also been observed with transverse arks, *Anadara transversa* within the laboratory. Transverse arks contained within a northern quahog, *Mercenaria mercenaria*, seed shipment were observed to migrate to the top of the quahog seed mass and even up the sides of the holding tray to the surface/water interface (Walker & Power, in press). Members of the Arcidae are reported to initiate gametogenesis and reach sexual maturity at a small size (Table 1). The blood arks mature at a similar age to *Anadara granosa* (Linnaeus 1758; Narasimham 1968) and *Senilia senilis* (Lamarck 1758; Yoloye 1974), but typically at a smaller size than most of the family (Table 1).

Temperature is one of the main exogenous factors controlling reproduction in marine invertebrates (Giese 1959; Sastry 1975). In Virginia, blood arks are reported to spawn in the summer months when water temperatures reach above 17°C (Chanley & Andrews

TABLE 2.

The sexual ratios and percentage hermaphroditism of various marine bivalves species from the Family Arcidae.

Species	Sample Size	Sexual Ratio (Female:Male)	% Hermaphroditism	Source
Anadara antiquata	1040	1.00:1.00		Toral-Barza & Gomez, 1985
Anadara granosa	300	1.00:1.00	0.003	Broom, 1983
Anadara ovalis	185	1.00:1.98		McGraw et al., 1998
Anadara ovalis	693	1.00:2.44	0	This study
Anadara rhombea	1155	1.00:1.27	0	Natarajan & John, 1983
Anadara scapha	235	1.00:1.47		Baron, 1992
Anadara senilis	100	1.00:0.34	0	Yoloye, 1974
Anadara senilis		1.00:1.00	0.004	Broom, 1985
Anadara subcrenata		1.00:1.00		Broom, 1985
Anadara transversa	199	1.00:0.53		Walker & Power (in press)
Anadara trapezia		1.00:1.00		Broom, 1985
Anadara tuberculosa	218	1.00:1.25		Cárdenas & Aranda, 2000
Anadara tuberculosa	1094	1.00:1.00		Cruz, 1984
Anadara tuberculosa		1.00:1.00		Dzyuba & Maslennikova, 1982
Noetia ponderosa	181	1.00:1.26		McGraw et al., 1998

1971; McGraw et al. 1998). In Georgia blood arks spawned earlier, from late spring through summer, presumably due to the coastal waters warming earlier in the year than in Virginia. By April–May 1995 and 1996, water temperatures were already above 20°C in the Skidaway River. In general, spawning periods and gametogenesis in marine bivalves start earlier and last longer in southern geographical areas than in northern ones (Eversole 1989; Thompson et al. 1996).

In this study, males dominated the population of blood arks with an overall sex ratio of 2.38:1.00 (M:F). This is in agreement with the observed sex ratio (1.98:1.00 M:F) reported by McGraw et al. (1998) for a Virginia population of 1-year-old blood arks (Table 1). An equal sex ratio has been observed in *Andara granosa* (Pathansali 1966; Broom 1983) and *Anadara subcrenata* (Lischke 1869; Ting et al. 1972); however, males dominated *Senilia senilis* populations (Yoloye 1974). For *Anadara scapha* (Linnaeus 1758), males were reported to dominate in the smaller size-classes while females were more frequent in the larger-size classes (Baron 1992). This may indicate a sex change but may also be a consequence of the differential energy requirements of males vs. females. No hermaphroditic *Anadara ovalis* were observed in the present study. Hermaphroditism is rare in the Family Arcidae (Table 2); *Anadara scapha* and *Senilia senilis* have been classified as a protandric hermaphrodite (Baron 1992; Yoloye 1974). The present study examined newly recruited blood arks, while in Virginia 1-year-old individuals were examined. Males typically dominate protandric bivalve species in the first year; whereas older age classes are generally equal. Blood arks can reach a maximum of 5 years and therefore these older age classes need to be examined to determine if this species is protandric.

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TEMPORAL FLUCTUATIONS (1989–1999) IN THE POPULATIONS OF *HALIOTIS FULGENS* AND *H. CORRUGATA* (GASTROPODA: HALIOTIDAE), AT ISLAS SAN BENITO (BAJA CALIFORNIA, MEXICO)

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ABSTRACT In our study area, green abalone (*Haliotis fulgens*) was more abundant than pink abalone (*H. corrugata*) until the end of the 1980s, but nowadays, both species have similar density levels and are close to values were recruitment failures may occur. In comparison to pink abalone, green abalone showed higher abundance of legal-sized stock and condition factors. Abundance of the legal-sized stock of both species has significantly decreased, but the density extracted by fishing remained the same. Overfishing of legal sizes and recruits, or poaching, may have not significantly contributed to the observed patterns. Only the proportion of recruits of pink abalone decreased significantly along the time and its fluctuation patterns support the idea that non-severe "El Niño" events may have a positive effect on recruitment. The lack of significant changes in recruitment of green abalone suggests that this effect may be species-specific. On the other hand, our results indicate that "El Niño" events lead to poor physiological condition. Mean condition factors of both species decreased significantly along the time, and are significantly related with the mean annual surface temperatures. The proportion of sexually mature organisms of both sexes, in both species, also decreased significantly along the time and could be a by-product of impoverished condition. Additionally, the sex ratio of green abalone varied significantly along the time, in favor to males.

KEY WORDS: abalone surveys, long-term dynamics, Baja California, *Haliotis fulgens*, *H. corrugata*

INTRODUCTION

The quantification of abalone and the assessment of its temporal fluctuations are often difficult tasks. Aggregated spatial distributions difficult the obtainment of precise abundance estimates at large spatial scales (McShane 1995), and even at small spatial scales estimates may have with high variance and low precision (McShane 1994).

The first attempts to quantify abalone in Baja California were done between 1968 and 1970, covering wide spatial scales along the coast (Guzmán-del-Próo 1992). Mean density estimates derived from these attempts had very low precision, due to habitat heterogeneity (Guzmán-del-Próo 1992). After 1988, abalone surveys were restricted to each fishing area, in order to calculate local yearly catch quotes, and regulate the fishery. We present the results of abundance surveys made between 1989 and 1999, for green abalone (*Haliotis fulgens*, Philippi 1854) and pink abalone (*H. corrugata*, Gray 1828), at Islas San Benito. These three islands are located at the current main abalone fishing area of Mexico.

This work brings information about one of the few long-term monitoring efforts applied to natural abalone banks in North America. Additionally to density values, we bring data that are rarely collected during surveys: namely, sizes, condition factors, sex ratios, and gonadal maturity of both species. We tried to assess the contribution of commercial extractions, overfishing of legal sizes, recruitment overfishing, and local climate forcing to the observed fluctuations.

MATERIALS AND METHODS

Study Area

Islas San Benito are located at the Pacific coast of Baja California, 40 km northwest from Cedros Island (Fig. 1a). They are away from continental runoff and pollution, and strong seasonal upwelling favor the growth of *Macrocystis pyrifera*, *Eisenia arborea*, and *Egregia menziessi* (González-Avilés & Shepherd 1996). The extension of the abalone fishing area is 6,862,155 m^2 (Arano-Castañon & Uribe-Osorio 1998). The fishermen cooperative "S.C.P.P. Pescadores Nacionales de Abulón" is the unique concessionaire of the abalone and lobster fishery in the area.

Survey Methodology

Surveys were conducted in the whole fishing area of the islands, when the fishery is closed (October to January), between 1988 and 1999. The coastline of the three islands has always been divided into 35 sections (≈500 m width) placed in the same location during all the surveys, using floats as markers. The offshore extension of each section has been fixed at the 30 m isobath; therefore, the length of each section varied between 88 m and 1473 m, perpendicular to the shore (Arano-Castañon & Uribe-Osorio 1998) (Fig. 1a).

Abalone divers from the fishermen cooperative collected all visible abalone inside each section, but the sampling procedure varied along the time (Fig. 1b):

1989 to 1995. Six to 12 sampling stations were randomly distributed inside each section, depending on the number of divers. A 4 m^2-quadrat was used as a sampling unit from:

1996 to 1999. Each section was divided into three bathymetric strata: 0–10 m, 10–20 m, and 20–30 m. Three, five, and two sampling stations were randomly located inside each bathymetric strata, respectively. A 10 m^2-quadrat was used as sampling unit.

Density Estimations

Only sampling stations located on rocky bottom were used fo density estimations. The mean density was calculated conside

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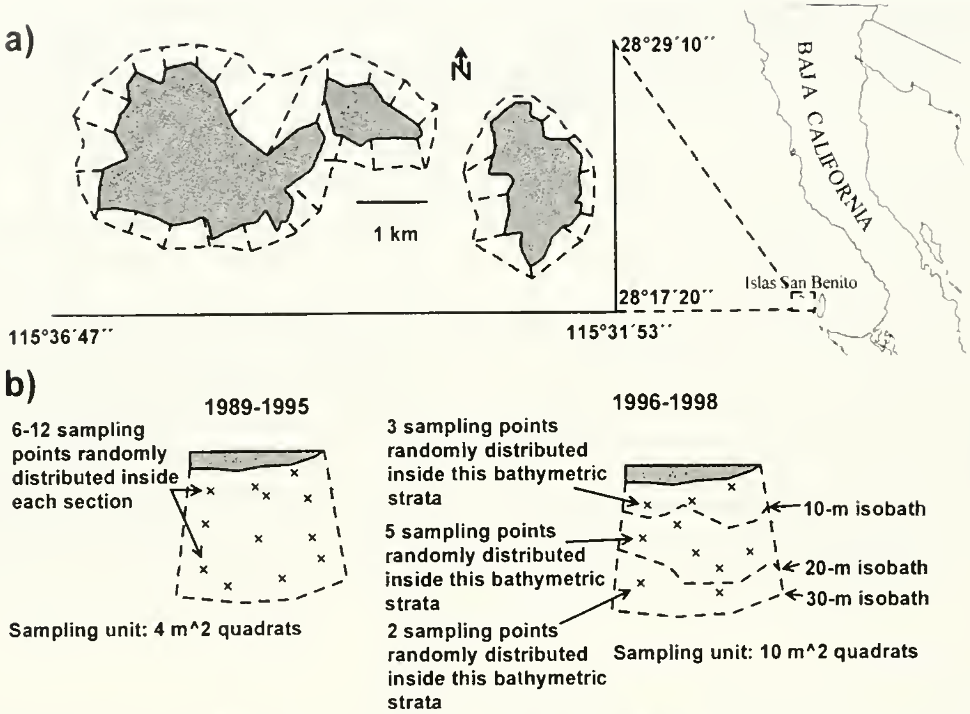
a)
N
28°29´10´´
BAJA CALIFORNIA
1 km
Islas San Benito
28°17´20´´
115°36´47´´
115°31´53´´
b)
1989-1995
6-12 sampling points randomly distributed inside each section
Sampling unit: 4 m^2 quadrats
1996-1998
3 sampling points randomly distributed inside this bathymetric strata
5 sampling points randomly distributed inside this bathymetric strata
2 sampling points randomly distributed inside this bathymetric strata
10-m isobath
20-m isobath
30-m isobath
Sampling unit: 10 m^2 quadrats

Figure. 1. (a) Location of the study area. The dashed line represents 30-m isobath. (b) Scheme of the sampling method.

all sizes. Estimations of the mean density of the legal-sized stock were done considering only sizes larger than the minimum legal size of capture (green abalone: 150 mm of shell length; pink abalone: 140 mm of shell length). Ninety-five percent confidence limits and standard errors of the total mean density, as well as 95% confidence limits of the mean density of the legal-sized stocks are shown. Significant temporal differences in the mean values were tested with ANOVA, in both species. We estimated the statistical power for the total mean density comparisons, at the given level of replication, and $\alpha = 0.05$ (Zar 1999).

Recruitment Estimations

Our survey methodology is inadequate to estimate recruitment accurately, but serious recruitment failures due to recruitment overfishing or environmental changes should be reflected in our data (Shepherd et al. 1998). From the 1989 to 1998 for size-frequency data, we calculated the percentage of recruits of both species, using the size criteria of Shepherd et al. (1998). Overall, significant differences between percentages (inside each species) were tested with Chi-squared analyses (Zar 1999). Paired comparisons are shown, to estimate statistical power (Zar 1999).

Assessing the Effect of the Fishery

The density extracted by fishing, between 1989 to 1996, was estimated for each species. The following data were used: local captures provided by the fishermen cooperative, legal-sized stock densities, local area of suitable habitat for both species (Rodriguez-Valencia et al. 1998), and mean weight of muscle (as described later). Significant temporal changes were tested by Chi-squared analyses.

Biametric Data

Biometric data of all captured abalone are available for the 1989 to 1998 surveys.

Sizes and Weights

Shell lengths of all captured abalone were measured to the nearest millimeter and muscles were weighed to the nearest gram. Each 5-mm size category contained at least three individuals. Since measuring and weighing is time-consuming, abalone were maintained in shadowed places and were covered with wet algae to prevent desiccation. Significant temporal differences in mean shell sizes were tested with ANOVA. Significant ascendant or descendant trends were tested by the significance of the slope (Beta) of simple linear regressions using the time as independent variable. Modal and maximum sizes of each species, at each survey, were compared using Chi-square tests.

Condition Factor

The condition factor of each sacrificed abalone was obtained dividing the muscle weight by the shell length, and the mean condition of each species at each survey was estimated. Significant temporal changes between mean condition factors were tested with ANOVA.

Sex Ratio and Gonadal Maturity

Sexes and gonadal maturity were determined to randomly selected abalone at each survey. Significant temporal changes in both variables were tested using Chi-square analyses.

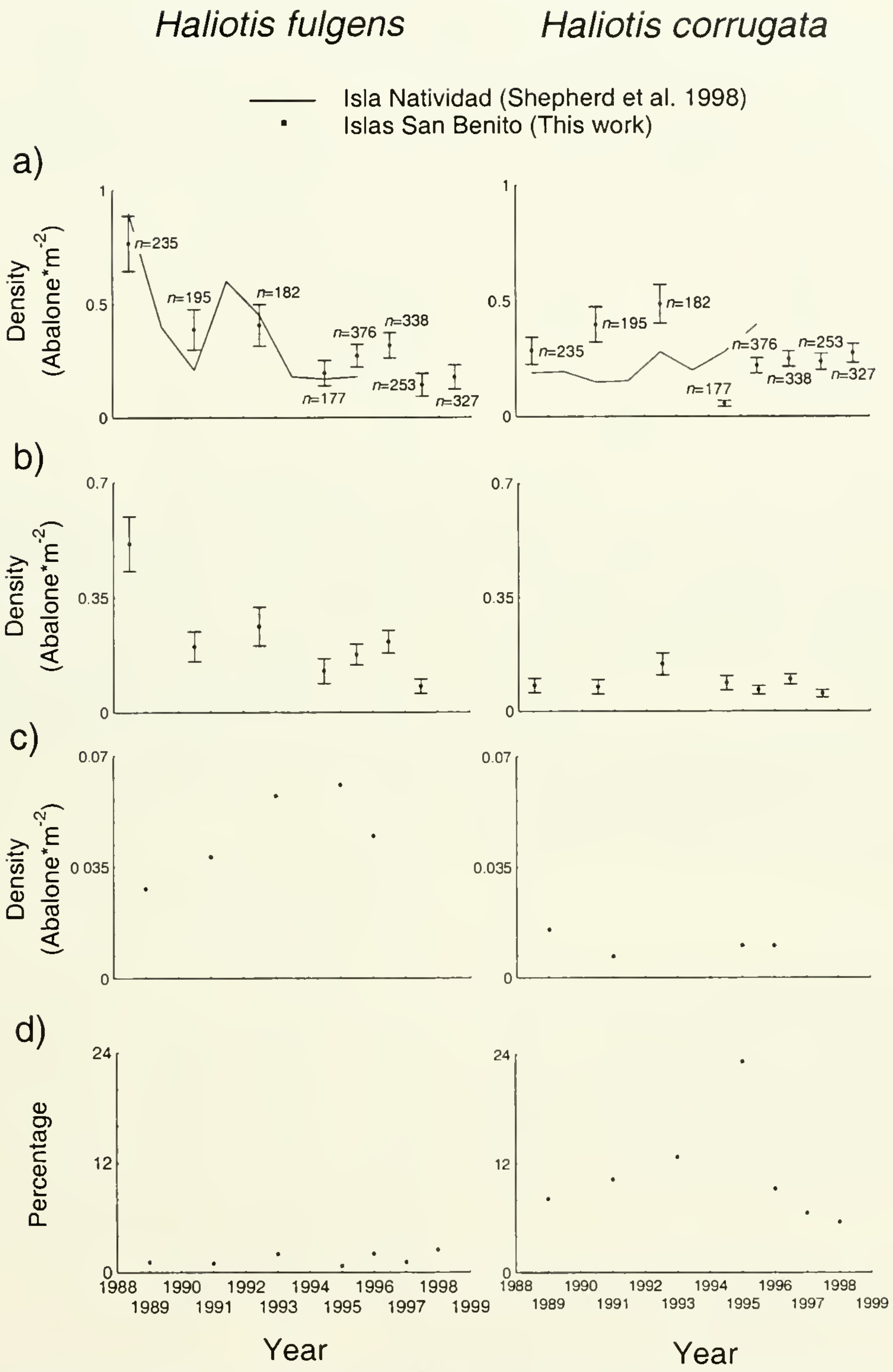

Figure. 2. (a) Temporal patterns of total mean densities and their 95% confidence limits. (*n*) represents the number of sampling stations located on rocky bottom; (b) Temporal patterns of the mean density of legal-sized stock and their 95% confidence limits; (c) Temporal patterns of the density extracted by fishing; (d) Percentages of recruits.

RESULTS

Patterns of the Total Mean Density

Green abalone was 2.5 times more abundant than pink abalone at the end of the 1980s, but at the beginning of the 1990s its density decreased, while that of pink abalone increased. After 1995, both reached their lowest density levels and followed comparable abundance trends (Fig. 2a). Temporal fluctuations are significant (Table 1). Modifications in sampling methods were more precise after 1995, because the confidence limits and standard errors of the means decreased (Fig. 2a and Table 2).

TABLE 1.

Assessment of significant temporal changes in the total mean density of *H. fulgens* and *H. corrugata* and estimates of the statistical power.

	H. fulgens					*H. corrugata*				
Comparison	$F_{(df.Effect, df.Error)}$	Effect MS	Error MS	ϕ	Power	$F_{(df.Effect, df.Error)}$	Effect MS	Error MS	ϕ	Power
1989 to 1991	$F_{(1,428)} = 22.9^{**}$	15.3	0.7	2.3	0.9	$F_{(1,428)} = 5.5^{*}$	1.3	0.2	1.0	0.6
1989 to 1993	$F_{(2,609)} = 16.9^{**}$	9.9	0.6	2.8	1.0	$F_{(2,609)} = 14.9^{**}$	8.4	0.6	2.6	1.0
1989 to 1995	$F_{(3,785)} = 24.7^{**}$	12.0	0.5	4.2	1.0	$F_{(3,785)} = 23.1^{**}$	11.1	0.5	4.2	1.0
1989 to 1996	$F_{(4,1160)} = 27.7^{**}$	11.3	0.4	5.2	1.0	$F_{(4,1160)} = 34.0^{**}$	11.3	0.4	5.1	1.0
1989 to 1997	$F_{(5,1497)} = 25.0^{**}$	9.4	0.4	5.5	1.0	$F_{(5,1497)} = 30.3^{**}$	10.2	0.3	5.5	1.0
1989 to 1998	$F_{(6,1749)} = 28.6^{**}$	9.9	0.3	6.4	1.0	$F_{(6,1749)} = 25.7^{**}$	8.6	0.3	6.0	1.0
1989 to 1999	$F_{(7,2076)} = 29.2^{**}$	9.6	0.3	7.0	1.0	$F_{(7,2076)} = 11.9^{**}$	1.9	0.2	3.9	1.0

* = $P < 0.05$.
** $P < 0.01$.

Patterns of the Mean Density of Legal-Sized Stocks and Density Extracted by Fishing

The proportion of harvestable sizes of green abalone has been twice as high as that of pink abalone (Table 3), and its density of legal-sized stock is significantly higher ($F_{(1,3499)} = 189.3^{**}$). The mean density of the legal-sized stock of both species has significantly varied and decreased along the time (green abalone: $F_{(6,\ 1744)} = 34.0^{**}$; pink abalone: $F_{(6,1744)} = 8.0^{**}$) (Fig 2b). On average, the fishery extracted 24% and 14% of the legal-sized stocks of green and pink abalone, respectively, corresponding to 15% and 7% of their total mean densities. The extraction by fishing has remained constant, since the densities extracted by fishing of both species remained constant along the time (green abalone: $\chi^2_{(4)} = 0.02$; pink abalone: $\chi^2_{(3)} = 0.004$), although that of green abalone seems to have increased (Fig 2c).

Recruitment Estimations

Recruits of pink abalone have been more abundant than green (Fig. 2d). Overall comparisons between the percentages of recruits of green abalone indicated non-significant changes ($\chi^2_{(6)} = 1.8$), but those of pink abalone varied significantly ($\chi^2_{(6)} = 19.5^{**}$), with increasing percentages until 1995 and decreasing percentages after. Paired comparisons indicated non-significant changes for green abalone, and significant changes for pink abalone (Table 4). The average power of the paired comparisons was 0.81 and 0.83, for green and pink abalone, respectively.

TABLE 2.

Standard errors of the total mean density estimations (Abalone*m^{-2}).

Survey	*Haliotis fulgens*	*Haliotis corrugata*
1989	0.061	0.030
1991	0.045	0.039
1993	0.046	0.042
1995	0.028	0.027
1996	0.026	0.017
1997	0.028	0.017
1998	0.025	0.018
1999	0.027	0.021

Patterns of Sizes and Condition

Mean sizes of both species showed significant temporal variations (Table 3) without trend (green abalone: $F_{(1,6)} = 1.2$; pink abalone: $F_{(1,6)} = 3.5$). Modal and maximum sizes showed non-significant temporal variations (Table 3).

The mean condition of green abalone was significantly higher than that of pink abalone during the studied period (1.6 vs. 1.1; $F_{(1,1177)} = 196.7^{**}$). No significant differences were detected between sexes, inside each species. Significant temporal changes in the mean condition of both species were detected (green abalone: $F_{(6,1032)} = 10.1^{**}$; pink abalone: $F_{(6,844)} = 3.0^{**}$), and both species showed significant decreasing trends (Fig. 3a). The mean condition of both species was significantly related with the mean annual surface temperature (Fig. 3b), which increased 2.1°C between 1989 to 1998, in the study area.

Patterns of Sex Ratio and Gonadal Maturity

The sex ratio of green abalone showed significant temporal changes in favor of males, while that of pink abalone remained close to 1:1, without significant changes (Table 5). The proportion of mature organisms of both sexes, inside each species, decreased significantly along the time (Table 4). In 1995, a conspicuous peak of mature organisms of both sexes was detected, in both species. Relationships between the proportion of mature organisms of green abalone and its mean condition factors and mean annual surface temperature were suggestive, but non-significant (Fig. 3 c–d). On the other hand, these relationships were significant for pink abalone (Fig. 3 c–d).

DISCUSSION

The Surveys

We have tried to follow the survey approach proposed by Hilborn and Walters (1992), Van der Meer (1997), and Gorfine et al. (1998), distributing sampling points in the whole capture area and applying low sampling effort at each point. The method used to estimate abalone abundance seems to be appropriate. However, main disadvantages are: (a) The small portion of potential habitat sampled (0.01%–0.02% between 1989 to 1995; 0.05%–0.07% be-

TABLE 3.

Sizes (minimum, maximum, mean and its 95% confidence limits, and mode; all in mm). Minimum size of capture = MSC. * = $P < 0.05$; ** = $P < 0.01$.

	Haliotis fulgens						*Haliotis corrugata*					
Survey	n	Min.	Max.	Mean	Mode	% > MSC	n	Min.	Max.	Mean	Mode	% > MSC
1989	721	47	203	152.7 ± 1.3	150	66.4	269	73	169	131.8 ± 1.8	130	28.3
1991	303	31	196	148.8 ± 1.9	140	51.8	311	65	173	125.9 ± 1.8	120	19.6
1993	269	56	193	152.3 ± 2.1	150	64.9	350	66	175	131.2 ± 1.6	130	29.7
1995	137	99	190	151.2 ± 2.8	160	65.0	159	75	180	133.0 ± 2.6	130	38.4
1996	1030	23	197	151.9 ± 1.1	150	63.9	827	29	187	131.6 ± 1.2	135	30.5
1997	1089	22	200	153.2 ± 1.0	160	66.7	847	46	190	134.2 ± 1.0	140	39.6
1998	364	40	197	150.2 ± 2.0	156	55.2	598	54	180	131.1 ± 1.1	135	23.4
1999	721	87	212	146.7 ± 2.6	138	42.4	269	48	177	135.5 ± 2.0	128	42.4
Temporal comparisons			$\chi^2_{(6)} = 0.6$	$F_{(7,4147)} = 5.4^{**}$	$\chi^2_{(6)} = 1.2$				$\chi^2_{(6)} = 1.9$	$F_{(7,3624)} = 11.3^{**}$	$\chi^2_{(6)} = 6.0$	

tween 1996 to 1998); and (b) The lack of use of specific methods to properly quantify juveniles (e.g., anesthetics (Prince & Ford 1985); sub-aquatic amplifiers (Shepherd & Turner 1985); suction method (McShane & Smith 1988). Differences in efficiency among divers were surely low, since only on-duty abalone divers participated, and they are highly efficient locating abalone (Prince & Guzmán-del-Próo 1993). Additionally, the use of quadrants and diving transects prevents over estimations and competition among divers (Findlay & Willerton 1996, McShane 1996). Shepherd et al. (1998) stated that Mexico is the only country making serious efforts to quantify the abundance of its abalone banks: nevertheless, surveys are frequently criticized by local cooperatives and academics.

TABLE 4.

Paired comparisons of the percentage of recruits of *H. fulgens* and *H. corrugata* between surveys and their statistical power.

Paired Comparisons	*H. fulgens*		*H. corrugata*	
	Z-value	Power	Z-value	Power
1989 vs. 1991	0.21	0.96	0.73	0.86
1989 vs. 1993	1.03	0.76	1.70	0.60
1989 vs. 1995	0.06	0.62	4.23*	0.99
1989 vs. 1996	1.40	0.68	0.42	0.92
1989 vs. 1997	0.24	0.94	0.72	0.85
1989 vs. 1998	1.70	0.60	1.26	0.70
1991 vs. 1993	0.62	0.81	0.86	0.84
1991 vs. 1995	0.21	0.95	3.62*	0.95
1991 vs. 1996	0.93	0.81	0.40	0.91
1991 vs. 1997	0.11	0.95	1.96*	0.55
1991 vs. 1998	1.05	0.71	2.42*	0.71
1993 vs. 1995	0.60	0.87	2.86**	0.83
1993 vs. 1996	0.22	0.95	1.70	0.60
1993 vs. 1997	0.89	0.74	3.34**	0.91
1993 vs. 1998	0.01	0.94	3.70**	0.96
1995 vs. 1996	0.74	0.90	4.91**	1.00
1995 vs. 1997	0.01	0.96	6.50**	1.00
1995 vs. 1998	0.89	0.82	6.60**	1.00
1996 vs. 1997	1.55	0.60	1.90	0.55
1996 vs. 1998	0.26	0.91	2.43*	0.73
1997 vs. 1998	1.61	0.63	0.66	0.88

* = $P < 0.05$.
** = $P < 0.01$.

Interpretation of Abalone Dynamics

Both species reflect the decline of the Mexican abalone fishery, observed since 1984 (Guzmán-del-Próo 1992; Shepherd et al. 1998), and their abundance patterns are similar to those reported for Isla Natividad (Shepherd et al. 1998). Their most recent density levels are close to values where recruitment failures may occur (Shepherd & Partington 1995) and a clear tendency to recover is still absent.

Vega et al. (1997) and Shepherd et al. (1998) proposed causing factors for the decline. Here we discuss the local role of some of these as follows: (a) overfishing of legal sizes and/or recruits; (b) negative effect of "El Nino" events over recruitment (independently of their intensity); and possible increases of recruitment during non-severe "El Niño" events; (c) physiological weakening during "El Niño" events.

Overfishing of Legal Sizes and/or Recruits

Overfishing can happen by fishermen cooperatives not respecting authorized fishing quotes, poaching, cooperatives fishing at erroneously calculated quotes, or the mixture of all of them. The first two choices have not occurred at Islas San Benito, since the local cooperative has been exemplarily adhered to the regulations and authorized quotes. Additionally, the location of the islands, and strict surveillance programs applied to owners and strangers resulting in rare poaching. The absence of significant temporal changes in the recruits of green abalone support our suppositions, and although that of pink abalone changed significantly, there is no reason to believe that only this species has been affected by poaching or recruitment overfishing. Changes in the percentage of recruits of pink abalone may be related to climatic changes, (see later). It seems that fishing quotes were erroneously estimated, since densities extracted by fishing remained constant, while the abundance of the legal-sized stock decreased significantly in both species.

Catches of green abalone have been higher than that of pink abalone, at Islas San Benito (Fig. 4). Pink abalone was the dominant species in the local catches between 1957 to 1984 and green abalone became dominant until 1985 (S. A. Guzmán-del-Próo, pers. comm.). Nowadays, green abalone is preferably fished for having a higher proportion of harvestable sizes and being more

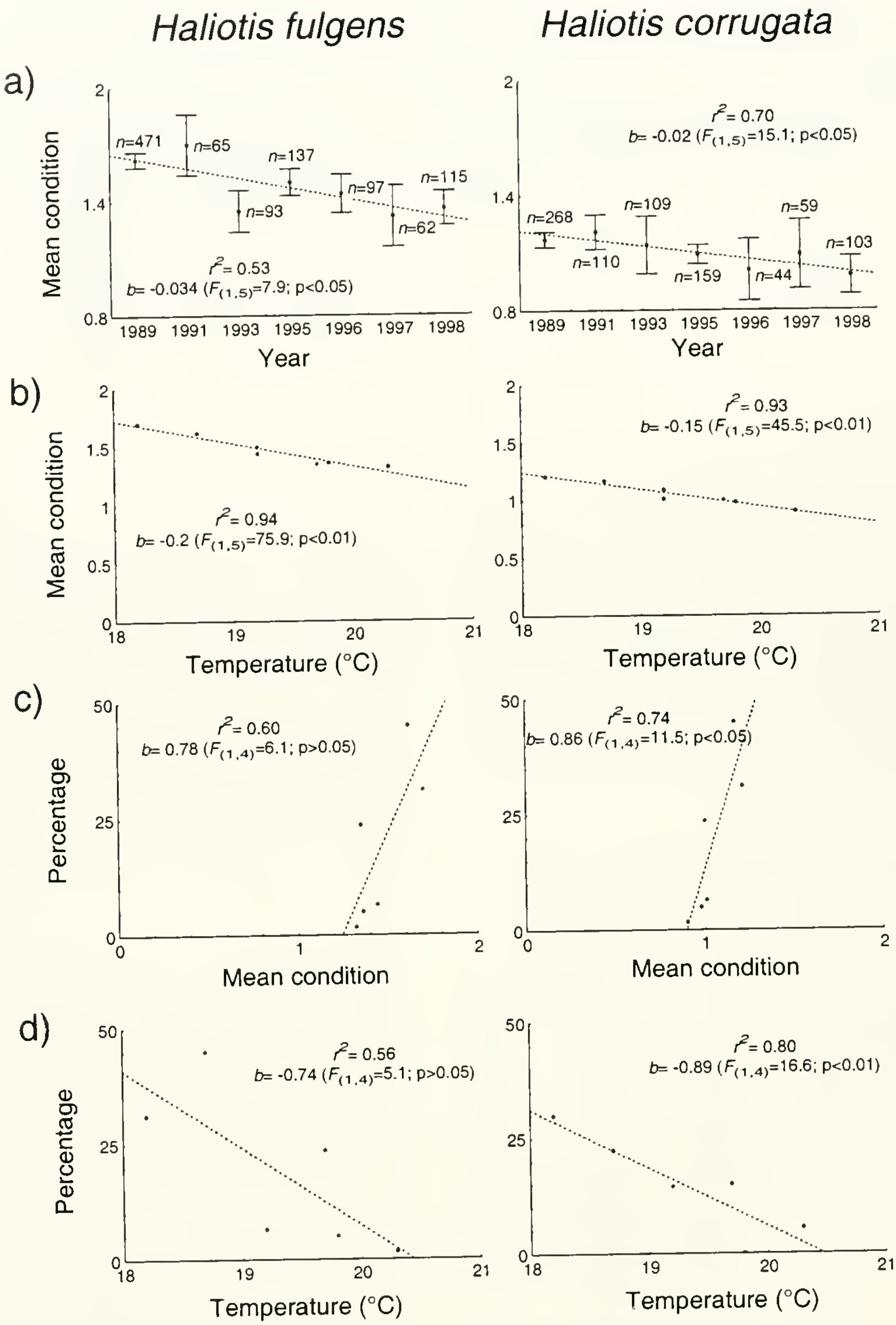

Figure. 3. (a) Temporal patterns of the mean condition factors. (*n*) represents the number of analyzed organisms; (b) Relationship between mean condition factors and mean annual surface temperatures; (c) Relationship between the percentage of sexual mature organisms and mean condition factors; (d) Relationship between the percentage of sexual mature organisms and mean annual surface temperatures.

revenue yielding by having larger sizes and shells that are more valuable. Fishing has been surely more important for the decline of green abalone than for that of pink abalone.

It would be desirable to have estimations of mortality by fishing, but this is out of our reach. We can only speculate that they would be lower than those at Isla Natividad (Shepherd et al. 1998), since captures of both species have been much higher at Isla Natividad (Fig. 4), but Isla Natividad and Islas San Benito have similar density levels. Additionally, quotes at Isla Natividad have been fixed considering an extraction of 30% of the legal-sized stocks (Shepherd et al. 1998) and this percentage at Islas San Benito has been lower.

TABLE 5.

Sex ratio (♀:♂) of *H. fulgens* and *H. corrugata*, at Islas San Benito.

Survey	*H. fulgens*	*H. corrugata*
1989	1:0.8	1:1
1991	1:0.7	1:1
1993	1:0.7	1:0.9
1995	1:1.3	1:1
1996	1:1	1:1.5
1997	1:1	1:1.5
1998	1:1.5	1:0.9
Temporal variations	$\chi^2_{(6)} = 20.7$**	$\chi^2_{(6)} = 8.5$

** $P < 0.01$.

Negative Effect of "El Niño" Events over Recruitment (Independently of their Intensity); and Possible Increases of Recruitment During Non-severe "El Niño" Events

According to Vega et al. (1997), increased water temperatures during "El Niño" events reduce the extensions of kelp beds and negatively affect the recruitment, because larvae are taken away from the banks by the currents. The frequent occurrence of "El Niño" events during the last decade (Fig. 5) should have continuously affected the recruitment, but our results of green abalone do not support this. The recruitment patterns of pink abalone support the hypothesis about a possible positive effect of non-severe "El Niño" events (Shepherd et al. 1998). Increasing recruitment was detected until 1995, because between 1981 and 1988 larvae settled under the influence of non-strong "El Niño" events (Fig. 5), but the 1981 to 1986 "El Niño" was one of the strongest ever observed (Arntz & Fahrbach 1996). Decreasing percentages were detected after 1996, because after 1989 larvae settled under the influence of frequent strong "El Niño" events (Fig. 5). It seems that the "El Niño" events determine a positive or negative effect over recruitment, but differences between species suggest that this could be species-specific. Data series longer than a decade are still necessary in order to have evidence that is more reliable.

Physiological Weakening During "El Niño" Events

Vega et al. (1997) and Shepherd et al. (1998) suggested that the reduction of food during warm periods could have lead to poor physiological condition and greater susceptibility to predation. The association between decreased physiological condition in both species and increased water temperatures support this. Local fishermen affirm that the local extensions of kelp beds declined due to the incidence of warm events, but we have no data to prove this. Kelp beds are also exploited by the local chemical industry, but records about the crop are rare.

The relationship between impoverished physiological condition and reduced proportions of sexually mature organisms was significant for only one species and suggestive for the other. Again, longer time series are needed. The remarkable peak in maturity detected in 1995 could have been produced by delayed spawning and/or shift in the reproductive season. Changes in the sex ratio of green abalone could be a by-product of changes in physiological condition, but it is hard to find a clear explanation. Guzmán-del-Próo (1992) also reported inter annual variations and deviations from the normal 1:1, in favor of females, in both species at other locations. This indicates that the sex ratio in abalone is also a dynamic parameter (Table 6).

Finally, as Shepherd et al. (1998) proposed, climatic variability, represented in our case by the surface water temperature, contributed to the decline of the populations by their weakened physiological condition. These effects have been underestimated, and although the environmental effect of sea temperature anomalies is considered in using the modified version of the Schaefer surplus production model (Shepherd et al. 1998), it seems that this caution has not been enough. Fluctuations in physiological condition should be taken into account when deciding the fishing quotes,

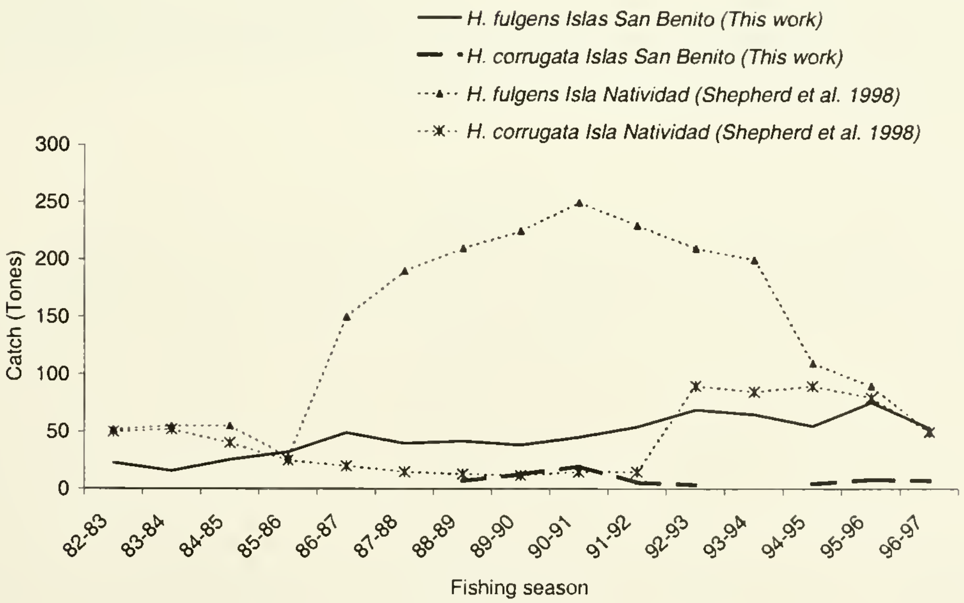

Figure. 4. Captures of *Haliotis fulgens* and *H. corrugata*.

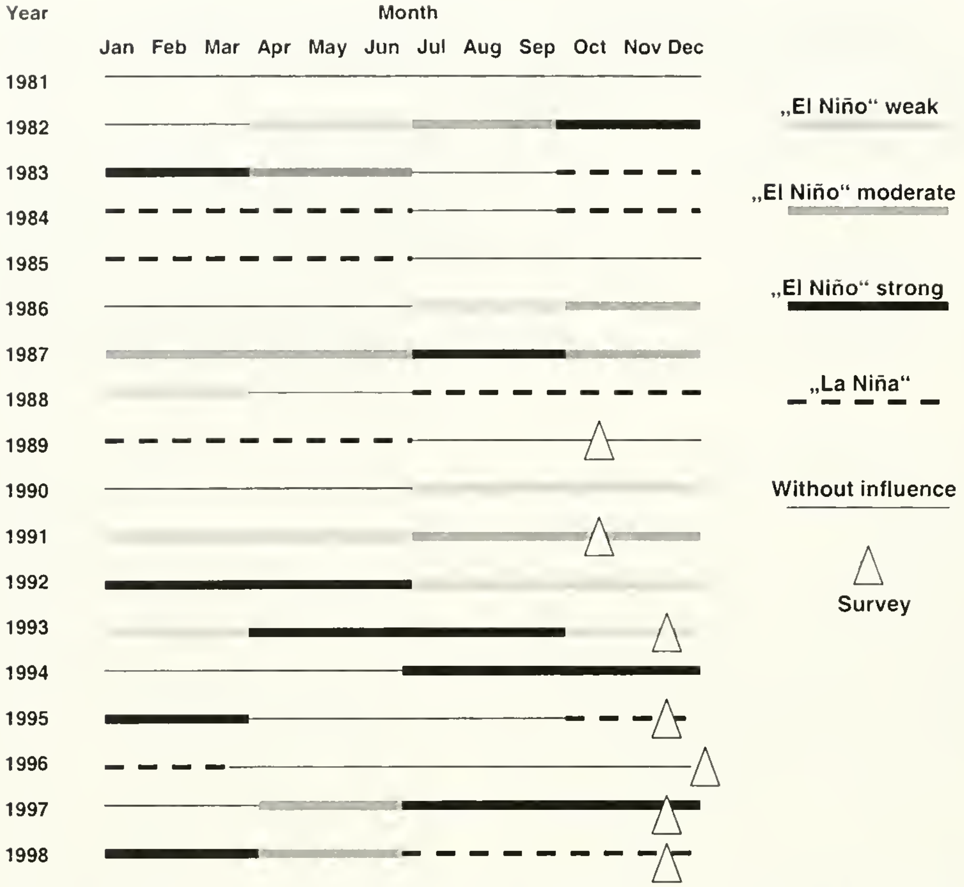

Figure. 5. Occurrence of warm and cold events during 1981 to 1998 (from http://www.cpc.ncep.noaa.gov/products/analysis_monitoring/ensostuff/ensoyears.html).

since they are fixed considering only abundance estimations and negotiation (Ponce-Diaz et al. 1998; Shepherd et al. 1998).

ACKNOWLEDGMENTS

We dedicate this work to the memory of our colleagues F. Salgado, G. Pérez, and A. Lelevier. We thank all members and directors of the cooperative "S.C.P.P. Pescadores Nacionales de Abulón" for their help and resources offered during the surveys, especially to D. G. Romero-Árce. We appreciate the support of F. Uribe, J. Cordova, J. L. Rivera, J. Castro, R. Sánchez, L. Vélez, M. Navarrete, J. Talavera, and O. Pedrín-Osúna during the surveys. We thank S. A. Shepherd, S. A. Guzmán-del-Próo, G. Davis, M. Strasser, and K. Reise for their valuable suggestions.

TABLE 6.

Percentage of sexual mature organisms of *H. fulgens* and *H. corrugata*, at Islas San Benito

	H. fulgens		*H. corrugata*	
Survey	♀	♂	♀	♂
1989	54.3	37.6	28.9	16.0
1991	44.4	22.2	31.3	28.4
1993	29.3	19.5	16.7	13.6
1995	96.0	92.9	85.2	77.8
1996	5.3	7.7	7.5	24.3
1997	3.2	0.0	3.0	9.1
1998	4.9	5.0	0.0	0.0
Temporal variations	$\chi^2_{(6)} = 206.7$**	$\chi^2_{(6)} = 231.5$**	$\chi^2_{(6)} = 208.9$**	$\chi^2_{(6)} = 160.4$**

** = $P < 0.01$.

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DEVELOPMENT OF THE NERVE GANGLIA OF ABALONE, *HALIOTIS ASININA* LINNAEUS

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ABSTRACT The development of cells in the ganglia during various ages of the abalone, *Haliotis asinina*, was studied. There were three types of neurosecretory cells (NS$_{1-3}$), four types of neurons (NR$_{1-4}$) and three types of neuroglia (NG$_{1-3}$). In the cerebral ganglia, NS$_1$ and NR$_1$ (giant neurons) first appeared in 1-month-old abalone while NS$_2$ and NS$_3$ first appeared in 3-month-old and 4-month-old abalone, respectively. These cells increased in number in 5- and 10-month-old abalone, reaching a maximum number at 12 months, and thereafter remained constant. In the pleuropedal ganglia, NS$_1$ and NR$_1$ first appeared in 1-month-old abalone, while NS$_2$ and NS$_3$ first appeared in 2-month-old and 4-month-old abalone, respectively. They increased in number in 4- and 7-month-old abalone, reaching a maximum at 11 months, and thereafter remained constant. In the visceral ganglia, NS$_1$ and NR$_1$ first appeared in 2-month-old abalone while NS$_2$ and NS$_3$ appeared later in 3-month-old and 5-month-old abalone, respectively. They increased in number at 4 months, reaching a maximum number at 11 months, and thereafter remained constant. NR$_2$, NR$_3$, NR$_4$ and NG were present in all ganglia early in development from one month onwards, and their numbers increased rapidly with age.

KEY WORDS: development, nerve ganglia, abalone, *Haliotis asinina*

INTRODUCTION

During neurogenesis of gastropods, the central ganglia of gastropods arise by proliferation and later delamination and/or invagination of the ectoderm. Cell division continues in the peripheral proliferative zones throughout embryogenesis, and post-mitotic cells then migrate inwardly to join the central ganglia which are formed nearby (Jacob 1984). Gangliogenesis in gastropods progresses from anterior to posterior with the cerebral ganglia developing first, followed by the pedal ganglia and then the more posterior ganglia of the abdominal loop (Kerkut & Walker 1975). The pattern of neurogenesis in the gastropod central nervous system resembles the proliferation of cells in the neural tube and the migration of the neural crest and ectodermal placode cells in the vertebrate nervous system but differs from the pattern described for other invertebrates (Jacob 1984).

Although the nervous systems of more derived species of gastropods as well as the development of neurons and individual transmitter systems have been studied (Lever et al. 1965; Coggeshall 1967; Kerkut & Walker 1975; Van Minnen and Sokolove 1984; Roubos et al. 1988; Carroll & Kempf 1994; Kruatrachue et al. 1994; Kempf et al. 1997; Marois & Carew 1997), the nervous development of prosobranchs has not been investigated in detail. Most studies of ganglia development in gastropods were conducted on opisthobranchs and pulmonates. In pulmonates, variation in morphology and lobulization of the ganglia is related to age and development (Kerkut & Walker 1975). Roubos et al. (1988) studied the development of neuroendocrine centers of *Lymnaea stagnalis* (Linnaeus) and found that the dorsal bodies and light green cells were already present in snails of 1 mm in shell length and that the caudo-dorsal cells first appeared in snails of 3 mm in shell length. The dorsal bodies and caudo-dorsal cells increased in number and size with increasing shell length. In *Achatina fulica* (Bowdich), the size of the ganglia and the number of nerve cells in the ganglia increased with increasing age. The prominent nerve cells in the ganglia were large cells and giant cells. The large cells were already present in all ganglia of the newly hatched snails, while the giant cells first appeared in 1-month-old snails. The neurosecretory cells in the cerebral ganglia of *A. fulica* first appeared in 2-month-old snails and increased in number and size with increasing age, reaching a maximum number in 8-month-old snails and thereafter remained constant in 9- to 12-month-old snails (Kruatrachue et al. 1994).

In general, the number of nerve cells in the ganglia of gastropods increases with age (Kerkut & Walker 1975; Lever et al. 1965; Coggeshall 1967). Coggeshall (1967) studied the opisthobranch snail, *Aplysia californica* (Cooper), and found that, during its maturation, the number of nerve cells in the ganglia increased by 40% and that the greatest number of large neurosecretory cells occurred in full grown animals. In the stylommatophoran snail, *Limax maximus* (Linnaeus), the morphology of the dorsal body cells also changed during maturation (Van Minnen & Sokolove 1984). The neurosecretory cells were small and released little secretory product in the immature and early male-phase animals. In contrast, these cells became larger and released large amounts of secretory product in the later female-phase animals (Van Minnen & Sokolove 1984). To the best of our knowledge, there is little information on the development of ganglia in prosobranch snails. Hence, the aim of the present investigation was to study the development of nerve ganglia of *Haliotis asinina* (Linnaeus), a common abalone species found along the coastal waters of Thailand.

MATERIALS AND METHODS

Ten adult *H. asinina* (average shell length 66.58 mm) (five males and five females) were obtained from the Marine Biological Station, Chulalongkorn University, Chonburi Province, Thailand. They were relaxed with 5% MgCl$_2$ for 3–4 hours prior to dissection. Dissections of the nervous system were done under an Olympus stereoscopic binocular microscope with a fiber optic dissecting light and drawings were made with the aid of a camera lucida.

To study the histological development of the nervous system of *H. asinina*, adult abalone and those from 1 to 12 months old were obtained. Ten animals from each age class were examined. They were relaxed and the cerebral, pleuropedal and visceral ganglia were dissected out and fixed in Bouin's fluid in 0.14 M NaCl f

24 hours, and washed with 70% ethyl alcohol. Then, they were dehydrated through a graded series of ethanol, cleared in dioxane, infiltrated and embedded in paraffin. Serial frontal sections of 5 μm thickness were cut and alternating sections were stained with hematoxylin and eosin, chrome-hematoxylin-phloxine (Gomori 1941) and paraldehyde-fuchsin (Gomori 1950). Sections were examined under an Olympus Vanox light microscope. Measurements (width, length and thickness) of the ganglia were taken from the median frontal sections (10 sections per ganglion; 10 animals for each age group). Neurons and neruosecretory cells in all ganglia were identified based on their histological characteristics (cell size and shape, nuclear size and shape) and staining affinities (Upatham et al. 1998; Kruatrachue et al. 1999; Thongkukiatkul et al. 2000). In addition, the numbers of cells in each ganglion of each age group were counted. For each animal, the cell count was done on the median frontal sections (10 sections per ganglion). The cell type and number were scored as follows:

Neurosecretory Cell (per section)		Neuron (per section)	
(−)	= 0 cell	(−)	= 0 cell
(+)	= 1–5 cells	(+)	= 1–10 cells
(++)	= 6–10 cells	(++)	= 11–20 cells
(+++)	= 11–15 cells	(+++)	= 21–30 cells
(++++)	= >15 cells	(++++)	= >30 cells

RESULTS

Figure 1 shows a diagramatic drawing of the gross anatomy of adult *H. asinina*. The nervous system consists of a pair of cerebral ganglia, a pleuropedal ganglion and a visceral ganglion.

Figure 2 shows the frontal sections of the cerebral (Fig. 2A), pleuropedal (Fig. 2B) and visceral ganglia (Fig. 2C) in adult *H. asinina*. There are 10 types of nerve cells in all ganglia of *H. asinina* (Figs. 2D, 3A), i.e., three types of neurosecretory cells (NS_{1-3}), four types of neurons (NR_{1-4}) and three types of neuroglia (NG_{1-3}) (Upatham et al. 1998; Kruatrachue et al. 1999; Thongkukiatkul et al. 2000). The NS cells were identified using special stains, i.e. chrome-hematoxylin- phloxine (Gomori 1941) and paraldehyde-fuchsin (Gomori, 1950).

The shape, size, and type of cells and their number in ganglia during various ages of developing abalone are summarized in Tables 1, 2 and 3.

Cerebral Ganglia

In 1-month-old abalone, the cerebral ganglion appeared as an elongated bean shape whose size was approximately 121 × 471 × 100 μm (Fig. 3B). Most of the ventral, dorsal and lateral parts of the ganglia had a thick cortex that contained 3-4 cell layers, while the medial part contained only 0–1 cell layers (Table 1). NS_1 cells first appeared in 1-month-old abalone; there were 1–2 cells per section. These cells were concentrated in the dorsal horn of the ganglion (Table 1). Most types of neurons (NR_{1-4}) were present, but NR_2 were the most numerous. NR_3 and NR_4 were moderate in number, while the NR_1 or the giant neurons were rarely found but when present were usually located in the dorsal horn similar to NS cells (Table 1). All types of NG were present but in a small number.

At 2–4 months, the ganglia appeared bean shape similar to those in 1-month-old abalone. The number of cell layers increased with age. The number of NS cells increased to about 2–5 cells per section (Table 1). Most of these were NS_1, while NS_2 were observed in 3-month-old and NS_3 in 4-month-old abalone. Most NS cells were concentrated in the dorso-lateral and dorso-medial, ventral and ventro-medial parts of the ganglia (Table 1). NR cells were similar in type and number to those in 1-month-old abalone. NG cells increased in number from one month onwards.

At 5 months, the size of the ganglion increased to 203 × 632 × 200 μm (Table 1). From 5 months onwards, the ganglion assumed a sickle shape (Fig. 3C). The number of cell layers in the cortex increased, especially in the ventral and dorsal parts. The number of NS cells increased to about 10 cells per section, and although all types of NS were scattered in all parts of the ganglia, most were still concentrated in the dorsal and ventral horns (Table 1). The number of NR increased with age, and the NR_1 count was approximately 11–20 cells per section (Table 1). They were present in the dorsal and ventral areas. At this age, the number of NG slightly increased.

From 6 to 10 months, the ganglia appeared sickle shaped but were larger and more elongated than those of 5-month-old abalone. The cortex in all areas thickened and the quantities and distribution of NS and NR cells were similar to those of 5-month-old abalone (Table 1).

At 11 months, the cerebral ganglia increased in size to about 377 × 810 × 300 μm (Fig. 3D). Other appearances were similar to those of 5- to 10-month-old abalone. However, the numbers of NS and NR_1 cells increased (Table 1). NG also increased with increasing age. When abalone were 12 months old, their ganglia (377 × 901 × 325 μm in size) were fully developed and appeared similar in all aspects to those of the adult abalone (Fig. 2A).

Pleuropedal Ganglia

In 1-month-old abalone, the pleuropedal ganglion appeared butterfly-shaped and about 189 × 418 × 150 μm in size (Fig. 4A). In the ventral and lateral parts of the ganglia, the cortex was thick and contained 2–5 cell layers (Table 2). The remaining parts of cortex were relatively thin. There were only about 1–2 NS cells per section. These cells were confined to the dorsal-sulcus of the ganglion; most of them being NS_1 (Table 2). There were all types of NR, but a few NR_1 and NR_4 were present in the dorso-medial part (Table 2). All types of NG cells were found in the ganglion at this age.

At 2–3 months, the size of the ganglia increased from 273 × 497 × 155 to 289 × 522 × 170 μm, but the shape was not altered (Fig. 4B). The number of cell layers in the cortex, NS and NR cells appeared to increase, and most cells were found in all parts of the cortex. However, NR cells were concentrated in the dorsal and dorso-lateral parts, while NS cells were concentrated in the dorso-medial and lateral sulci (Table 2).

At 4–6 months, the pleuropedal ganglia was still butterfly-shaped but increased in size from 337 × 556 × 200 μm to approximately 488 × 707 × 250 μm (Fig. 4B), and the cortex became much thicker. The number of NS cells (mostly NS_1) increased to about 20 cells per section and a larger number were found in the dorso-lateral and ventro-lateral parts (Table 2). NR cells increased in number with increasing age and were found in the ventro-medial, ventro-lateral and ventral sulci (Table 2).

At 7 months, the ganglia were H-shaped and increased in size to about 544 × 1615 × 315 μm (Fig. 4C). The number of cell layers increased and NS cell (mostly NS_1) number was about 30–40 cells per section; these cells were distributed in all areas (Table 2). NR cells (mostly NR_1) increased in number in comparison to earlier stages. From 8 to 10 months, pleuropedal ganglia were similar in shape to those of 7-month-old abalone (Table 2).

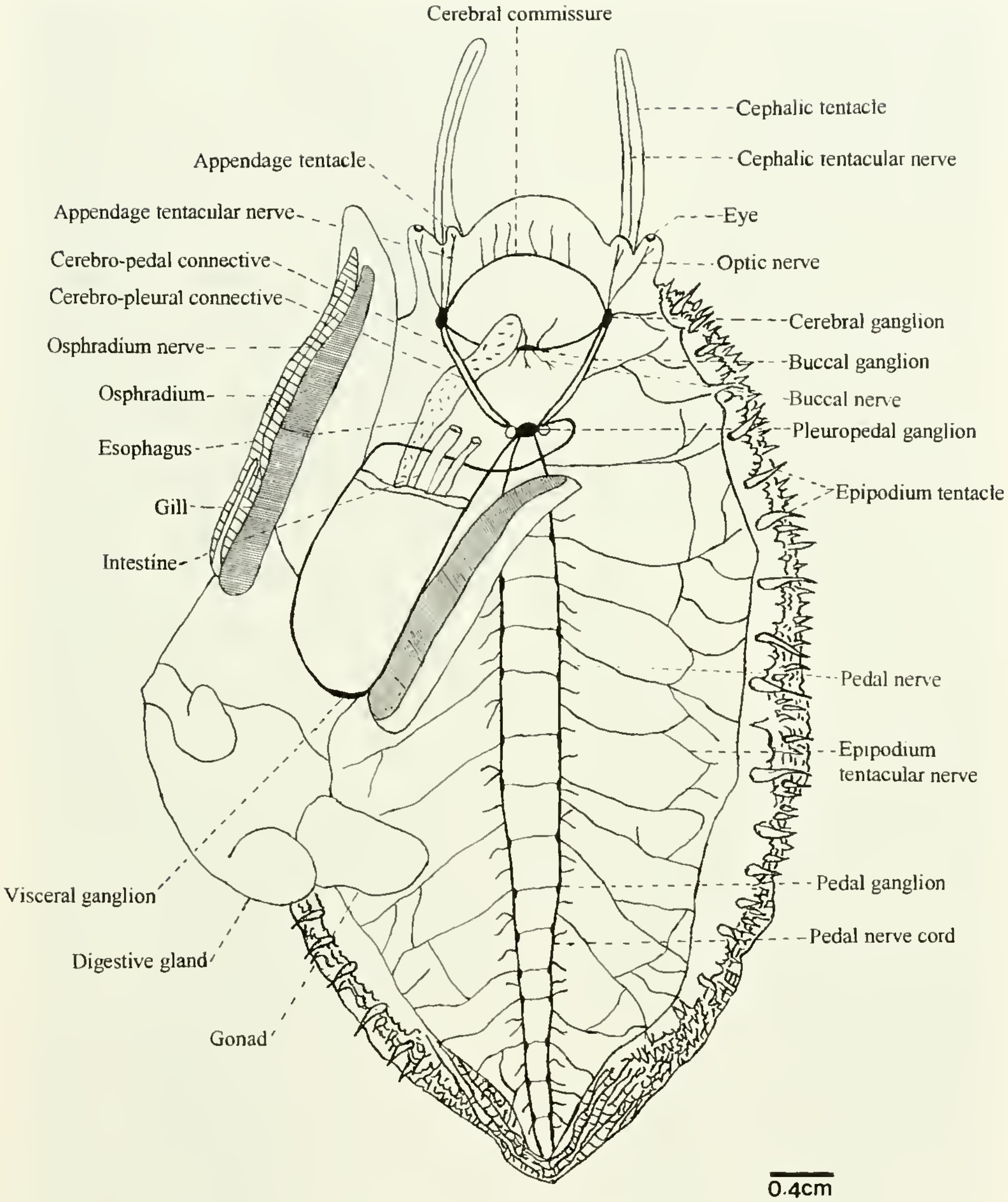
Cerebral commissure
Cephalic tentacle
Cephalic tentacular nerve
Appendage tentacle
Appendage tentacular nerve
Eye
Optic nerve
Cerebro-pedal connective
Cerebro-pleural connective
Cerebral ganglion
Osphradium nerve
Buccal ganglion
Osphradium
Buccal nerve
Esophagus
Pleuropedal ganglion
Gill
Epipodium tentacle
Intestine
Pedal nerve
Epipodium tentacular nerve
Visceral ganglion
Pedal ganglion
Digestive gland
Pedal nerve cord
Gonad
0.4cm

Figure 1. Diagramatic drawing of the gross anatomy of adult abalone showing the ganglia and their peripheral nerves (modified from Crofts, 1929).

At 11 months, the ganglia increased in size to 589 × 2508 × 470 μm; the ventral and dorsal horns were elongated (Fig. 4D). The number of cell layers in the cortex increased. The number of NS cells was about 60 cells per section (Table 2). NR cells and NG cells were distributed in all areas; their numbers increased with increasing age. At 12 months, the pleuropedal ganglia (589 × 2543 × 500 μm in size) were fully developed and appeared similar to those of the adult abalone (Fig. 2B).

Visceral Ganglia

In 1-month-old abalone, the visceral ganglion was as small as 37 × 72 × 30 μm and bean-shaped (Fig. 5A). The cortex had only one layer of cells (Table 3). NS cells and NR_1 cells had not yet appeared. In contrast, the remaining types of NR (NR_{2-4}) were present but still few in number (Table 3). All types of NG were observed.

From 3 months onwards, the ganglion was dumbbell-shaped (Figs. 5B–5D) and its size increased with increasing age (Table 3). The cortex was thicker, especially the lateral part. NS_1 cells first appeared in 2-month-old abalone, and their number was about 1–2 cells per section (Table 3). They were present in the left lateral, left latero-dorsal and left latero-ventral parts. There were all types of NR, but NR_1 and NR_4 were rarely observed.

At 3–10 months, the visceral ganglion increased in size from 118 × 488 × 50 μm to about 160 × 770 × 110 μm (Fig. 5B). The number of cell layers in the cortex increased. The number of NS cells increased to about 20 cells per section, and they were distributed in the right lateral part (Table 3). NR cells were similar in number and distribution to those at 3 months (Table 3).

At 11 months, the ganglion (160 × 889 × 110 μm) increased in length, but still had a similar width to that of 10-month old abalone (Fig. 5C). NS cells (mostly NS_1) increased in number and were

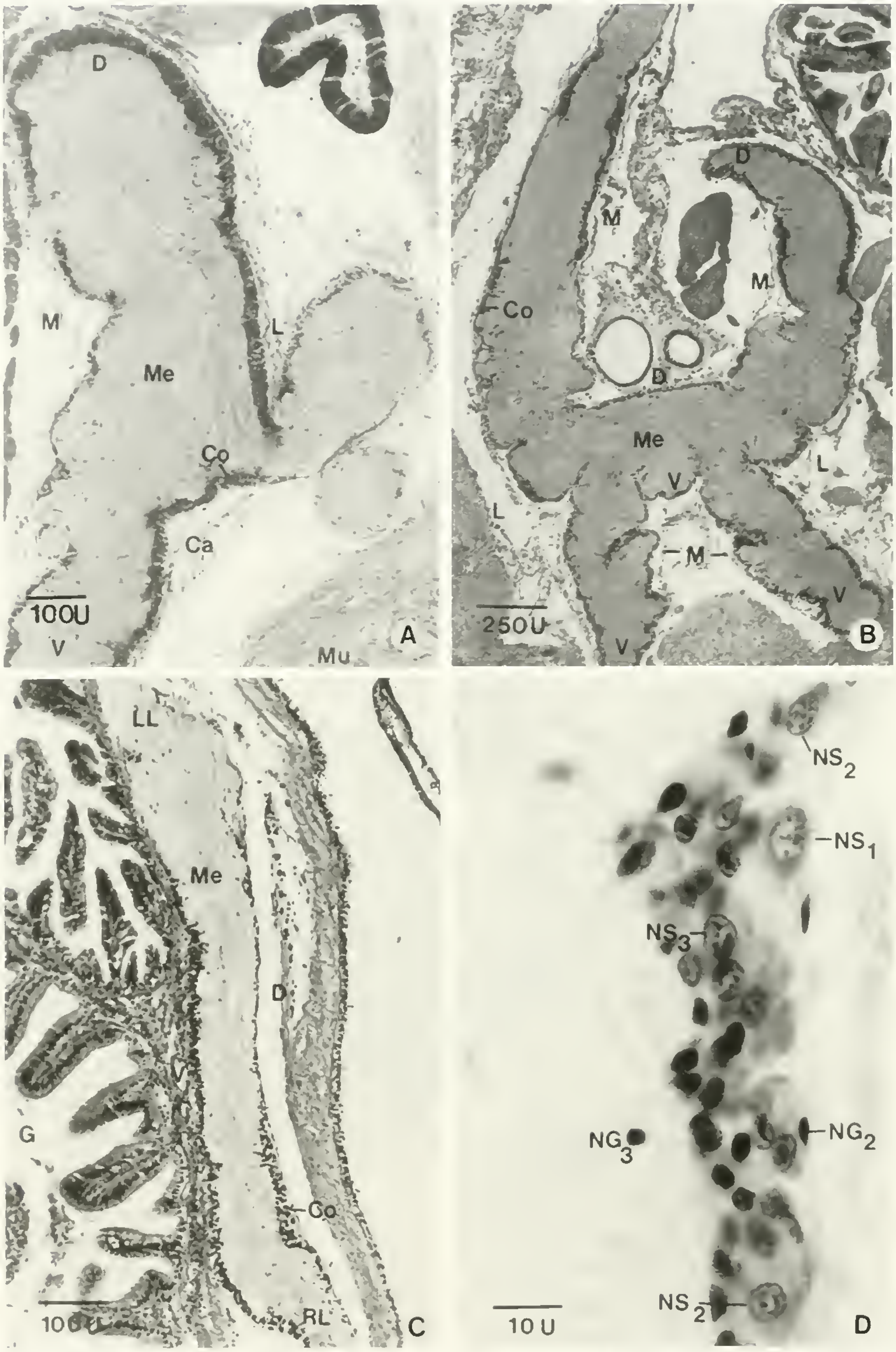

Figure 2. Photomicrographs of the frontal sections of ganglia of *H. asinina.* (A) A low-power micrograph of a cerebral ganglion, showing thick cell layers on the ventral (V) and dorsal (D) sides. Ca-capillary, Co-cortex, L-lateral, M-medial, Me-medulla, Mu-muscle. (B) A low-power micrograph of a pleuropedal ganglion showing thick cell layers on the dorsal (D) and lateral (L) sides. Co-cortex, M-medial, Me-medulla, V-ventral. (C) A low-power micrograph of a visceral ganglion showing thick cell layers on the ventral side. LL-left lateral, RL-right lateral, G-gill, Co-cortex, Me-medulla. (D) A high-power micrograph of a pleuropedal ganglion showing various types of nerve cells in the cortex region. NG_2-type 2 neuroglia, NG_3-type 3 neuroglia, NS_1-type 1 neurosecretory cell, NS_2-type 2 neurosecretory cell, NS_3-type 3 neurosecretory cell.

distributed in all areas of the ganglia (Table 3), but were concentrated mostly in the right lateral part (Fig. 5D). At 12 months, the ganglion (165 × 939 × 150 μm in size) appeared similar to those of adult abalone (Fig. 2C).

DISCUSSION

During postembryonic development of the nervous system of gastropods, the nerve cells and neurosecretory cells increase in size

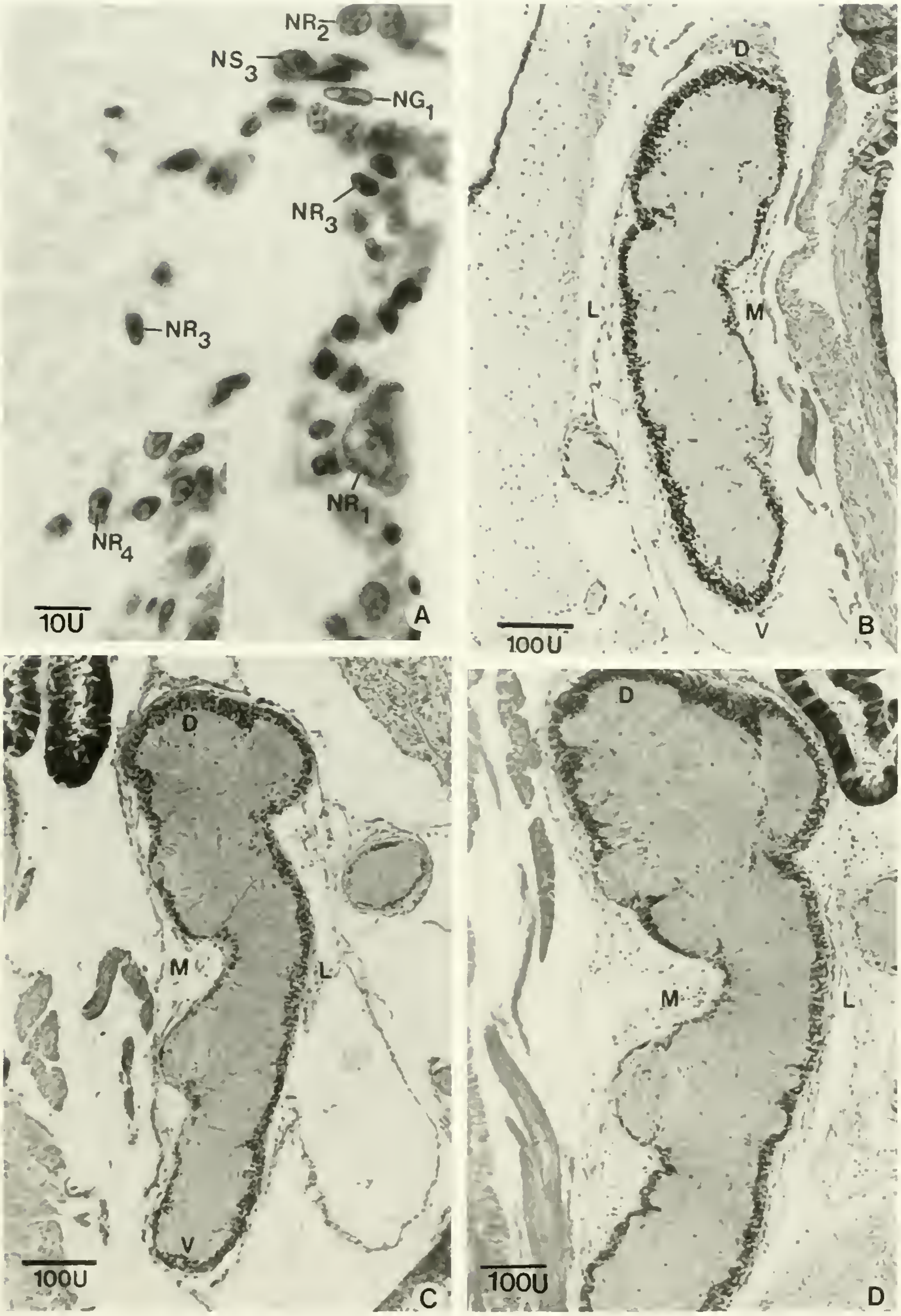

Figure 3. (A) High-power micrograph of cerebral ganglia showing type 1 neuroglia (NG_1), types 1-4 neurons (NR_{1-4}) and type 3 neurosecretory cell (NS_3). (B) Frontal sections of cerebral ganglia in 1- to 4-month-old abalone that appeared bean-shaped. (C) In 5- to 10-month-old abalone, the ganglion appeared as a sickle shape. (D) In 11-month-old abalone, the ganglion greatly increased in size. D-dorsal, L-lateral, M-medial, V-ventral

and number (Bullock & Horridge 1995), for example, the neuroendocrine cells of *L. stagnalis* increase in number and size with increasing shell length (Roubos et al. 1988). In addition, Lever et al. (1965) showed the same result in the cerebral and parietal ganglia of *Biomphalaria glabrata* (Say). Similarly, Kruatrachue et al. (1994) reported that the number and size of neurosecretory cells in the cerebral ganglia of *A. fulica* increased with increasing age. A similar trend was observed in *H. asinina* in the present study. Furthermore, our histological study indicates that the cerebral, pleuropedal and visceral ganglia appeared as definite organs with specific shapes in 1-month-old abalone. Later there were changes in the size but not so much shape of these ganglia; and the numbers of neurons and neurosecretory cells in all ganglia markedly increased with increasing age.

TABLE 1.

Key events during the development of a cerebral ganglion in *H. asinina*.

Month	Shape	Size (μm) W × L × T	Average Number of Cell Layers				Average Number of NS/ Section	Location of NS	Relative Number of NS[a]			CH-P	PF	Relative Number of NR[b]				Location of NR$_1$
			Ventral (V)	Dorsal (D)	Medial (M)	Lateral (L)			NS$_1$	NS$_2$	NS$_3$			NR$_1$	NR$_2$	NR$_3$	NR$_4$	
1	Bean shape	121 × 471 × 100	4	3	0–1	2–3	1–2	D	+	–	–	–	–	+	++++	++	++	D
2	Bean shape	182 × 607 × 100	5	4	0–3	1–3	2–5	D,DL,DM	+	–	–	–	–	+	++++	++	++	D,DM
3	Bean shape	182 × 644 × 110	5	4	0–3	1–3	2–5	D,DL,DM, V,VM	+	+	–	–	–	+	++++	++	++	D,DM,V
4	Bean shape	184 × 648 × 150	6	7	0–3	2–4	10	D,DL,DM, V,VM	+	+	+	–	–	+	++++	+++	++	D,DM,V
5	Sickle shape	203 × 632 × 200	6	7–8	0–3	1–4	20	In all areas	++	++	+	+	+	++	++++	+++	++	D,DM,DL,VV M,VL
6	Sickle shape	203 × 637 × 213	4–5	6–8	0–3	1–3	20	In all areas	++	++	+	+	+	++	++++	+++	++	Same
7	Sickle shape	206 × 808 × 220	4–5	5–7	1–2	1–3	20	In all areas	++	++	+	+	+	++	++++	+++	++	Same
8	Sickle shape	273 × 735 × 240	4–5	5–7	0–3	1–5	20	In all areas	++	++	+	+	+	++	++++	+++	++	Same
9	Sickle shape	286 × 690 × 250	4–5	5–7	0–3	1–5	20	In all areas	++	++	+	+	+	++	++++	+++	++	Same
10	Sickle shape	294 × 787 × 280	4	6	0–3	1–4	30	In all areas	+++	+++	++	+	+	+++	++++	++++	++	Same
11	Sickle shape	377 × 810 × 300	4	5–7	1–3	1–4	30–40	In all areas	+++	+++	++	+	+	+++	++++	++++	++	Same
12	Highly elongated, convoluted	377 × 901 × 325	4–6	5–7	1–3	1–5	30–40	In all areas	+++	+++	++	+	+	+++	++++	++++	++	Same

[a] (–) = 0 cell/section, (+) = 1–5 cells/section, (++) = 6–10 cells/section, (+++) = 11–15 cells/section, (++++) = >15 cells/section

[b] (–) = 0 cell/section, (+) = 1–10 cells/section, (++) = 11–20 cells/section, (+++) = 21–30 cells/section, (++++) = >30 cells/section

n = 10 animals per month; 100 sections per ganglion

W = width; L = length; T = thickness

(CH-P = chrome-hematoxylin-phloxine, PF = paraldehyde-fuchsin, NR = neuron, NS = neurosecretory cell)

TABLE 2.

Key events during the development of a pleuropedal ganglion in *H. asinina.*

Month	Shape	Size (μm) W × L	Average Number of Cell Layers				Average Number of NS/Section	Location of NS	Relative Number of NS[a]			CH-P	PF	Relative Number of NR[b]				Location of NR_1
			Ventral (V)	Dorsal (D)	Medial (M)	Lateral (L)			NS_1	NS_2	NS_3			NR_1	NR_2	NR_3	NR_4	
1	Butterfly shape	189 × 418 × 150	2–4	0–3	1–3	0–5	1–2	DS	+	–	–	–	–	+	+++	++	++	DM
2	Butterfly shape	273 × 497 × 155	2–4	1–5	1–2	1–5	2–3	DS,DM,LS	+	+	–	+	–	+	+++	++	++	DM
3	Butterfly shape	289 × 522 × 170	4	1–5	1–3	2–4	2–3	DS,DM,LS	+	+	–	+	–	+	+++	++	++	DM,DL,D
4	Butterfly shape	337 × 556 × 200	5	2–5	2–3	2–7	20	DS,DM,LS,DL, VL	++	+	+	+	+	++	+++	+++	++	DM,DL,D,V M,VL,VS
5	Butterfly shape	455 × 572 × 230	5	2–5	2–3	1–7	20	DS,DM,LS,DL, VL,V	++	++	+	+	+	++	++++	+++	++	Same
6	Butterfly shape	488 × 707 × 250	5	2–5	2–3	1–7	20	DS,DM,LS,DL, VL,V,D	++	++	+	+	+	++	++++	+++	++	Same
7	H shape	544 × 1615 × 315	4–6	2–5	5	3–10	30–40	In all areas	+++	++	++	+	+	+++	++++	++++	++	Same
8	H shape	546 × 1652 × 340	3–5	5	2–3	4–7	30–40	In all areas	+++	++	++	+	+	++	++++	++++	++	Same
9	H shape	572 × 1661 × 390	4–5	2–5	3–4	1–8	30–40	In all areas	+++	+++	++	+	+	++	++++	++++	++	Same
10	H shape	580 × 1700 × 430	4–5	2–5	3–4	2–7	30–50	In all areas	++++	+++	++	+	+	+++	++++	++++	++	Same
11	Highly elongated, enlarged	589 × 2508 × 470	5–6	4–6	5	3–10	60	In all areas	++++	++++	+++	+	+	+++	++++	++++	+++	In all areas
12	H shape, similar	589 × 2543 × 500	5	5–6	3–4	7	60	In all areas	++++	++++	+++	+	+	+++	++++	++++	+++	In all areas

[a] (–) = 0 cell/section, (+) = 1–5 cells/section, (++) = 6–10 cells/section, (+++) = 11–15 cells/section, (++++) = >15 cells/section

[b] (–) = 0 cell/section, (+) = 1–10 cells/section, (++) = 11–20 cells/section, (+++) = 21–30 cells/section, (++++) = >30 cells/section

n = 10 animals per month; 100 sections per ganglion

W = width; L = length; T = thickness

CH-P = chrome-hematoxylin-phloxine, PF = paradehyde-fuchsin, NR = neuron, NS = neurosecretory cell

TABLE 3.

Key events during the development of a visceral ganglion in *H. asinina*.

Month	Shape	Size (μm) W × L × T	Average Number of Cell Layers				Average Number of NS/ Section	Location of NS	Relative Number of NS[a]			CH-P	PF	Relative Number of NR[b]				Location of NR$_1$
			Ventral (V)	Dorsal (D)	Medial (M)	Lateral (L)			NS$_1$	NS$_2$	NS$_3$			NR$_1$	NR$_2$	NR$_3$	NR$_4$	
1	Bean shape	37 × 72 × 30	1	0–1	1	1–2	–	–	–	–	–	–	–	–	++	++	+	–
2	Bean shape	48 × 74 × 35	1–2	1–2	1	1–3	1–2	LL	+	–	–	–	–	+	++	++	+	LL,LLV
3	Dumbbell shape	118 × 488 × 50	1–2	1–2	1	1–4	2–3	LL,LLV,LLD	+	+	–	–	–	+	++	++	+	LL,LLV
4	Dumbbell shape	123 × 494 × 55	1–2	1–2	1	2	10	LL,LLV,LLD	+	+	–	–	–	+	++	++	+	LL,LLV, LLD
5	Dumbbell shape	151 × 607 × 65	1–2	1–2	1	1–3	10	LL,LLV,LLD, RL	++	+	+	–	–	+	++	++	+	LL,LLV, LLD,RL
6	Dumbbell shape	155 × 923 × 65	1–3	1–2	1–2	1–3	10	Same	++	+	+	+	+	++	++	++	+	Same
7	Dumbbell shape	168 × 640 × 80	1–3	1–2	1–2	1–3	10	Same	++	+	+	+	+	++	++	++	+	Same
8	Dumbbell shape	161 × 640 × 90	1–3	1–2	1	1–3	10	Same	++	+	+	+	+	++	++	++	+	Same
9	Dumbbell shape	160 × 700 × 110	1–3	1–2	1–2	1–3	10	In all areas	++	+	+	+	+	++	+++	++	+	Same
10	Dumbbell shape	160 × 770 × 110	1–3	1–2	1–2	1–3	20	In all areas	+++	+	+	+	+	++	+++	++	+	Same
11	Dumbbell shape	160 × 889 × 110	1–3	1–2	1–2	1–3	20	In all areas	+++	+	+	+	+	++	++++	+++	++	In all areas
12	Dumbbell shape	165 × 939 × 150	1–3	1–2	1–2	1–3	20	In all areas	+++	++	+	+	+	++	++++	+++	++	In all areas

[a] (–) = 0 cell/section, (+) = 1–5 cells/section, (++) = 6–10 cells/section, (+++) = 11–15 cells/section, (++++) = >15 cells/section

[b] (–) = 0 cell/section, (+) = 1–10 cells/section, (++) = 11–20 cells/section, (+++) = 21–30 cells/section, (++++) = >30 cells/section

n = 10 animals per month; 100 sections per ganglion

W = width; L = length; T = thickness

CH-P = chrome-hematoxylin-phloxine, PF = paradelhyde-fuchsin, NR = neuron, NS = neurosecretory cell

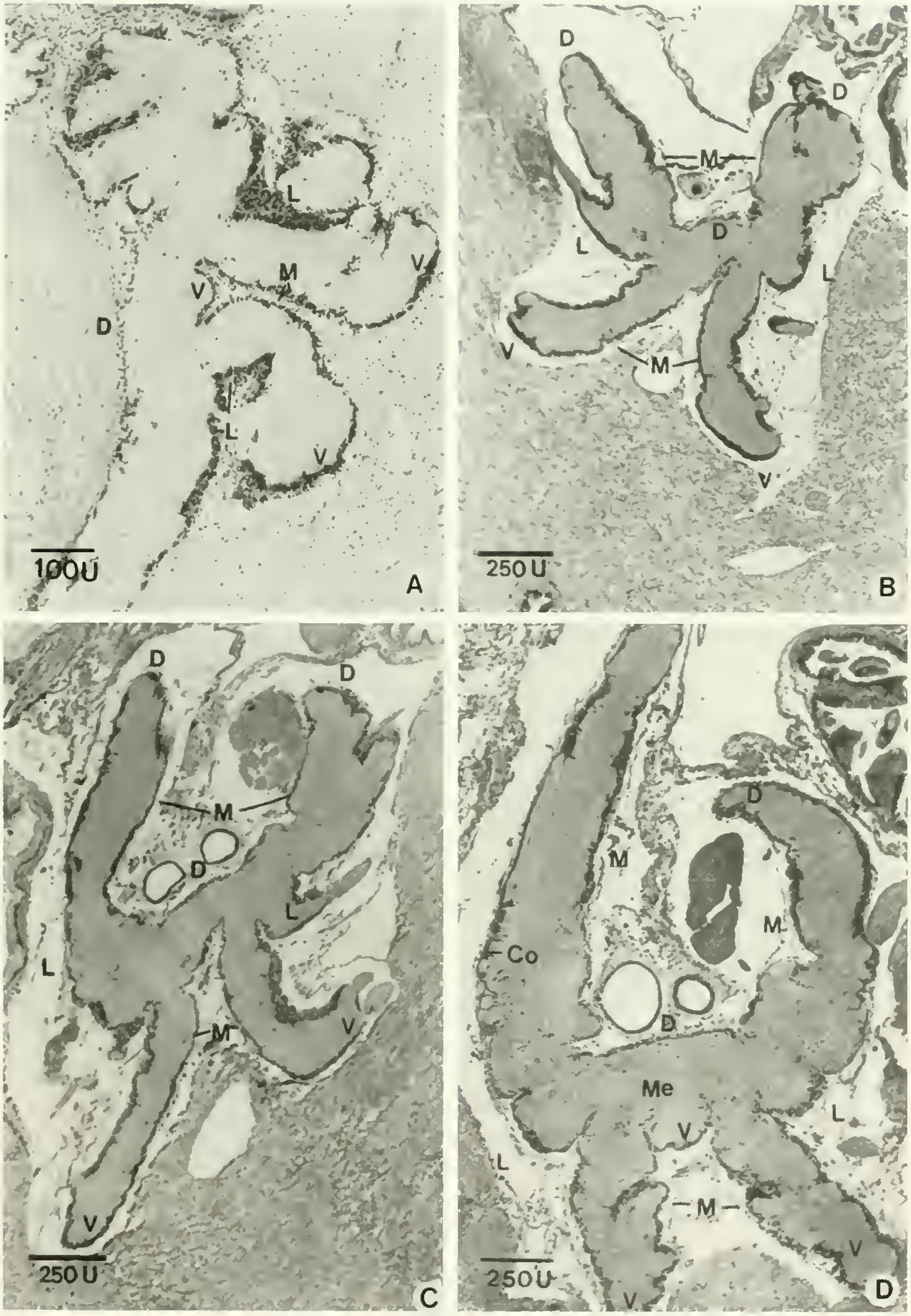

Figure 4. Low-power micrographs of frontal sections of pleuropedal ganglia. (A) In 1- to 2-month-old abalone, the pleuropedal ganglion had a butterfly shape. (B) In 3- to 6-month-old abalone, the ganglion still had a butterfly shape. (C) In 7- to 10-month-old abalone, the ganglion had an H shape. (D) In 11-month-old abalone, the ganglion was larger in size but still had an H shape. Co-cortex, D-dorsal, L-lateral, M-medial, Me-medulla, V-ventral

There have been many reports on the functions of neurosecretory cells in the cerebral ganglia of snails. It has been found that some snails have factors that stimulate growth rate (Geraerts & Algera 1976) and shell regeneration (Dillaman et al. 1976). Thongkukiatkul et al. (1998) reported that neurosecretory cells in the cerebral ganglia of *H. asinina* were positively stained with anti-human GH and anti-human insulin. In this study, it was observed that the neurosecretory cells in the cerebral ganglia first appeared in 1-month-old abalone and increased in number with increasing age. A large number of neurosecretory cells were found in 5- and 10-month-old abalone that are assumed to be juvenile and pre-adult stages, respectively. They formed maximum numbers that were observed and appeared adult-like in 12-month-old abalone that reached the adult stage. It seems, therefore, that the increase in the number of NS cells is correlated with the increase in abalone growth.

Similar studies of other species of gastropods suggest that the number and staining properties of neuroendocrine cells in ga-

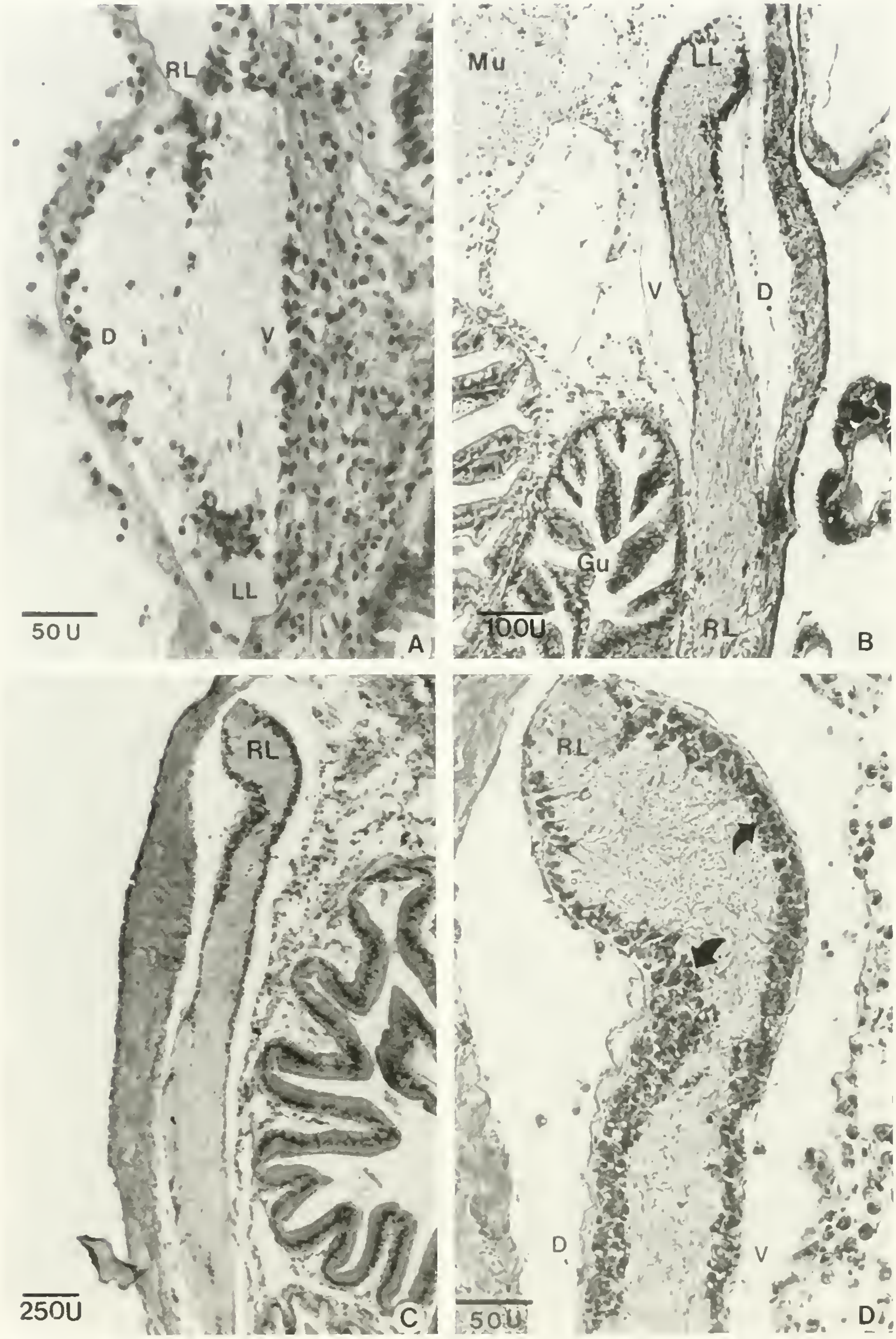

Figure 5. Frontal sections of visceral ganglia. (A) In 1- to 2-month-old abalone, the visceral ganglion was small and had a bean shape. (B) In 3- to-10-month-old abalone, the ganglion was large and assumed a dumbbell shape. (C) In 11-month-old abalone, the ganglion increased only in length. (D) High magnification of (C) showing thick cortex containing all types of nerve cells (arrows). D-dorsal, Gu-gut, LL- left lateral, RL-right lateral, Mu-muscle, V-ventral

ropedal and visceral ganglia are related to the gonadal maturation (Coggeshall 1967; Dogterom et al. 1983; Van Minnen & Sokolove 1984; Smith 1967). In our study it was observed that the neurosecretory cells in the pleuropedal and visceral ganglia first appeared in 1-month old-abalone. The number of neurosecretory cells in the pleuropedal ganglia increased in 4- and 7-month-old abalone, while in the visceral ganglia it increased in 4-month-old abalone. They reached a maximum number in 11-month-old abalone. The development of these neurosecretory cells may correlate with the development of the reproductive organs. Thongkukiatkul et al. (1998) reported that neurosecretory cells in the pleuropedal and visceral ganglia of *H. asinina* were stained by anti-human LH, while only those in the pleuropedal ganglion were stained by anti-human FSH.

It was reported that early spermatocytes and spermatids of *H. asinina* appeared at 4 months, and early oocytes (OC_{1-2}) at 6–7 months (Sobhon et al. 1999), while fully mature spermatozoa appeared in the gonads as early as 6–7 months. At this age, there were already a large number of neurosecretory cells in the visceral and pleuropedal ganglia of the abalone. Moreover, Sobhon et al. (1999) showed that a large number of mature oocytes of the reproductive cycle of *H. asinina* occurred at 10 to 11 months, the age at which the neurosecretory cells in the pleuropedal and visceral ganglia reached a maximum number and appeared adult-like. Our observations were supported by Yahata (1973) who demonstrated that the pleuropedal and visceral ganglia might produce and release factors that could induce spawning.

In the present study, the number of giant neurons (NR_1) increased following the development of the ganglia. In the cerebral ganglia, they increased in number in 5- and 10-month-old abalone that were assumed to be the juvenile and pre-adult stages. They reached a maximum number and appeared adult-like in 12-month-old abalone. NR_1 first appeared in the dorsal horn of the pleuropedal ganglia, later they were regularly found in both dorsal and ventral horns. Thus, NR_1 may proliferate relatively later than the other types of neurons (NR_{2-4}) that are abundant at an early age. This may be related to active movement due to muscular activity exhibited by abalone, as they become older.

NR_1 were more abundant in the pleuropedal ganglia than in the cerebral ganglia of *H. asinina* (Upatham et al. 1998). These cells are very large, multipolar and pyramidal in shape. Compared with the classification of neurons in the nervous system of higher vertebrates, NR_1 are similar to such large motor cells as the ventral horn motor cells of the spinal cord and Purkinje cells of the cerebellum in vertebrates. As such, they may be involved in controlling and coordinating motor activities, especially that of the pedal muscle.

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INDUCTION OF SPAWNING AND EARLY DEVELOPMENT IN *Fissurella picta* (MOLLUSCA: ARCHAEOGASTROPODA) FROM SOUTHERN CHILE

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ABSTRACT Specimens of *Fissurella picta* were collected from the rocky intertidal coast near Valdivia, southern Chile, and used for studies of spawning. Males responded to artificial stimuli more frequently than females. Potassium chloride was the most effective agent for inducing spawning, whether injected into the mantle cavity, added to the seawater, or a gonad macerate, or combined with a period of exposure to air. Injection of hydrogen peroxide or addition of prostaglandin to ultraviolet-irradiated seawater in the experimental containers also induced males and females to spawn. Oocytes of *F. picta* (mean diameter 178 μm) were surrounded by two membranes, the vitelline and albumen membranes, and a gelatinous outer coat, which was lost a few minutes after spawning. The first polar body was released 15–20 minutes after fertilization, and various cleavage stages were observed during the following 7 hours. Trochophore larvae were observed after 15 hours and early veligers 21 hours after fertilization (temperature 17.5 ± 1°C, salinity 29–30‰). The hatched veliger larva began torsion and a free-swimming mode of life after 40 hours. The results showed the possibility to obtain the larval development in *F. picta* under laboratory conditions, providing a significant progress for its future culture.

KEY WORDS: development, spawning, gastropod, *Fissurella*, induction, reproduction

INTRODUCTION

Species belonging to the genus *Fissurella* (Bruguière 1789), known locally as "lapas", sustain a multispecies artisanal fishery in Chile. In spite of the economic importance of this resource, there is very little published information about the biology of the genus. During the last years, research on *Fissurella* species in Chile has been directed to establish the scientific and technological foundations to culture them, by studying their reproductive biology and embryo-larval development. In this sense, trials of gamete release under laboratory conditions have been conducted in view of culture. Spawning induction in *F. cumingi* (Vega & Osorio 1995; Vega et al. 1996) has had relative success in males, by using low concentrations of hydrogen peroxide. Huaquín et al. (1998) obtained gamete emission in *F. crassa* by intravisceral injection of KCl. The spawning induction methods used in *F. picta* (thermal, electric, and osmotic shock) have not produced positive results (González et al. 1999). Considering the ecological (Moreno & Jaramillo 1983) and economic (Bretos 1988) importance of *F. picta* at southern Chile, the objective of this study is to obtain gametes from *F. picta* Gmelin (1791) by various spawning induction techniques, and to describe embryonic and larval development in this species, in a population from southern Chile. Such information will greatly facilitate attempts to grow this species in culture, and contribute to efficient management of aquaculture sites in the future.

MATERIALS AND METHODS

A total of 451 specimens of *Fissurella picta* were collected from a wild population from the rocky intertidal shore at Playa Rosada and La Misión, near Valdivia (39°48′S, 73°24′W; Fig. 1), when gonads were fully mature, between October 1996 and December 1997. Only individuals of shell length greater than 40 mm were taken, to ensure that most of them were sexually mature (Bretos et al. 1988).

In the laboratory the shells were scrubbed and thoroughly washed with filtered seawater to remove epibionts, and the limpets were transferred to 30-liter plastic tanks containing continuously aerated filtered seawater (0.45 μm). The tanks were maintained under ambient conditions of photoperiod, temperature (10–20°C) and salinity (29–30‰), according to the season. In order to keep the temperature constant, the plastic tanks were placed in a 500-liter fiberglass container filled with circulating, temperature-controlled freshwater.

The experimental animals were fed ad libitum with the algae *Macrocystis pyrifera* (Linnaeus) C. Agardh 1820 and *Ulva lactuca* Linnaeus 1771, collected from the same place as the limpets. These algae are known to support growth in *F. picta* (Castro & Iglesias 1995). Occasionally *Mazzaella laminarioides* (Bory) Fredericq 1993, was also added, because it forms part of the natural diet of the limpet.

In order to determine the sex of the specimens of *F. picta*, a sample (0.1 ml) of gonad tissue was removed by aspiration through an intravisceral-pedal puncture. The sex of the individual is easily established from the color of the gonad tissue (Bretos et al. 1983).

The stage of gamete development was determined by optical microscopy and characterized as follows: oocytes mature or undergoing vitellogenesis, presence of gelatinous coat, amount of yolk, motility and appearance of sperms. The diameter (μm) of the oocytes was measured with an ocular micrometer (Carl Zeiss) in 4–7 females taken at random from each sample within the size range collected. The counting of sperms and estimation of motility was done with a blood-cell counting camera (Malassez).

Induction of Spawning

Limpets were maintained for 7 days in the laboratory, without food for 24 hours before the following stimuli were applied:

Temperature Stimuli

The animals were submitted to water temperatures from 10–24°C in increments of 3°C (12-15-18-21°C) each 15–20 min.

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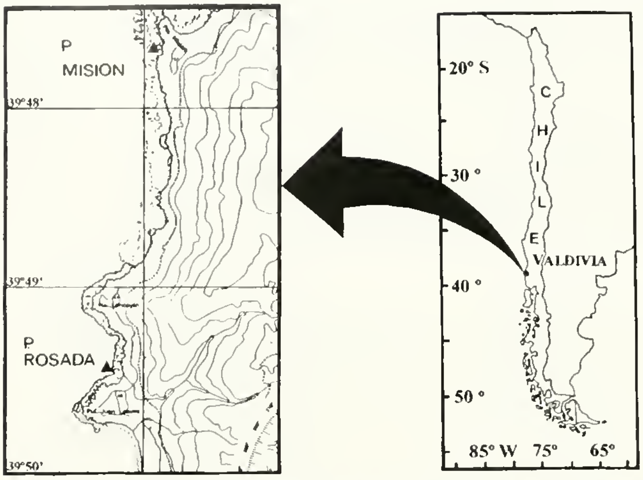

Figure 1. Map of Valdivia region, showing the locations of the two sites used to collect *Fissurella picta* during this study.

Chemical Stimuli

(a) Potassium chloride: an intravisceral-pedal injection (0.5 ml of 0.5, 10, 20, and 100 mM), or added to the seawater (concentration 10 mM).
(b) Hydrogen peroxide: an intravisceral-pedal injection (0.5 ml of a 50 mM solution) or added to the seawater (concentration 8 mM).
(c) Dopamine: an intravisceral-pedal injection (0.5 ml of a 2-mM solution).
(d) Dopamine plus hydrogen peroxide: added to the seawater (concentrations 2 mM and 8 mM, respectively).
(e) Dopamine plus seawater treated with UV radiation: added to the seawater (concentration 1 and 2 mM; irradiation period 40 minutes).
(f) Synthetic prostaglandin (α postrol, PG): an intravisceral-pedal injection (0.5 ml of a 0.04 mg/ml solution).
(g) Synthetic prostaglandin plus seawater irradiated with UV light: added to the seawater (concentration 0.04 mg/ml; irradiation period 40 minutes).

Physical Stimulation

The animals were exposed to air for 15–20 minutes.

Other Stimuli

A combination of chemical and physical stimulation was used, together with the addition of macerated gonad tissue to the seawater. Stimuli for the induction of spawning were applied to groups of 4 to 10 limpets, males and females separately, each group being maintained in 4 liters of filtered seawater gently circulated (0.5 l/min).

Any limpet failing to respond to a spawning stimulus within two or three days was replaced. The nonparametric Wilcoxon test ($P <$ level 0.05) was used to compare responses of male and female limpets to the various spawning stimuli, using the program Statistica (Windows version 4.2).

Fertilization and Development

After the initiation of spawning, individual limpets were placed in small containers with 1 liter filtered seawater. Triplicate samples of 1 ml were taken from each suspension of gametes. Oocytes were

TABLE 1.

Effect of different inductors on spawnings in *Fissurella picta*.

Inductor	No of Individuals	No of Trials	No Successful Trials with Males	No Successful Trials with Females	Date
Temperature Stimuli					
Temperature (10–24°C)	78	9	2	0	Oct 1996, Dec 1996, Feb 1997
Chemical Stimuli					
KCl (injection)					
0.5 mM	22	3	0	0	Feb 1997, Apr 1997
10 mM	40	5	3	1	Oct to Dec 1996, Oct 1997
20 mM	16	2	0	0	Oct 1997
100 mM	16	2	0	0	Oct 1997
H_2O_2					Dec 1996, Oct Nov Dec 1997
8 mM (seawater)	96	12	1	1	Jan to Apr 1997
50 mM (injection)	16	2	1	1	Nov 1997
Dopamine 2 mM (injection)	9	1	0	0	Nov 1997
Dopamine + H_2O_2 (seawater)	5	1	0	0	Nov 1997
Dopamine + seawater treated with UV	26	4	1	0	Nov 1997
PG (injection)	24	3	1	0	Apr to Nov 1997
PG (injection) + seawater treated with UV	32	4	0	0	Feb to Nov 1997
PG (seawater) + seawater treated with UV	24	3	1	1	Nov 1997
Other stimuli					
KCl (seawater) + macerated gonad	24	3	2	1	Nov 1997
KCl (seawater) + exposed to air	8	1	1	0	Nov 1997
Seawater treated with UV	15	2	0	0	Dec 1996, Oct to Nov 1997
TOTAL	**451**	**57**	**13**	**5**	

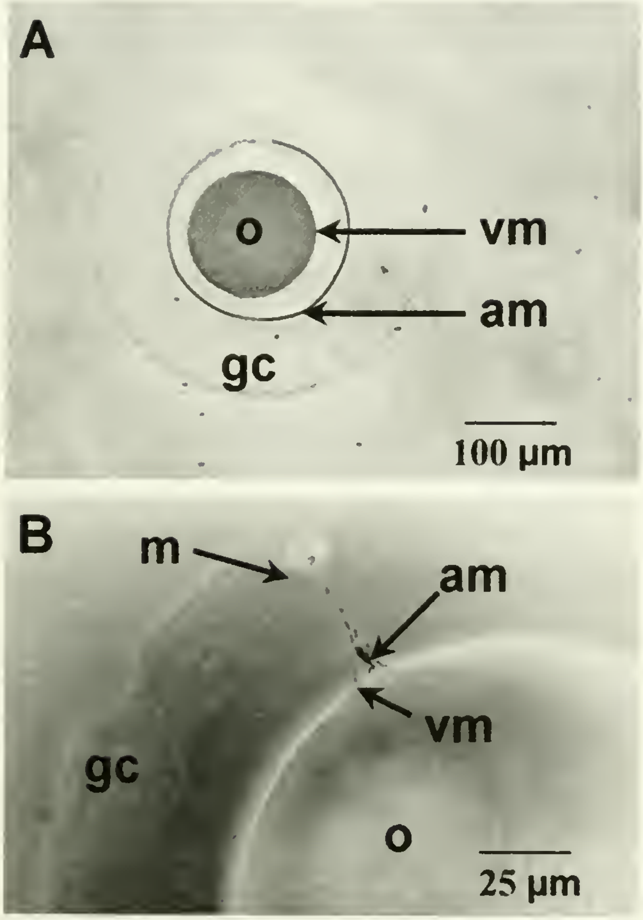

Figure 2. A. Oocyte in vivo of *Fissurella picta* with its membranes, at the light microscope. am: albumen membrane; gc: gelatinous coat; o: oocyte; vm: vitelline membrane. B. "Micropyle" inside the gelatinous coat of the oocyte of *Fissurella picta*. am: albumen membrane; m: micropyle; o: oocyte; vm: vitelline membrane; gc: gelatinous coat.

counted under a stereomicroscope, a mean value obtained, and the total number of gametes calculated. Sperms were counted with a Malassez camera.

Sub-samples of oocytes taken during spawning were fixed in 3% glutaraldehyde, washed in phosphate buffer and distilled water, and mounted under cover slips for examination and photomicrography (Zeiss Axiomat microscope).

The remaining oocytes were screened (150 μm mesh), washed several times in sterile filtered seawater and maintained in the 1-liter containers to await fertilization. The sperm suspensions were also screened (45 μm) before use to get fertilization, gamete suspensions from individual limpets were used in ratio 10–15 sperms per oocyte.

After fertilization had been confirmed by the appearance of the first polar body, each batch of eggs was washed several times in sterile seawater to remove the excess of sperms, then transferred to a 2 liter container of sterilized filtered (0.45 μm) seawater and kept in darkness with constant aeration. Every 24 hours afterwards, two thirds of the water was replaced. Temperature was maintained at 17.5°C ± 1°C and salinity at 29–30‰. Replicate samples were taken every 15 minutes for the first 3 hours after fertilization, every 30 minutes from 3 to 24 hours post-fertilization, and every hour thereafter until the liberation of the larvae.

The embryos and larvae were screened with different mesh sizes appropriate for their size. The samples were examined under a light microscope (Olympus) and obtained from a camera (Samsung NF-E80SN) which replaced one of the eyepieces of the microscope. Selected images were captured (ATI program), digitized (BMP format) and processed by image analysis (Scion Image PC).

The criterion for determining the stage of development for any given sample was that at least 60% of the individuals should have attained the stage in question.

RESULTS

Induction of Spawning

Data from the 57 spawning induction trials, undertaken principally in October and November 1997 are presented in Table 1. More males responded than females (Wilcoxon test; $N = 16$; $Z = 2.201$; $P < 0.05$). Potassium chloride was the most effective inducing agent (10 mM), whether administered by injection, added to the medium with macerated gonad tissue, or combined with air exposure. A combination of UV-irradiated seawater with either dopamine or prostaglandin (PG) also induced spawning in males.

On one occasion with hydrogen peroxide injection, and on another with PG added to the medium together with UV-irradiation of the seawater, as many males responded as females did. Only once did a male spawn in response to PG. Dopamine was not effective for inducing spawning in males, except when combined with UV-irradiated water.

Characteristics of Gametes

Mature oocytes had two external membranes, the vitelline membrane and the albumen membrane, outside which was a gelatinous coat (Fig. 2A). The gelatinous coat was often lost, but when it was present the micropyle originating from the albumen membrane was clearly visible (Fig. 2B). The albumen membrane, which in Figure 2B had yet to expand, was derived from the oocyte and had the appearance of a clear gelatinous fluid.

Oocyte diameter varied from 117–327 μm (mean 178 μm), but those with a gelatinous coat reached a diameter of 385 μm.

Fertilization and Development

During December 1996, October and November 1997, a total of 10 trials of artificial spawning were successfully completed (Table 2); these were of three types: (a) use of gamete suspensions obtained by dissecting the gonads (OT) of 10 *F. picta* individuals; (b) oocytes from dissected ovaries together with sperm from induced spawning (OI) of 10 animals; and (c) oocytes and sperm both obtained from spontaneous spawning (SS) in 10 specimen.

The spawned oocytes of *F. picta* frequently required several seconds before complete hydration and expansion of the albumen membrane. During this time eggs were observed to be surrounded by large numbers of sperm, and occasionally the sperm head could be seen within the micropyle.

At 17.5°C, fifteen to twenty minutes after fertilization the first polar body was visible, having the appearance of a small, translucent, refringent granule (Fig. 3A). The polar body was located immediately adjacent to the vitelline membrane within the extraembryonic fluid of the albumen membrane of the egg.

After 90 minutes the first two holoblastic segmentation divisions had given rise to four blastomeres of equal size (Fig. 3B,C

The third oblique plane of division resulted in the formation eight blastomeres (Fig. 3D), of which four were very large macromeres, located at the vegetative pole of the embryo and containing yolk reserves, and the remaining four were small micromeres located at the animal pole.

After 4 to 7 hours successive divisions of the blastomeres had given rise to a blastula, a compact, multicellular sphere that gently rotated within the membrane, suggesting the presence of cilia on the external surfaces of the blastomeres. Embryonic development was highly synchronized, but became less so during the larval stages. Fertilized eggs obtained by OT and OI (Table 2) developed only until blastula stage.

The trochophore larva, exhibiting a ring of prototroch cilia, appeared 15 hours after fertilization, and was completely enveloped by the albumen membrane (Fig. 4A). Survivorship was about 50% of fertilized eggs in SS trials (Table 2).

Larvae became an early veliger, turning round within the albumen membrane, 21 hours after fertilization. Forty hours after fertilization, the albumen membrane had disappeared and the veligers had emerged (Fig. 4B) to spend a short period moving through the water column. The pretorsional larva exhibited the protoconch, an expanded velum, the foot, and the rudimentary

TABLE 2.

Fissurella picta. Stages of development obtained in laboratory at 17.5 ± 1°C and 29–30 ‰ salinity.

Fertilization	Stages	Time	Diameter (µm)
OT	Egg	First minutes	180
OT	First polar body	15–20 min.	180
OT	2 Blastomeres	<60 min.	180
OT	4 Blastomeres	80–90 min.	180
OI, SS	4–8 Blastomeres	80–90 min.	180
OI	Blastula	4–7 hours	180
SS	Trochophore	15 hours	180
SS	Early veligers	21 hours	180
SS	Hatched veligers	40 hours	200

OT = Dissected ovary and testicle
OI = Dissected ovary plus sperm from induced spawnings
SS = Spontaneous spawning in females and males.

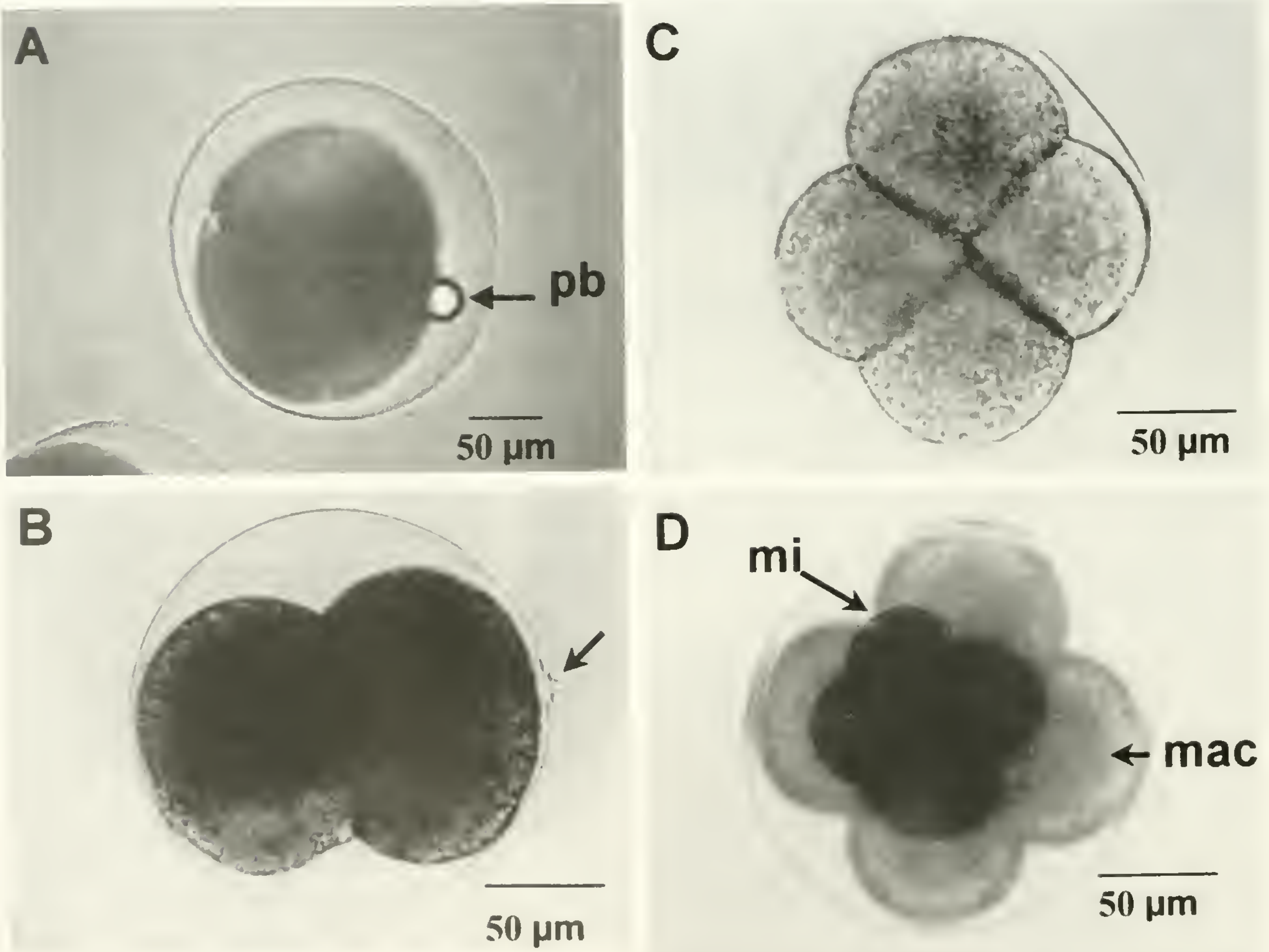

Figure 3. Embryonic development stages of *Fissurella picta* within the albumen membrane. A. Fertilized egg where a polar body (pb) is visible. B. Two-blastomere stage. Arrow shows remnants of micropyle in the albumen membrane. C. Four-blastomere embryo. D. Embryo with unequal eight blastomeres. View from animal pole. mac: macromeres; mi: micromeres.

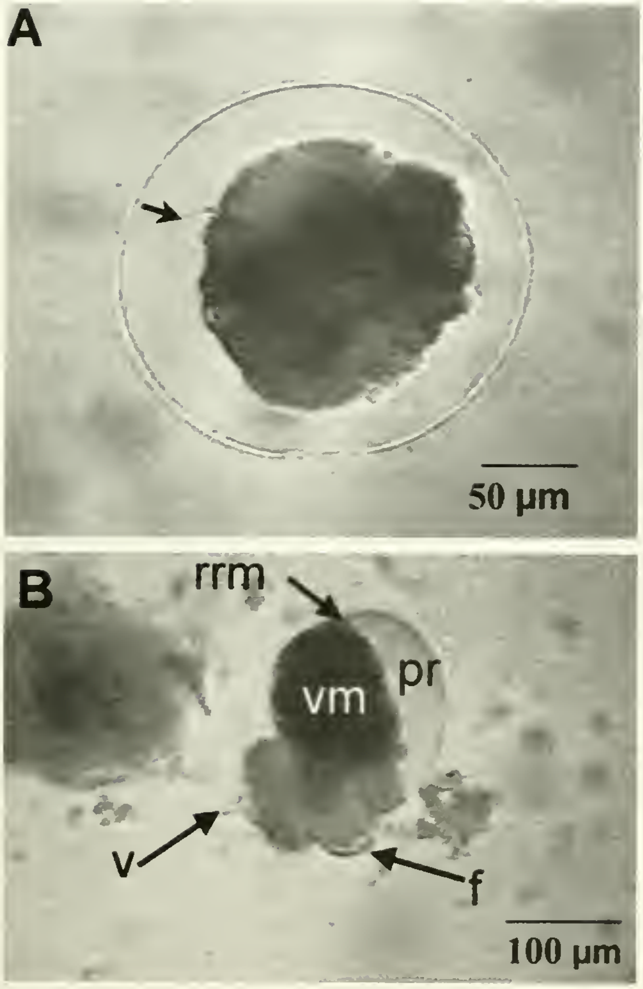

Figure 4. Larval development stages in *Fissurella picta*. A. Trochophore larva. Arrow shows ring of prototrochal cilia. B. Pretorsional hatched veliger larva.f: foot; pr: protoconch; rrm: rudimentary retractor muscle; v: velum; vm: vitelline membrane.

retractor muscle. The visceral mass was well developed and the yolk reserves and velar cilia diminished.

About a 30–40% of embryos obtained by SS trials survived to veliger stage. Development did not continue, and larvae ultimately died.

DISCUSSION

Induction of Spawning

The spawning stimuli used in this study were similar to those used by other authors (Morse et al. 1977; Morse & Morse 1984; Uki & Kikuchi 1984; Pechenik 1986; Martínez et al. 1996) to obtain gametes from various marine invertebrates. In the case of *F. picta*, the maximum maturity condition in the gonads detected in this study during the spring (September to December) would facilitate the spontaneous gamete release to get fertilization and development in a more successful way.

Our results suggest that induction of spawning in *F. picta* was successful, but more effective with males than with females. Similar data have been obtained in previous studies on *F. maxima* and *F. cumingi* (Vega & Osorio 1995). The artificial spawning induction methods tested (thermal, electric, and osmotic shock) by González et al. (1999) in *F. picta* did not produce positive results; this report suggests that a combination of various inductive stimuli should be required to obtain positive responses, as has been shown in the present study.

Potassium chloride injections, either alone or combined with other stimuli (exposure to air, gonad maceration), and hydrogen peroxide induced spawning in *F. picta*. Potassium chloride acts at the cellular level and has been extensively used to induce metamorphosis in marine invertebrate larvae (Yool et al. 1986; Bahamondes-Rojas & Tardy 1988; Bahamondes-Rojas 1990) as well as for the induction of spawning.

Hydrogen peroxide induces spawning in gravid male and female abalones *Haliotis rufescens* and *Nordotis gigantea*, the mechanism possibly being direct stimulation of the enzymatic synthesis of prostaglandin endoperoxidase (Morse et al. 1977; Tanaka 1979; Hahn 1989). Our data support those of Vega et al. (1996), who successfully induced spawning in male *Fissurella cumingi* with low concentrations of hydrogen peroxide (3 mM when added to the medium, and 5 mM when combined with gonad maceration).

It is also known that seawater irradiated with ultraviolet light induces spawning in many mollusks (Kikuchi & Uki 1974; Kagawa & Nagahama 1981), but our data are not in agreement. Nevertheless, we found ultraviolet irradiation to be effective when combined with PG or dopamine, presumably as a result of synergistic action. Moss et al. (1995) induced spawning in Haliotis iris with two agents, hydrogen peroxide and seawater irradiated with ultraviolet light, the former being more effective.

The role of prostaglandin remains unclear, although its presence and biosynthesis have been established (Ogata et al. 1978; Nomura & Ogata 1976). We found PG to be a successful agent for inducing spawning in male *F. picta*, but in females the mechanism may be different. Thus Martínez et al. (1996) suggested that in the hermaphrodite scallop *Argopecten purpuratus*, dopamine and PG may be involved in the release of oocytes.

The fact that most of the trials were successful only in males may be attributable to different mechanisms or maturation rates in male and female gonads. Our microscopic observations demonstrated that gonad maturation was almost continuous in males, whereas in females there was a latent period during the vitellogenic phase.

One outcome from this experiment should nevertheless be emphasized. Spawning inducers such as KCl and H_2O_2 are inexpensive, easy to use and are widely used for the control of reproduction in molluscs such as fissurellids.

Sex and Gametes

Fissurella picta is an archaeogastropod which, like all fissurellids, does not exhibit external sexual dimorphism (McLean 1984). There is no evidence for hermaphroditism or sex reversal (Bretos et al. 1983). The technique we used for determining the sex of each specimen was non-invasive, successful and simple.

There have been few studies of the life cycle (e.g., spawning, fertilization, and age of sexual maturation) in fissurellids. Ward (1966) described the reproductive cycle of *F. barbadensis*, and demonstrated the presence of oocytes of 80–180 μm in diameter (including gelatinous coat). This species is known to have a pelagic phase of two to three days duration (Lewis 1954; Lewis 1960). In *Diodora aspera* spawned eggs are 160–198 μm in ameter, without the gelatinous coat (Hadfield & Strathmann 1996). In *F. maxima* (Bretos et al. 1983) the oocyte diameter lies between 120 and 280 μm, and that of *F. crassa* is approximately 300 μm, excluding the gelatinous coat (Huaquín et al. 1998). Our values for oocyte diameter in *F. picta* are similar to the maximum values recorded for other Chilean species. Nevertheless, differences observed from tropical little sized species such as *F. barbadensis* and *Diodora apertura* may be related to differences in reproductive strategy e.g., time to reach sexual maturity.

In *Diodora aspera* the external gelatinous coat which envelops the oocyte appears to break open only in response to a mechanical action (Hadfield & Strathmann 1996), whereas the internal membrane disappears as a result of the action of enzymes produced by the larva. In Patella (Fretter & Graham 1962) the gelatinous coat of the oocyte disappears a short time after spawning, as in *F. picta* (this study).

The presence of a canal, the micropyle, in the gelatinous coat of the oocyte, and the presence of sperm cells within it, has been observed in *F. crassa* (Huaquín et al. 1998), *Diodora aspera* (Hadfield & Strathmann 1996) and *F. picta* (Fig. 2B of this article).

Fertilization and Development

Knowledge of larval development of Chilean fissurellids is limited mainly to a few unpublished observations; all of them obtained by spontaneous gamete release and fertilization at the laboratory. Vega and Osorio (1995), and Vega et al. (1996) determined that the duration of the pelagic larval phase in *F. cumingi* is 3–5 days under controlled conditions (16–19.8°C), whereas in *F. latimarginata* at 13°C the veliger stage and metamorphosed larva occur 6–7 days after fertilization (Pereira & Quezada 1996).

According to González et al. (1999), who also got spontaneous gamete release and fertilization, the initial trochophore stage was observed in *F. picta* at 72 hours, and swimming trochophore hatched at 96 hours at 10°C. In the present study at 17.5°C, trochophores of *F. picta* were obtained 15 hours after fertilization within the albumen coat, and veligers hatched out of this coat after 40 hours. Our data are not consistent with those of González et al. (1999), probably owing to the difference in temperature. It has been described for *Haliotis* and other species, that the temperature can hasten or delay development (Hahn 1989), and this has probably happened in *F. picta*.

The veligers which were liberated in this study showed morphogenetic movements associated with torsion before settlement and metamorphosis took place. In this regard, our observations agree with those of Page (1997) for the archaeogastropod *Haliotis kamtschatkana*, demonstrating cephalo-pedal and viscero-pallial rotation in pretorsional larvae.

The fact that development of *F. picta* took place in the laboratory during this study suggests the potential for future cultivation of this species. Nevertheless, it will be necessary to elucidate the principal biological factors and ontogenetic mechanisms required to improve survival rates and to identify the conditions required for settlement and growth of a large number of larvae.

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TYRIAN PURPLE FROM MARINE MURICIDS, ESPECIALLY FROM *PLICOPURPURA PANSA* (GOULD, 1853)

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ABSTRACT A review of the literature discloses that most marine snails of the family Muricidae produce in the hypobranchial gland a viscous secretion containing, besides mucus and biologically active compounds, minute amounts of chromogens. These chromogens develop enzymatically and under the influence of light and oxygen into a purple pigment known as "Tyrian Purple", "Royal Purple" or shellfish purple. In the hypobranchial gland the enzyme purpurase is kept apart from the chromogens, so that no pigments are formed under normal conditions. Different species of muricids produce different pigments, depending on the number and concentration of different chromogens and on the varying light intensity and oxygen availability during pigment formation. The main pigments obtained from the hypobranchial gland from muricids are indigoids. The pigment of *P. pansa* is mainly 6,6′-dibromindigo with smaller amounts of 6-bromoindigo and 6,6′-dibromoindirubin, similar to that of *Murex brandaris*.

KEY WORDS: "Tyrian Purple", gastropoda, muricidae, *Plicopurpura pansa*, hypobranchial gland

INTRODUCTION

A review of the hypobranchial gland of muricids, its secretions, including "Tyrian purple", seemed justified in view of the growing interest in natural dyes and marine products with pharmacological properties.

The majority of purple producing marine snails belong to the family of Muricidae and most, if not all, produce a colorless secretion in the hypobranchial gland, which turns purple on exposure to air and light (Fretter & Graham 1994).

In antiquity, the purple from the muricids *Murex trunculus*, *M. brandaris*, and *Purpura haemastoma* was produced in the eastern Mediterranean countries – now called, Crete, Lebanon, and Israel. Through the Phoenicians the art of purple production was spread from the Mediterranean to West Africa and Ireland (Jackson 1917). Purple dyes were used extensively by Egyptians and subsequently by Greeks and Romans. In view of the enormous quantity of marine snails needed to produce a minute amount of the dye, the scarcity of the animals, and the high costs of production, Tyrian purple was at that time a most expensive luxury article. In addition there was the symbolic importance of purple as a sign of royalty, power and wealth, and the belief that it could possess magic and supernatural powers (Reinhold 1970). At that time it was the only known fast vat dye, other than indigo. With the Arab conquest of Palestine in 638 A.D., and finally with the fall of Constantinople in 1453 A.D. the use of Tyrian purple became, with a few exceptions, extinct in the Old World (Herzog 1919; Born 1936b; Clark et al. 1993). Through archaeological studies it was confirmed that during the Middle Ages on the west coast of France the muricid *Nucella lapillus* was used as a source for purple (Gruet 1993). From the 16th to the 18th century the artisanal use of purple for marking linen was widespread in Ireland, South Wales and Cornwall, as well as in Scotland, France, Norway and other parts of Europe (Cole 1685; Jackson 1917).

In Japan, the muricid *Rapana bezoar* was of importance in ancient dyeing processes (Baker 1974). On the Japanese peninsula Shima, professional seafood collectors stained their diving suits, made of cotton, with the purple from marine snails believing that it contained supernatural powers (Yoshioka 1974).

The use of muricids for dyeing on the pacific coast of the Americas dates at least from pre-Columbian times. In the same way as the now extinct Mediterranean purple industry the exploitation of the dye of marine snails led also on the pacific coast of the Americas to a product of high economic value.

Today, however, there is not much general interest existing in Tyrian purple derived from marine snails, since similar pigments can be obtained from synthetic substitutes at much less cost (Born 1936c). However, two remarkable exceptions have to be mentioned: (a) the dark violet-blue tekhelet color, which is relevant to Jewish religious rituals derived from the Mediterranean muricids *Murex erinaceus* and *M. trunculus*, and b) on the Pacific coast from Peru to Mexico, the hypobranchial secretion of the muricid *Plicopurpura pansa* (Gould 1853) has been exploited since pre-Columbian times by Indians for dyeing cotton yarn, which until now is subsequently woven into traditional dresses (Martens v. 1874, 1898; Schunck 1980a; Nuttall 1909; Jackson 1917; Born 1936c; Gerhard 1964; Turok et al. 1988; Yoshioka 1974; Thompson 1994; Garay 1996; Sandberg 1997). v. Martens (1898) pointed out that the use of the pigments from *P. pansa* for dyeing in Central America must have had a very long and pre-Columbian tradition and were not brought by the Spanish conquistadors from Europe to the New World. Its presence in archaeological textiles and pictures confirmed his finding.

The carnivorous muricid *Plicopurpura pansa* (Gould 1853), according to Kool (1993) conspecific with *Purpura pansa* (Gould 1853), inhabits intertidal rocks exposed to the open sea with high impact waves. The range of *P. pansa* extends at the Pacific from the north-west coast of Mexico (Baja California Sur) (Clench 1947; Keen 1971) to northern Peru (Peña 1970; Paredes et al. 1999).

Hypobranchial or Mucous Gland

Since the mid 18th century the hypobranchial gland of muricids has attracted the interest of natural scientists, investigating its functional role, and the astonishing production of Tyrian purple. Fretter and Graham (1994) consider the main function of the hypobranchial gland to be a secretor of mucus for trapping and cementing particulate matter sucked into the mantle cavity with the respiratory water current, prior to its expulsion.

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The hypobranchial or mucus gland is an elongated epithelial structure located in the dorsal mantle cavity between the gills and recto-genital organs, immediately below the shell. In the gland three distinct anatomical and functional areas have been described: two lateral regions composed of eight distinct cell types, among them many active secretory cells (mucocytes), ciliated on the ventral surface, and possessing pores through which the mucus and other secretory products are released into the mantle cavity, and a central area where the formation of the "purple" precursors takes place and where secretory products accumulate prior to their release from the snail (Bolognani Fantin & Ottaviani 1981; Roller et al. 1995). The purple precursors (tyrindoxyl sulphate) and the enzyme (aryl sulfatase = purpurase) that induce the transformation of the purple precursors into pigments are only localized in the median zone of the hypobranchial gland (Erspamer 1946) and are kept separate, so that no reaction occurs. Mollusk purple as such does not occur in the live animal, but it is formed during a sequence of chemical reactions from the secretions produced by the animal. When the animal contracts vigorously the cells are massively liberated, burst open by mechanical or osmotic pressure, and their contents dispersed into the mucus (Lacaze-Duthiers 1859). These observations were later refined by the histological work of Bernard (1890), who found a well-developed innervation in the gland, suggesting a role in perception (Verhecken 1989).

The pharmacological action by extracts of the hypobranchial gland was discovered by Dubois (1909), and he described for the first time their toxic and paralyzing action in both warm- and cold-blooded species. The secretion of the hypobranchial gland from a large number of muricids contains, besides mucus, the precursors of the purple dye, proteins (aryl sulfatase, purpurase), and toxins and narcotizing agents, like serotonin (5-hydroxytryptamine), murexine (urocanylcholine,), choline ester and biogenic amines (Erspamer 1952; Erspamer & Benati 1953; Whittaker 1960; Malaszkiewicz 1967; Huang & Mir 1971; Roseghini et al. 1996; Shiomi et al. 1998).

The secretion from the hypobranchial gland of *P. pansa* can be obtained by "milking" without harming the animals. It is a milky-white liquid, which turns on exposure to air and light, at first yellow, then greenish, bluish and finally purple ("Tyrian purple"). During personal field observations (unpublished) we observed that *P. pansa* uses the secretion to immobilize prey (*Nerita* sp., *Littorina* sp.) in the intertidal zone, and does not resort to drilling through the shells of other snails. Additionally interesting to notice is the fact that during the predation no purple color is formed on the prey, despite the presence of oxygen and intense light radiation.

The chromogens containing the hypobrancial secretions seem to be purely incidental, and their functional role, if any, is presently unknown (Clench 1947). The volume of secretion obtainable from *P. pansa* depends not only on the size and sex of the animals, the time interval between the each "milking", but also on the season. Its production and use may be in proportion to the type of food the snails feed on. From small animals of less than 2 cm shell length can be obtained about 0.5 ml of secretion, from 5–6 cm large animals up to 4 ml (Rios-Jara et al. 1994). It has to be kept in mind however, that in this volume only a minute proportion consists in the dye precursors.

Chemistry of Tyrian Purple Formation

Several preliminary studies on the chemical composition of the pigments of *P. pansa* are available. The comparison between the chemical composition of the hypobranchial secretion of other muricids will lead to a better understanding of the metabolic pathways that lead to the final production of Tyrian purple.

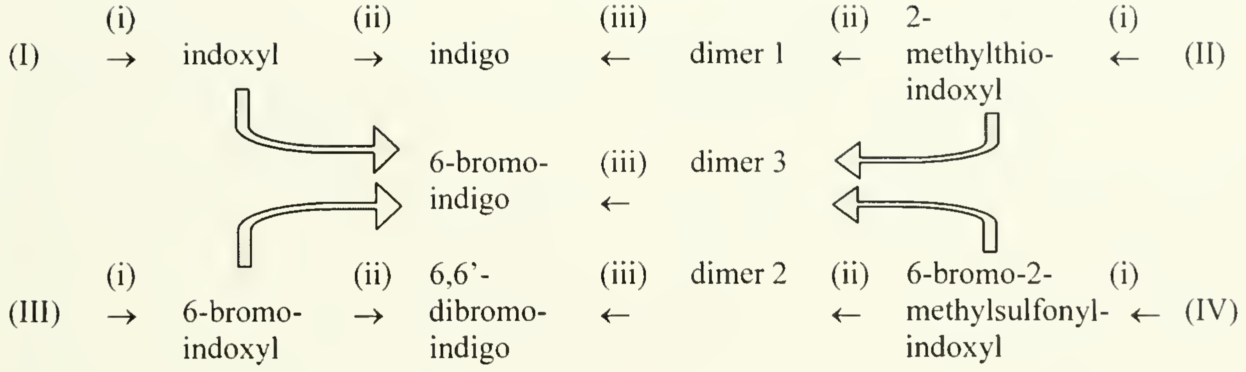
Since the re-discovery by Cole (1685) of "Tyrian Purple" from *Nucella (Purpura) lapillus* a number of researchers have worked on the determination of the chemical composition of the secretion of the hypobranchial gland. Most remarkable, considering the limited knowledge of organic chemistry at the beginning of the 19th century, is the analytical work by Bartolomeo Bizio about the origin and properties of Tyrian purple from the Mediterranean muricids *Murex trunculus* and *M. brandaris* (Ghiretti 1994). When collecting the glandular secretion of the snails he made the important observations, first, that as soon as the colorless fluid is exposed to light and air it becomes immediately yellow and greenish, and soon afterwards it turns into deep emerald green, blue, deep blue and finally reaches the purple color. Next, that during the production of the purple dye, a highly odorous compound is released. In comparing the color differences between the purple from *Murex trunculus* and *M. brandaris* he discovered that they are species specific. Bizio also determined that Tyrian purple is a substance with chemical properties similar to indigo. Schunck (1879) isolated and crystallized the pigment from the "ink" of *Nucella (Purpura) lapillus*, and determined the chemical properties. He called the pigment punicin. To obtain 7 mg of punicin he extracted the hypobranchial gland of 400 animals, after which he reports "my patience was exhausted". Friedländer (1909) isolated 1.4 g of the pure pigment from 12,000 hypobranchial glands from *Murex brandaris*, and showed that it was 6,6′-dibromoindigo.

Recently, using advanced analytical methods, Fouquet (1970), Baker and Duke (1973), Michel et al. (1992) and Koren (1994, 1995) among others, have confirmed that the major pigment from all studied muricids is 6,6′-dibromoindigo.

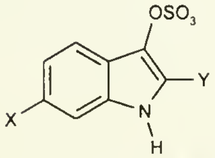
Different species of muricids produce different color qualities of the dye, depending mainly on the number and concentration of the different chromogens. Fouquet (1970) found four different chromogens in the hypobranchial gland of *M. trunculus*: I) indoxyl sulfate, II) 2-methylthio-indoxyl sulfate, III) 6-bromoindoxyl sulfate, and IV) 6-bromo-2-methylsulfonyl-indoxyl sulfate, and he described the chemical pathway leading to Tyrian purple: The first step in the purple production is hydrolysis of the sulfate group with purpurase (aryl sulfatase). Indoxyl sulfate (I) and 6-bromoindoxyl sulfate (III) are then oxidized by oxygen to give indigo and 6,6′-dibromoindigo, respectively. With 2-methylthio-indoxyl sulfate (II) and 6-bromo-2-methylsulfonyl-indoxyl sulfate (IV) oxidation is followed by dimerisation and the dimer is photolysed in light to give indigo and 6,6′-dibromoindigo respectively together with methanethiol or dimethyl disulfide. These reactions as described by Foquet (1970) are shown in Figure 1.

At the time of Fouquet's studies the possibility of cross-coupling of the indoxyls which accounts for the large percentage of 6-bromoindigo in the pigment of *M. trunculus*was was unknown.

The composition of the chromogens of other muricids is less complicated. *Thais clavigera*, *T. bronnii*, *Dicathais orbita*, *M. brandaris* and *N. lapillus* contain 6-bromo-2-methylthio-indoxyl sulfate (IV); *M. erinaceus* contains a single different chromogen and *Purpura haemastoma* and *Rapana bezoar* contain two other different chromogens, but the chemical structures are not known (Baker 1974; Hiyoshi & Fujise 1992). The reaction pathways of



Reagents: (i) aryl sulfatase, (ii) oxygen, (iii) light.



X=H, Y=H, (I) indoxyl sulfate
X=H, Y=SCH_3, (II) 2-methylthio-indoxyl sulfate
X=Br, Y=H, (III) 6-bromoindoxyl sulfate
X=Br, Y=SO_2CH_3, (IV) 6-bromo-2-methylsulfonyl-indoxyl sulfate

Figure 1. The chromogens from *Murex trunculus* and their reactions to give indigoid pigments (Fouquet, 1970).

6-bromo-2-methylthio-indoxyl sulfate (tyrindoxyl) to give indigoid pigments are shown in Figure 2.

The composition of the different chromogens is not only dependent on the species of muricids, but also environmental, and physiological condition of the animals. The light intensity and oxygen availability also play a role during pigment formation. According to historical reports the best seasons to exploit the purple snails in the Mediterranean are autumn and winter. During summer the animals are hidden and in spring they lay eggs, at which time the hypobranchial secretion presumably is losing its coloring power and is not suited for color production (Born 1936; Cardon & du Chatenet 1990). Fouquet (1970) cites Schaefer (1941 "Neuere Ansichten über den antiken Purpur" Chemiker Zeitung, 273) and O. von Fürth (1903 "Vergleichende chemische Physiologie der niederen Tiere". Verlag G. Fischer, Jena, page 377) who stated that there are seasonal changes in the chromogens of snails from the "trunculus" and "brandaris" types, due to age, gender, and food.

Chemistry of Tyrian Purple from P. pansa

For more than a hundred years the chemical composition of "Tyrian Purple" from *P. pansa* has attracted the interest of chemists. Edward Schunck (1880a) obtained a sample of cotton yarn dyed on the west coast of Nicaragua with the extract of *Purpura patula* (now *P. pansa*). From 24 g of dyed material he obtained 99 mg of pure crystalline pigment with all the properties of punicin, which he had earlier obtained from *Purpura capillus (Nucella lapillus)* (Schunck 1879). Thirty years later it was shown by Friedländer (1909), that Schunck's punicin was 6,6'-dibromindigo. In 1922 Friedländer obtained from Mexico a sample of yarn dyed with the excretion of *P. aperta* (the zoological description has to be *P. pansa*, since *P. aperta* does not occur on the Pacific coast of the Americas). The analysis of the dye showed no differences in solubility, color, and absorption spectrum between the dye from *M. brandaris*, which he had analyzed before and for this reason he concluded with some certainty the dye of *P. pansa* consists mainly of 6,6'-dibromoindigo (Friedländer 1922).

Saltzman (1992) showed that the reflectance spectrum of cloth dyed with the "ink" of *P. pansa* had a maximum absorption at 520 nm. Very similar results obtained Withnall et al. (1993) and Clark et al. (1993) for synthetic 6,6'-dibromoindigo. Mass spectrometry was used by McGovern et al. (1991) to confirm that the major colorant of the cotton sample from Saltzman, was 6,6'-dibromoindigo with traces of 6-bromoindigo. It was found that direct introduction of the cotton sample into the mass spectrometer led in addition to the detection of large amounts of 6-bromoindigo also to some indigo. This artifact arose from interaction of the cotton fiber and 6,6'-dibromoindigo at the high injection temperatures, leading to debromination and the formation of indigo. The problem can be avoided by extraction of the dye from the fiber with hot quinoline, or with dimethyl sulfoxide (McGovern et al. 1991). Using this extraction methodology, it was observed that apart from the major 6,6'-dibromoindigo component there were also smaller amounts of monobromoindigo and indigo which have been previously obscured by other low molecular weight materials.

The analytical technique of choice for the characterization of mixtures of indigoid dyes is the application of the High Performance Liquid Chromatography (HPLC), pioneered by Wouters and Verhecken (1991). This technique allows the characterization of pigments by retention time and absorption spectrum. Koren (1994) applied this technique to a sample of Dr. Saltzman's material containing the Mexican purple from Oaxaca. He could only detect 6,6'-dibromoindigo. A recent chemical study of the pigments of *P. pansa* confirmed the finding that the main component of the dye is 6,6'-dibromoindigo (90%); with 9% monobro

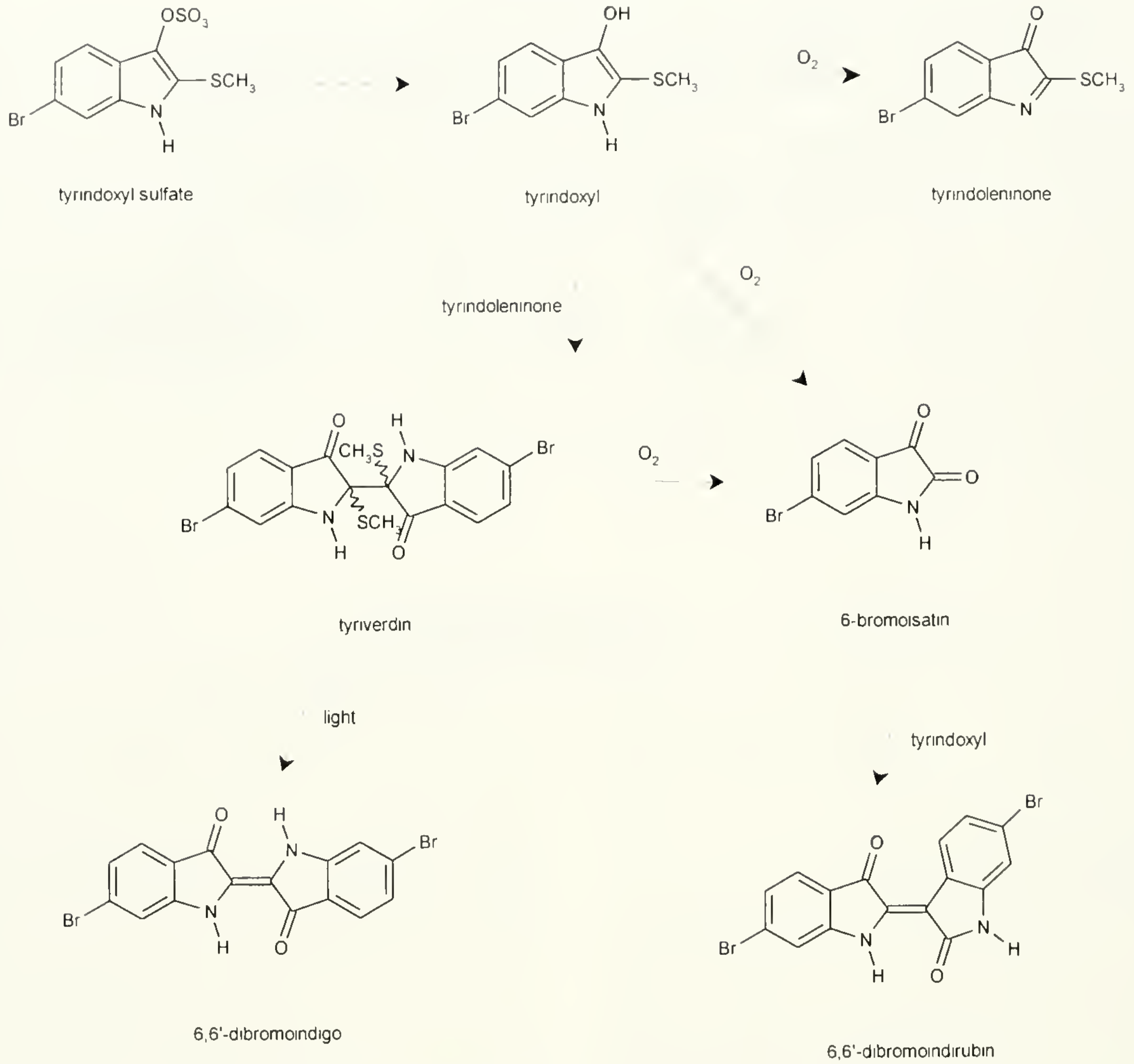

OSO3
SCH3
Br
N
H
tyrindoxyl sulfate
OH
tyrindoxyl
O2
O
tyrindoleninone
tyrindoleninone
O2
CH3S
tyriverdin
6-bromoisatin
light
tyrindoxyl
6,6'-dibromoindigo
6,6'-dibromoindirubin

Figure 2. The production of indigoids from tyrindoxyl sulfate in "brandaris-type" mollusks.

digo, and 1% dibromoindirubin (Withnall et al., unpublished). Results obtained by different authors (Wouters 1992; Cooksey et al. 1992; Koren 1995, Withnall et al. unpublished) using HPLC to determine the chemical composition of the indigoid constituents of the purple dye from various muricids are shown in Table 1.

Since the values in Table 1 were obtained using a variety of HPLC protocols, close comparison is not justified, but some trends can be noted: *P. pansa* belongs to the *M. brandaris* group containing no indigo in the pigment, some 6,6′-dibromoindirubin, and showing a higher proportion of 6-bromoindigo than the average.

TABLE 1.

The composition (in %, obtained through HPLC analysis) of the indigoid constituents of the purple dye from various muricids.

	Indigo	Indirubin	6-Mono Bromo Indigo	6,6′-Dibromo Indigo	6,6′-Dibromo Indirubin	Reference
Murex brandaris	0	0	3	83	14	Wouters (1992)
Thais haemastoma	0	0	3	91	6	Wouters (1992)
Nucella lapillus	0	0	3	88	9	Cooksey et al., (1992)
Nucella lapillus	8	0	1	77	14	Withnall et al., (unpublished)
P. pansa	0	0	9	90	1	Withnall et al., (unpublished)
P. pansa	0	0	16	77	7	Wouters (pers. com).
Murex trunculus	55	7	35	3	0	Wouters (1992)
Murex trunculus	3	0	15	63	2	Koren (1995)

Textile Dyeing with "Tyrian Purple"

In using the "ink" for dyeing materials two significant differences have to be mentioned between the Mediterranean muricids and *P. pansa*: (a) the Mediterranean snails have to be killed to obtain the chromogens, whereas *P. pansa* can be "milked" to obtain the dye without harming the animals; and (b) the "milk" from the *P. pansa* can be applied directly on textiles where the final pigments are formed in the presence of light and oxygen.

In textile dyeing, there are two methods for dyeing with mollusk purple. The most simple is to have the chromogens react in the presence of light and air to obtain directly the final pigment on the fiber, as is the case with *P. pansa*. Since the molecular structure of mollusk purple is indigoid, there exists also the possibility of starting the dyeing process with the final oxidized purple colorant by reducing it in an aqueous alkaline bath, and applying the highly water soluble, and almost colorless leuco-form as a vat dye, just like indigo. Exposure to air oxidizes the leuco-form back to the indigoid pigment (Verhecken 1993; Clark et al. 1993; Schweppe 1998). In sunlight, the brominated leuco-indigoids can be photodebrominated, leading to 6-bromoindigo or indigo after aerial oxidation and changing the purple color to blue. This chemistry of reduction and photodebromination of 6,6′-dibromoindigo, was first described by Driessen (1944), as shown in Figure 3.

DISCUSSION

Different species of muricids produce different color qualities of the ink (Born 1936a), depending on the number and concentration of different chromogens. According to Verhecken (1993) depending on the precursors and chemical reactions for the formation of the pigments, two groups of dyes from marine muricids can be distinguished: the "trunculus type" where light is not necessary, and the "brandaris type" requiring both light and oxygen. Since for the formation of the dye of *P. pansa* light and oxygen is necessary, the dye of *P. pansa* according to this definition belongs to the "brandaris type". Another fact supports this characterization: the pigments of the "brandaris type" contain mainly 6,6′-dibromoindigo. The pigments of *P. pansa* contain 90% 6,6′-dibromindigo, confirming that the snails are forming part of the "brandaris type". This is in contrast to the purple pigment derived from *M. trunculus*, which is exceptional in containing non-

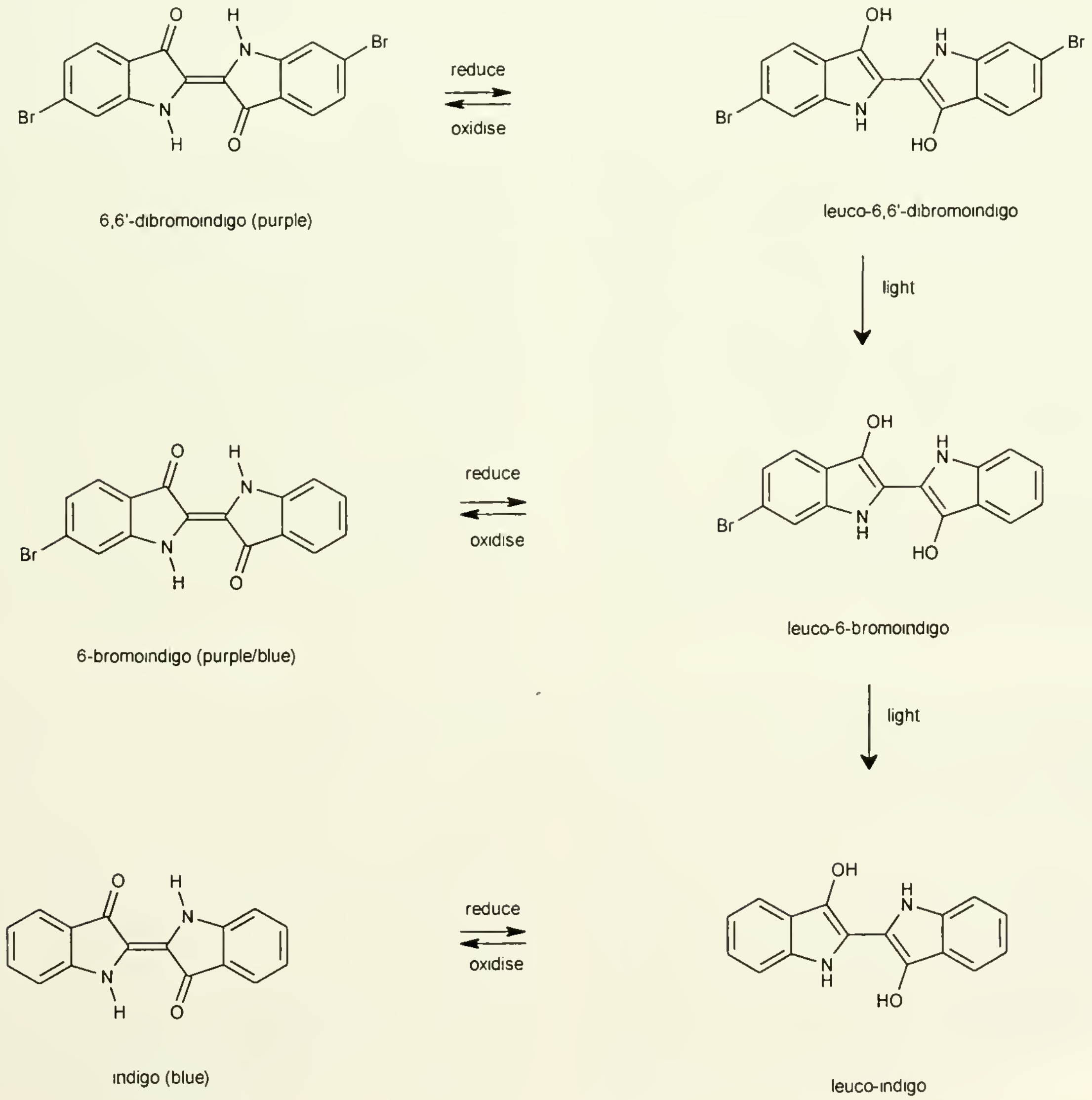


Figure 3. The reduction and photodebromination of 6,6′-dibromoindigo.

brominated precursors, leading to widely varying mixtures of indigoid pigments, including indigo and indirubin (Malaszkiewicz 1967).

The biosynthesis of the chromogens in the hypobranchial gland of muricids originates from tryptophan, an essential amino acid for animals, which is enzymatically split into indole and pyruvate. After a sequence of chemical reactions different intermediates are formed from indole, which lead finally to the colorless precursor of indigoid pigments tyrindoxyl sulfate, and subsequently through an enzymatic reaction with aryl sulfatase to give the yellowish intermediate tyrindoxyl (Fouquet 1970). In the presence of oxygen the red tyrindoleninone is formed, which reacts together with tyrindoxyl to give the greenish tyriverdin. In the presence of light tyriverdin is photolysed to give dimethyl disulfide and the purple, insoluble pigment 6,6′-dibromoindigo (Verhecken 1989). Additionally, from photolysis of tyrindoxyl or tyriverdin in the presence of oxygen, 6-bromoisatin can be formed which reacts with tyrindoxyl to 6,6′-dibromoindirubin (Withnall et al., unpublished). Oxygen and the light intensity during the chemical reactions of the intermediate substances determine the final composition of the pigments. The higher content of 6-bromoindigo than average in the pigments of *P. pansa* may result from the development of the dye under conditions of high light intensity. Under these conditions, any leuco-6,6′-dibromoindigo would be photodebrominated to give leuco-6-bromindigo, which on aerial oxidation would give 6-bromindigo. Under high intensity light conditions, the photoelimination of dimethyl disulfide from tyriverdin to yield 6,6′-dibromindigo will proceed rapidly, leading to a low concentration of tyriverdin. Consequently, oxidative cleavage of tyriverdin, a bimolecular reaction, to give 6-bromoisatin is a minor reaction pathway, leading to a low concentration of 6,6′-dibromoindirubin in the purple pigments of *P. pansa* (Withnall et al., unpublished).

The importance of the chromogens in the metabolism of the purple snails is unclear. The presence of the enzyme aryl sulfatase, which presumably occurs in all muricids (Erspamer 1946), supports the hypothesis that the chromogens could serve as a storage for the highly unstable indoxyls, which are formed enzymatically by the aryl sulfatase from sulfate esters (Fouquet 1970). Additional attention needs the question about the biological function of the indoxyls and their substituted bromo and methylthio analogs. It could be possible that these bromo and thio substituted indoxyls, like the iodine derivatives of tyrosine could act as hormones in the metabolism of the snails. Since the chromogens, besides mucus and bioactive substances, have their origin in a specialized area of the hypobranchial gland, it is feasible that the gland could have additionally inner secretory activities (Fouquet 1970).

We observed during field work that *P. pansa*, above sea level uses the secretion to immobilize their prey, without the formation of purple pigments (unpublished personal observations). This supports the finding that under normal circumstances the enzyme purpurase is kept apart from the chromogens, and therefore no pigments are formed, despite the presence of oxygen and light (Verhecken 1989). Additionally, in preliminary, yet unpublished personal studies, we could show, that the secretion from *P. pansa* is toxic to nauplii of Artemia, and has gram negative and gram positive antibacterial properties.

From snails of less than 2 cm shell length can be obtained about 0.5 ml of secretion and from large animals 5-6 cm up to 4 ml (Rios Jara et al. 1994). About one liter of secretion is required to dye about 200 g of cotton (Acevedo Garcia et al. 1993; Michel Morfin 2000). Since the average size of *P. pansa* is about 3 cm and a collection of more than 1 ml secretion per animal seems difficult, the enormous number of at least 1,000 snails has to be "milked" to obtain 1 liter of secretion to dye only 200 g of material. Since too frequent "milking" does harm to the animals it was the right decision of the Mexican government to permit only Indian communities the traditional exploitation of *P. pansa* for its pigments and to declare it a protected species.

In contrast with the Mediterranean region, where the use of purple from marine snails has long been forgotten and the craft of dyeing today cannot exactly be reconstructed, in remote Pacific regions of Mexico (in the States of Oaxaca and Michoacan) and with the Indian community of the Borucas in Costa Rica (Turok 1999) its use is continuing now and represents the survival of a knowledge of considerable antiquity. However, as Thompson (1994) observed that this old tradition will be lost in the future. As Thompson (1994) notes "In the early 20th century in Mexico shellfish purple was in much more widespread use than it is now. The beliefs, languages, and crafts of the Mexican Indians are fast disappearing. The progressive 'westernization' of rural Mexico has led people in many villages to abandon their traditional textiles and customs, in favor of factory-made cloth and western-style clothes which are readily available everywhere. Cultural and social decay is continuing to the point that the demand for traditional textiles has almost vanished. Weavers in a few villages formerly noted for their excellent textiles have turned to making more 'commercial' articles, for sale to people, such as tourists, outside their culture—a classic manifestation of the 'airport art' phenomenon."

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A CYTOLOGICAL STUDY OF THE MANTLE EDGE OF *HALIOTIS TUBERCULATA* L. (MOLLUSCA, GASTROPODA) IN RELATION TO SHELL STRUCTURE

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ABSTRACT The mantle cytology and ultrastructure of the abalone *Haliotis tuberculata* were investigated and different structures are detailed in relation to shell structure. The mantle edge of the abalone (3–4 mm near the shell edge) was chosen because it corresponds to the most active area in shell formation. The mantle is composed of a thin lamella of connective tissue wrapped in monolayered epithelia, the inner epithelium (facing the body cavity) and the outer epithelium (facing the shell). At the edge, the mantle ends with the division of the edge in two folds. The main features of glandular and nonglandular cells were characterized. The inner epithelium, responsible for ion exchanges with the external environment, is composed of three glandular cell types and ciliated or unciliated nonglandular cells. The periostracal groove, where the periostracum originates, shows high metabolic activity, with many secretions inside both from glandular and nonglandular cells. Numerous cilia have been observed in relation to these secretions. The outer epithelium could be divided into two distinct areas: a first short area in the outer part surface of the outer fold at the mantle edge (approximately 250–300 μm long), called the tubular area, and a larger area which extends on the whole shell surface beyond the tubular zone. These two areas are both folded, but in the tubular area folds form tubules. Moreover, some alimentary pigments have been observed to be associated with the tubular zone. Both structural characteristics of these two distinct areas as well as features of the cells indicate that they are responsible for the formation of the two distinct layers of the shell: the tubular zone (the calcite prismatic layer) and the outer epithelium (the aragonite nacreous layer), respectively.

KEY WORDS: mantle, ultrastructural study, shell formation, marine gastropod, *Haliotis*

INTRODUCTION

The shell of the mollusc abalone, *Haliotis tuberculata*, is made up of three distinct layers (Nakahara et al. 1982; Wilbur & Saleuddin 1983): the outer layer or periostracum, which is a tanned proteinaceous layer, a middle prismatic layer (calcite), and an inner nacreous layer (aragonite). The calcified layers are a tight association of calcium carbonate minerals with organic matrix. Despite numerous studies of the mineralization processes in molluscs (reviewed by Wilbur 1964; Wilbur 1972), the precise mode of formation of shell is far from clear. The organic matrix plays an important role in mineralization, providing sites for crystal nucleation and a template for crystal growth and orientation. Each layer of the shell corresponds to a different composition of this organic matrix. The precursors of this organic matrix are first secreted by the mantle into a small cavity between the mantle and the shell (the extrapallial space). Some studies on mollusc mantle structure show that a regionalization of the mantle exists in relation with the secretion of the three shell layers (Beedham 1958; Kawaguti & Ikemoto 1962b; Wilbur & Saleuddin 1983).

Whereas the ultrastructure of the molluscan mantle edge, especially the bivalves, has been studied in great detail, the epithelium of the inner (facing the mantle cavity) and the outer (facing the shell) surface of the mantle has received considerably less attention. In molluscs, the structure of the mantle, and more particularly the mantle edge, shows a high degree of variation. In bivalves, the mantle edge is generally composed of three main folds (Jabbour-Zahab et al. 1992; Morrison 1993): an inner muscular lobe, a middle lobe believed to be sensory, and an outer lobe (Richardson et al. 1981). It is generally held that the periostracum in bivalves is secreted by the cells lining the surface of the outer lobe. In some gastropods, the mantle is also arranged into lobes, however, in numerous species the mantle lobes can be reduced in size and number (e.g., *Biomphalaria glabrata*, Bielefeld et al. 1993a; Bielefeld et al. 1993b), or are virtually lacking (e.g., *Littorina littorea*, Bevelander & Nakahara, 1970). An additional feature of gastropods is the presence of one or more groups of marginal glands embedded in the connective tissue (Bevelander & Nakahara 1970; Saleuddin 1975).

Because no study of abalone mantle has been undertaken to our knowledge, we investigated the histology and ultrastructure of the whole mantle, and more particularly near the shell edge, which is considered to be the more active part of the mantle in the biomineralization processes. Then we attempted to relate features of the cells to formation of the different shell layers: the periostracum, the calcitic prismatic, and the aragonitic nacreous layers.

MATERIALS AND METHODS

Source and Maintenance of Animals

Adult (6–7 cm in length) and juvenile (<1 cm in length) abalones, *H. tuberculata*, were purchased from a French farm (ECHINOXE, West coast of Cotentin, Manche, France). Animals were maintained at the laboratory in tanks, in artificial, aerated seawater at 18–20°C.

Histology

Pieces of the mantle (ca. 5 mm^2) were cut near the edge of the shell, after the central foot of the abalone was removed. For this study, the most active parts of the mantle were used: the two frontal lobes and the right mantle near the shell edge of the abalone. For fixation of the mantle, tissue was placed in Bouin's mixture overnight (Martoja & Martoja 1967). Sections of 5 μm in thickness were made according to the usual paraffin method. St ing with hemalum picro-indigo carmine was found to be excellent in differentiating epithelial cells, muscle, and collagen fibers.

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Identification of Cell Types: Transmission Electron Microscopy (TEM)

For TEM studies, different parts of the mantle near the edge were used. Preparations were fixed in a cocktail containing 3% glutaraldehyde, sodium cacodylate buffer at pH 7.0, and 0.35 M saccharose, overnight at +4°C. Then preparations were postfixed in a solution of 2% OsO_4 for 1 h at room temperature and rinsed twice in double-distilled water (2 × 10 min). Preparations were then placed in uranyl acetate aqueous solution (1%) during 1 h at room temperature and in the dark. Preparations were then rinsed twice in double-distilled water (2 × 10 min). Preparations were dehydrated through a graded ethanol series and then embedded in Araldite. Thin sections were cut on a Reichert ultramicrotome, stained with uranyl acetate (5% in ethanol 50°C, w/v) and lead citrate, and examined in an electron microscope (Philips EM 201 under 80 kV, Centre de Microscopie Electronique de Jussieu–Pierre et Marie Curie University, Paris, France).

RESULTS

Gross Morphology of the Mantle

The mantle of abalone is a thin lamella that originates at the base of the foot muscle. The mantle covers the visceral mass and extends to the shell edge so as to cover the shell surface. As we investigated the structure of abalone mantle in relation with the biomineralization processes, we focused on the mantle near the shell edge. Indeed, scanning electron microscope (SEM) study of the shell surface has demonstrated that the more active area of shell growth (in length and in width) corresponds to a band of 2–4 mm in length near the shell edge (unpubl. data). For this study, mainly adult abalones were used, except for Figure 9, which shows a juvenile abalone.

Figure 1a corresponds to a transverse section through the mantle edge of *H. tuberculata.* At the shell edge, the mantle is composed of a thin lamella of connective tissue covered by a monolayer of epithelial cells. In the connective tissue, hemolymph lacunae are found; hemocytes are known to participate in the biomineralization processes, and more precisely in shell regeneration. A particular feature with the muscle fiber repartition is the formation of a dense network of fibers parallel to the outer epithelium (OE) mantle surface, whereas muscle fibers, mixed with collagen fibers, remain with no specific orientation near the inner epithelium (IE). Such muscle repartition is probably responsible for the tight contact with the inner shell and for the mantle's retractive ability in response to mechanical stimuli. Because the extrapallial space (between the shell and the mantle) is very thin and contact with the external medium may be limited, this particular organization of muscle fibers may contribute to the adherence of the mantle onto the shell. The epithelium is separated from the connective tissue by a basal membrane. The epithelium in contact with the shell is called the OE, whereas the IE, in contact with seawater, faces the visceral mass. The mantle edge ends with the periostracal groove (PG) separating the mantle into two folds: the inner fold (facing the visceral mass) and the outer fold (facing the shell). For a better understanding, we have divided the mantle epithelium into different functional areas (Fig. 1b).

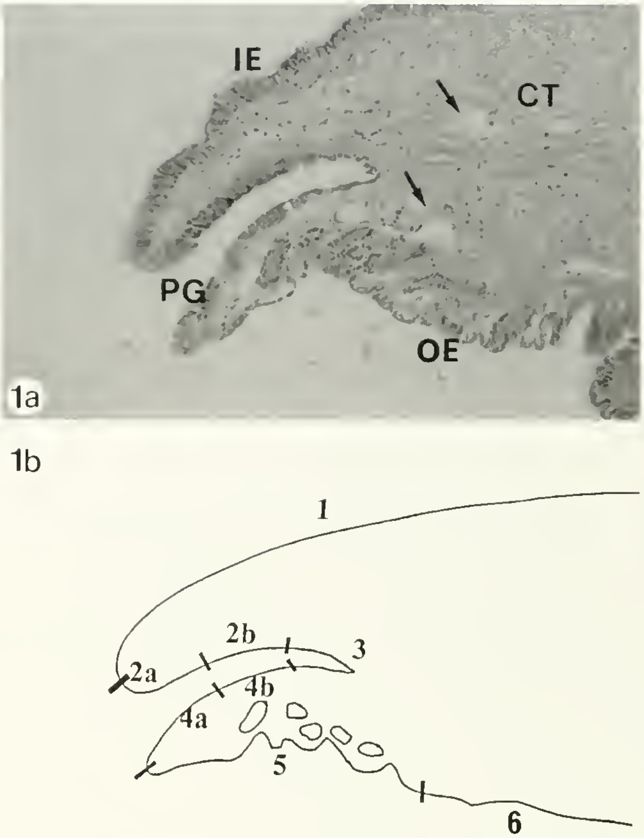

Figure 1. (a) Light micrograph of the mantle edge structure in *Haliotis tuberculata*. CT, connective tissue; IE, inner mantle epithelium (facing the body); OE, outer mantle epithelium (facing the shell); PG, periostracal groove. The hemolymph lacunae are designated by the black arrows (×160). (b) Subdivision of the mantle edge into different functional areas: 1, inner epithelium; 2, epithelium of the outer face of the inner fold; 3, bottom of the periostracal groove; 4, epithelium of the inner face of the outer fold; 5, tubular area of the outer epithelium; and 6, outer epithelium, which extends on the whole shell surface beyond area 5.

The Inner Mantle Epithelium: Area 1

The inner mantle epithelium generally consists of palisadic cells. The apical border of the cells exhibits a prominent border of microvilli, 2–5 μm high (Fig. 2a). Cells are interconnected by intercellular junctions comprising an apical desmosome and septate junctions. A distinguishing feature of the inner epithelial cells is the highly convoluted interdigitations of the plasma membranes below the apical desmosome (Fig. 2b). Under the basal lamina, a thin sheet of collagen fibers is observed (orientated parallel to the basal lamina). Two major cell types can be identified, depending on the secretions of their cytoplasm: epithelial glandular cells (where the cell cytoplasm is filled with secretory granules) and epithelial nonglandular cells.

Epithelial nonglandular cells are generally similar in size. Cilia are occasionally observed, interspersed between the microvilli (Fig. 2a), but the general characteristics of these cells remain similar along the mantle IE. These epithelial cells contain a basal nucleus in which most of the heterochromatin is concentrated in the periphery. A Golgi complex is often observed in the vicinity of the nucleus. Mitochondria are mainly located near the nucleus as well as in the apical part of the cell cytoplasm, even though some have been observed in the whole cytoplasm. Numerous vacuoles are located in the apical part of the cells. Some vacuoles contain some dark-staining contents, which are sometimes heterogeneous

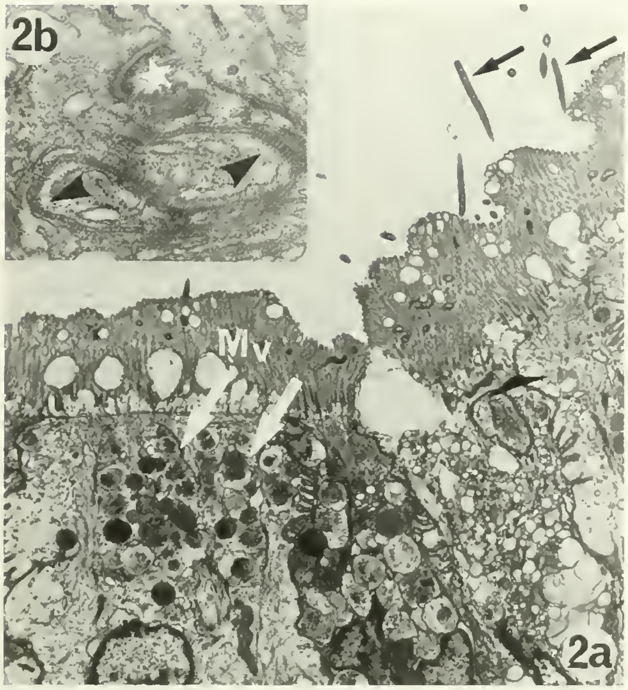

Figure 2. Area 1. (a) Inner mantle epithelium (TEM) with nonglandular cells. Cilia (black arrows) are interspersed with microvilli (Mv). Some vesicles with pigments are observed in the apex of the cells (white arrows) (×4,500) (b) Detail (TEM) of highly convoluted plasma membranes below the microvillar border. Cells are separated from each other by intercellular junctions comprising a desmosome (white star) and septate junctions (black arrowheads) (×20,000).

and could be related to some kind of pigments. Tonofilament bunches are observed mainly orientated in the axis of the cell (Fig. 2b).

A general feature of the glandular cells (or secretory cells) is their goblet shape. The narrow openings of glandular cells can sometimes be seen. The main features of these cells are the occurrence of numerous secretory granules in their cytoplasm and a basal small nucleus surrounded by a more or less developed rough endoplasmic reticulum (Figs. 3, 4, and 5). Three glandular cell types could be distinguished depending on their secretions. Type A glandular cells (Fig. 3) contain clear ovoid granules, closely packed and homogenously stained, which tend to coalesce. Type B glandular cells (Fig. 4) contain distinct, darkly staining granules, which have a denser core in some cells. The membrane of type B granules is generally still distinct when they are discharged. Type C glandular cells (Fig. 5) contain some distinct, membrane-bound granules, with rosette-like mucous granules. Type C cells have peculiar secretory granules of heterogeneous stages: in a clear and finely granular matrix an array of complex chains of electron-dense material is found. The basal nucleus is surrounded by a well-developed rough endoplasmic reticulum of enlarged cisternae filled with finely granular material. Between the rough endoplasmic reticulum and the secretory granules, Golgi stacks occur. The immature granules from the trans Golgi only contain finely granular material, then the dense complex progressively condenses and develops.

Figure 6 resumes the main features of the IE: a monolayered epithelium which constitutes ciliated (Ci), or nonciliated, nonglandular, and glandular cells (A, B, C types) with microvillous border (Mv).

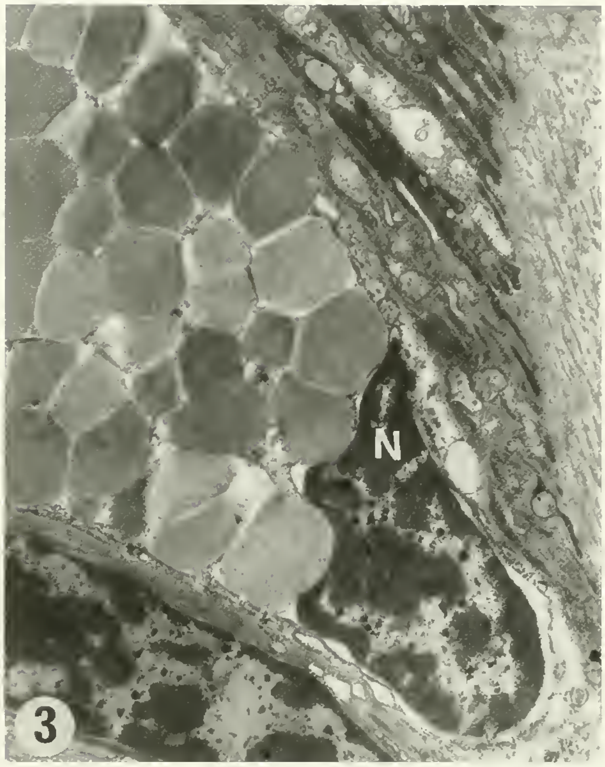

Figure 3. Area 1. Detail of type A glandular cells in the inner mantle epithelium (TEM): The cytoplasm is filled with ovoid granules, closely packed, homogeneously stained. N, nucleus (×14,000).

The Periostracal Groove: Areas 2a, 2b, 3, 4a, 4b

We divided the periostracal groove, which runs parallel to the mantle edge, into three different parts of spatial and equal importance, depending on the structure of the cells. The first part (a) corresponds to the opening of the periostracal groove directly in contact with external seawater: areas (2a) and (4a) are, respectively, the epithelium of the inner and outer folds of the periostracal groove. Principal features of the cells are very similar to those of cells of the IE: in the 4a area, characteristics of the IE are less marked (plasma membrane is not as convoluted as in the 2a area, and pigment granules are less representated than in the 2a area). The second part (b) corresponds to the middle of the periostracal groove: areas (2b) and (4b) are, respectively, the epithelium of the inner and outer folds of the periostracal groove. A principal feature of this area is the presence of a group of ciliated cells of the epithelium 4b (outer fold, Fig. 7) and the presence of numerous secretions from nonglandular cells of the epithelium 2b (inner fold, Fig. 7). All cilia are orientated to the opening of the periostracal groove and some secretions are often found associated with the cilia. Principal characteristics of the cells are quite similar to that of cells previously described.

In the periostracal groove, the microvillous border is not equivalent in the inner and outer folds. In the epithelium of the outer fold (4a, 4b), microvilli are dense and arranged in a brush border, whereas the cells of the inner fold (2a, 2b) are lined by microvilli irregularly arranged. A lot of material seems to be discharged by these cells, as clear vesicles with a fine granular content. We can also observe some secretions from nonglandular cells in the microvilli in area 2b (in the inner fold).

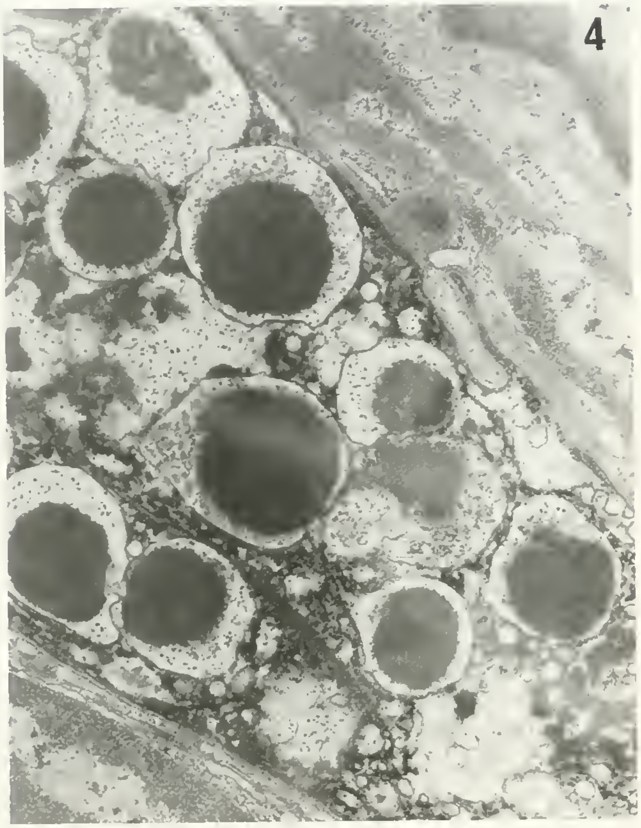

Figure 4. Area 1. Detail of type B glandular cells in the inner mantle epithelium (TEM): The cytoplasm is filled with distinct, darkly staining granules, which have a denser core in some cells (×30,000).

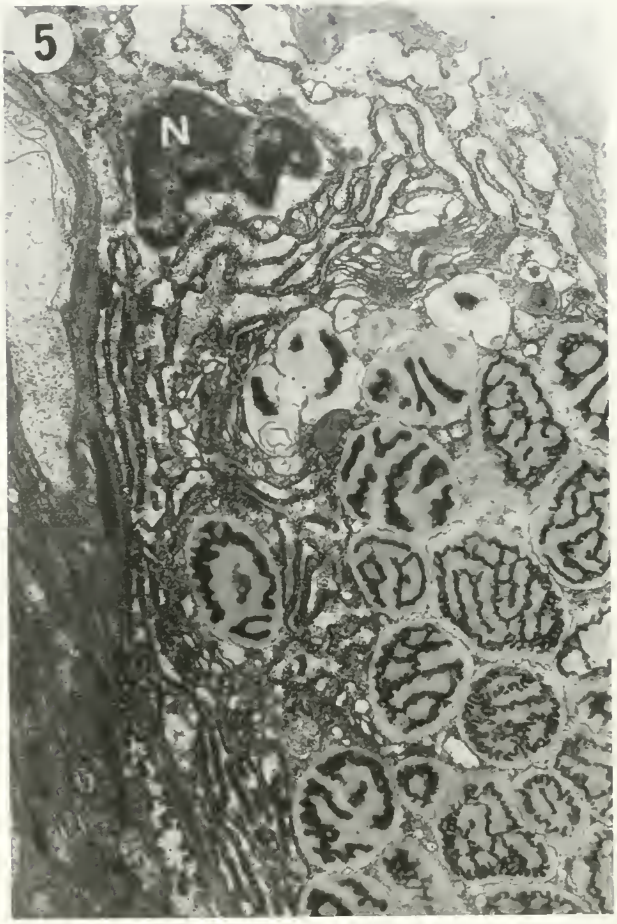

Figure 5. Area 1. Detail of type C glandular cells in the inner mantle epithelium (TEM): The cytoplasm is filled with distinct, membrane-bound granules, which have a heterogeneous content. N, nucleus (×14,000).

In areas (a) and (b) of the inner and outer fold, we can notice the presence of numerous B-type glandular cells. A-type glandular cells have been observed in some sections in the epithelium of both outer and inner fold. However, C-type glandular cells have only been found in the epithelium of the inner fold.

At the bottom of the periostracal groove (area 3), the cells become gradually cuboidal. Essentially, nonglandular cells are observed. Cilia and cell secretions could not be observed (Fig. 8). The inner and outer folds appear morphologically equivalent.

Fine Structure of the Outer Mantle Epithelium: Areas 5 and 6

In *H. tuberculata*, two distinct areas of the outer mantle epithelium can be distinguished: a short area near the periostracal groove (area 5) and the other part of the outer mantle epithelium (area 6). The structure of the cells and the structure of the folds in the OE determine this separation. Area 5 corresponds to a very folded epithelium, which forms some tubules when this area is quite developed. In scanning electron micrographs of the outer mantle epithelium, this area corresponds to an alveolous structure in external view: this area has been called the tubular zone (TA, Fig. 9). In area 6, the OE displays folds that are quite parallel to the mantle margin (Fig. 9).

In area 5 the tubular zone is more or less developed, depending on the collected specimens in a same size class. To observe the well-developed tubular zone, some juveniles have been observed by TEM (Fig. 10). The principal features of these cells are their cuboidal shape, a high nucleoplasmic ratio, and numerous round-shaped mitochondria. In Figure 10, tubules are cut transversally, and we can observe in the lumen of each tubule some small microvilli and some secretions. In this area, pigments are found travelling from hemolymph lacunae to tubules: they correspond to an obvious red and/or green line along the border of the OE in live specimens. The pigments are carried by "migrating cells" (likely hemocytes), which have a central nucleus and a cytoplasm filled with four or five vacuole sites containing a large central core of heterogeneous granules (Fig. 11).

Figure 12 summarizes the main features of the tubular zone: a monolayered epithelium (which is very folded) composed of cuboidal cells and tubules (T) containing microvilli (Mv), alimentary pigments (Pi) in vacuoles, and some secretions in the lumen (Lu).

In area 6, a different structure than the OE has been observed: cells are low columnar (<10 μm) (Fig. 13) to very high columnar (>15 μm) (Fig. 14), depending on studied specimens in the same size class. Meanwhile, the OE is always folded. In some specimens with low columnar cells (Fig. 13), numerous "empty" cells have been observed and the other cells have a cytoplasm where few organelles are present, corresponding to low cell activity. In other specimens, the OE is very high and narrow columnar (Fig. 14): numerous mitochondria, Golgi apparatus, and inclusions are found in the cytoplasm of these cells (Fig. 15). In such cells, all organelles, and in particular, all Golgi complexes, are found orien-

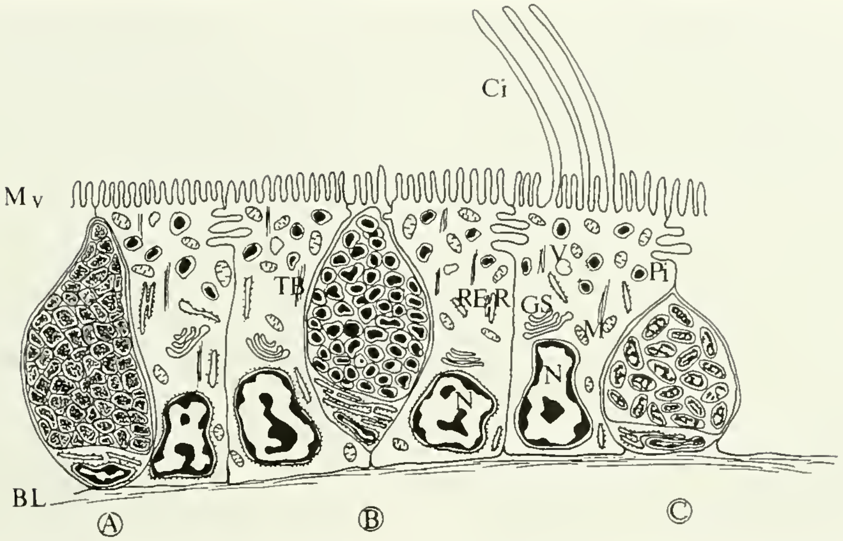

Figure 6. Area 1. Schematic drawing of the main features of the cells of the inner epithelium. In this area, three types of glandular cells are present: types A, B, and C (encircled A, B, C), with nonglandular cells, with or without cilia (Ci). BL, basal lamina; Ci, cilia; GS, Golgi stacks; M, mitochondria; Mv, microvilli; N, nucleus; RER, rough endoplasmic reticulum; Pi, pigments; TB, tonofilament bunches; V, vesicle (not to scale).

tated in the longitudinal axis of the cells (Fig. 15). The microvillous border is well developed in very high columnar cells and numerous protruding cell processes are found associated with this microvillous border (Fig. 14). In low columnar cells the microvillous border is quite nonexistent excepted in fold of the IE; however, in the cells of the OE, some distensions of the membranes are generally observed and they are often associated with mitochondria (Fig. 15).

The schematic Figure 16 resumes the main characteristics of the OE far from the periostracal groove: very high columnar cells containing numerous mitochondria (Mi), Golgi apparatus (G), and microvillar border (Mv) with protruding processes, orientated in the longitudinal axis of cells.

DISCUSSION

Many publications describe shell formation in molluscs and some of them reported the structure of the mantle and its role in shell formation. But the majority of these studies dealt with bivalves (Kawaguti & Ikemoto 1962a; Kawaguti & Ikemoto 1962b; Neff 1972; Bubel 1973a, 1973b, 1973d; Garcia-Gasca et al. 1994). Most of the work on gastropods has been done on the terrestrial snail *Helix* and on freshwater gastropods (Zylstra et al. 1978, Bielefeld et al. 1993a, Bielefeld et al. 1993b); only one reference to a marine gastropod was found: *Littorina littorea* (Bevelander & Nakahara, 1970). Although shell production is often assumed to be quite similar among molluscs, there are important morphologic

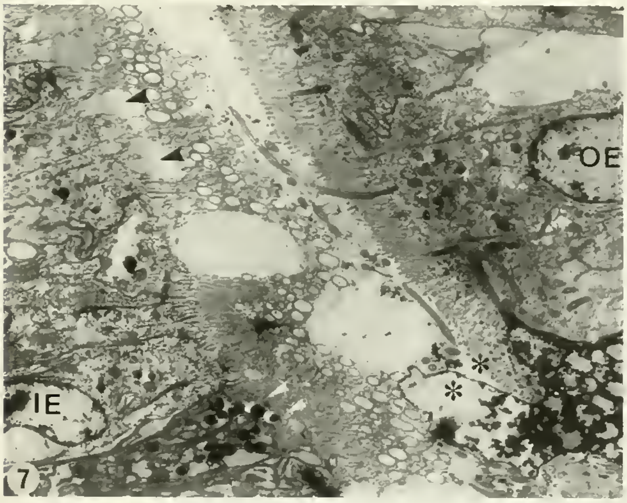

Figure 7. Area 2b–4b of the periostracal groove (TEM): Numerous cilia are interspersed in the microvillar border of the epithelium of the outer fold (4b). The microvillar border of the inner fold (2b) is irregular and some cell protruding processes can be observed (black or white arrowheads). Some secretions of the B type glandular cells are associated with cilia (*). IE, inner mantle epithelium; OE, outer mantle epithelium (×9,000).

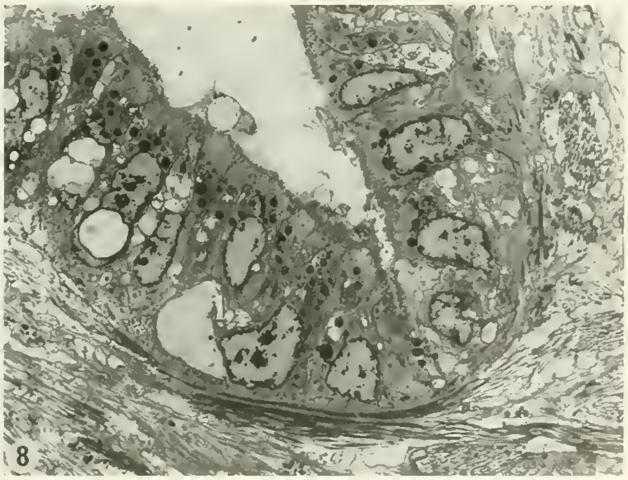

Figure 8. Area 3. Bottom of the periostracal groove (TEM). The cells are cuboidal. Only few secretions and no cilia can be observed in this part of the periostracal groove (×4,000).

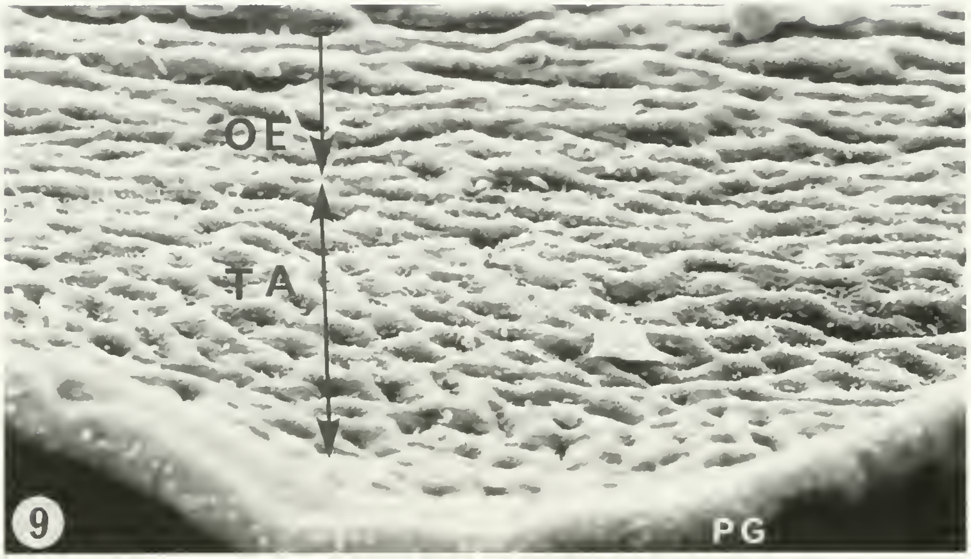

Figure 9. Areas 5 and 6. External structure of the outer mantle epithelium (SEM). Near the periostracal groove (PG), the tubular area (TA) (area 5) appears as honeycomb lobe at the surface of the mantle (double black arrow), whereas the outer epithelium (OE) beyond area 5, covering the whole shell surface (area 6) (single black arrow) have some folds parallel to the periostracal groove. This tubular area (area 5) can be distinguished from the outer epithelium covering the remainder of the mantle (area 6) (×350).

differences of the mantle between bivalves and gastropods due to different shell structure and morphology (Hedegaard, 1997).

As Martin et al. (1983) have demonstrated with their study of the gonads in *Haliotis rufescens*, two cell types can be defined depending on their cytoplasm content: glandular (or secretory) cells and nonglandular cells. In *H. tuberculata*, ultrastructural studies have allowed us to determine three types of glandular cells with regard to their secretion vesicles content. Type A and B glandular cells are present in the whole epithelium, whereas type C cells only occur in the IE and in the inner fold epithelium of the periostracal groove. Type A glandular cells correspond to a typical mucous cell. Type A and B glandular cells are present in various species (for example: *Lymnaea stagnalis*, Zylstra et al. 1978). Type C glandular cells are rarely represented in molluscs and have already been described in the gastropod *Rhodope* sp. (Haszprunar & Künz 1996). Glandular cells, and notably type B, are highly represented in the periostracal groove. While in the IE glandular cells play a role in the protection of the mantle (Lemaire-Gony & Boudou 1997), various authors report the presence of glandular cells, which might facilitate the movement of the periostracum out of the groove or protect the site of periostracum formation against invading water (Bielefeld et al. 1993a; Bielefeld, 1993b; Garcia-Gasca et al. 1994). Some ultrastructural studies demonstrated that glandular cells play a role in periostracum formation (Tsujii 1968a; Tsujii 1968b; Bevelander & Nakahara 1969a; Bevelander & Nakahara 1969b; Nakahara & Bevelander 1971): this role is to be assumed in *H. tuberculata*. The role of glandular cells in mineral deposition is controversial in the studies: Tsujii (1960) suggests that in *Pinctada martensii*, glandular cells in the OE do not participate in shell formation, whereas Beedham (1958) (studying *Anodonta cygnea*, *Mytilus edulis*, and *Ostrea edulis*) believes that secretion may be incorporated in the organic matrix of the shell.

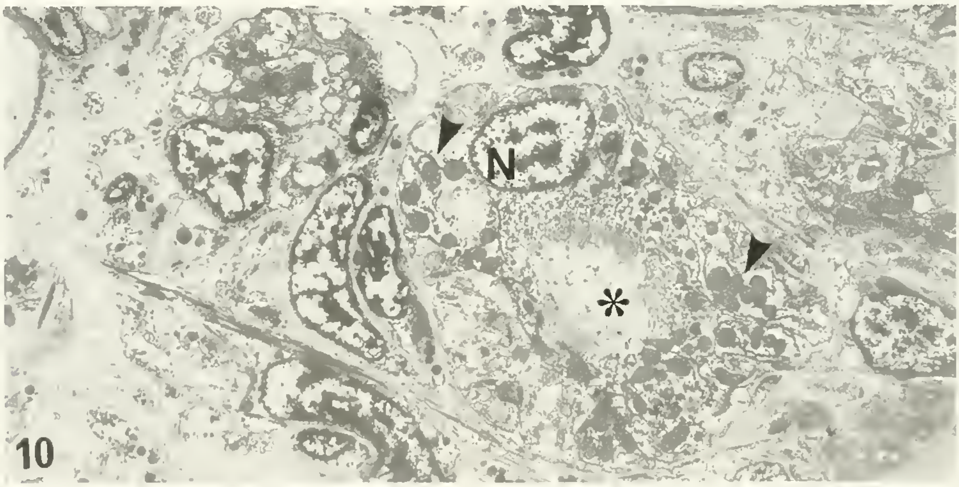

Figure 10. Area 5. Tubular area of the outer mantle epithelium (TEM) in a juvenile abalone. Structure in tubules are shown transversally cut. Cells contain a central nucleus (N) and numerous mitochondria can be observed (arrow heads). In the lumen of the tubule some secretions are present (*) (×4,000).

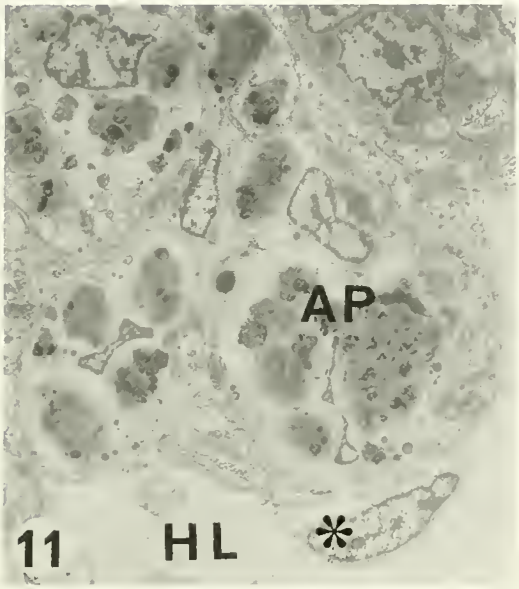

Figure 11. Area 5. Tubular area of the outer mantle epithelium (TEM). Alimentary pigments (AP) are found in vacuoles of wandering hemocyte-like cells. HL, hemolymph lacunae; hemocyte (*) (×3,000).

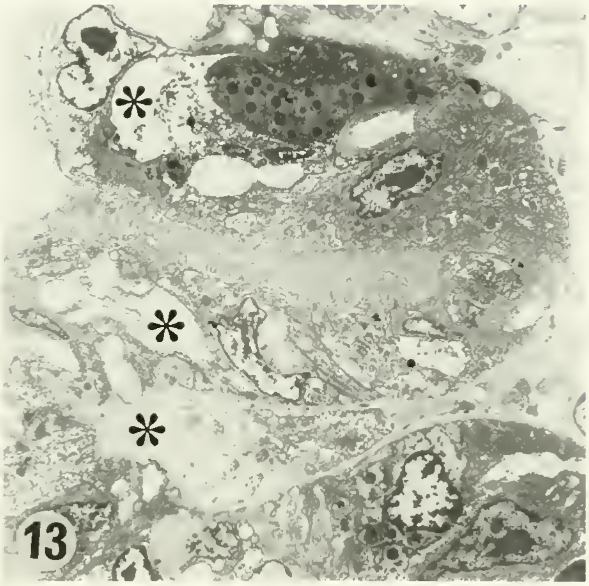

Figure 13. Area 6 of the outer mantle epithelium (TEM). Detail of one fold: Numerous empty cells can be observed (*) (×3,000).

Hillman (1961) suggests that not all glandular cell types could interfere with the biomineralization processes. The organic matrix of the shell mineral layers (which are calcite and aragonite) contains some mucopolysaccharides (Garcia-Gasca et al. 1994). As a consequence, the participation of glandular cells in shell formation could not be ruled out in *H. tuberculata*, and further studies need to be undertaken to determine the nature of each glandular cell and the role of glandular cells in shell formation.

In *H. tuberculata*, the mantle edge ends with a periostracal groove, parallel to the mantle surface, which divides the mantle into two folds: the inner and outer folds. In all molluscs, the periostracum is secreted by the mantle edge, but the mode of formation and the morphological structure of the mantle edge vary from species to species. Generally, in molluscs, the periostracal groove divides the mantle into folds, although it is virtually lacking in some species such as the gastropod *L. littorea* (Bevelander & Nakahara 1970). The mantle edge of bivalves generally consists of three folds compared with the usual two folds among (freshwater or terrestrial) gastropods. In bivalves, the periostracum originates from epithelial cells lining the inner surface of the outer fold of the periostracal groove and/or from a row of basal cells at the bottom of the groove (Kawaguti & Ikemoto 1962a; Dunachie 1963; Neff 1972; Bubel 1973a; Bubel 1973c; Bubel 1973d; Saleuddin 1974; Petit et al. 1979). In some gastropods, such as *Helix* sp., the periostracal groove is described as a single layered, flattened

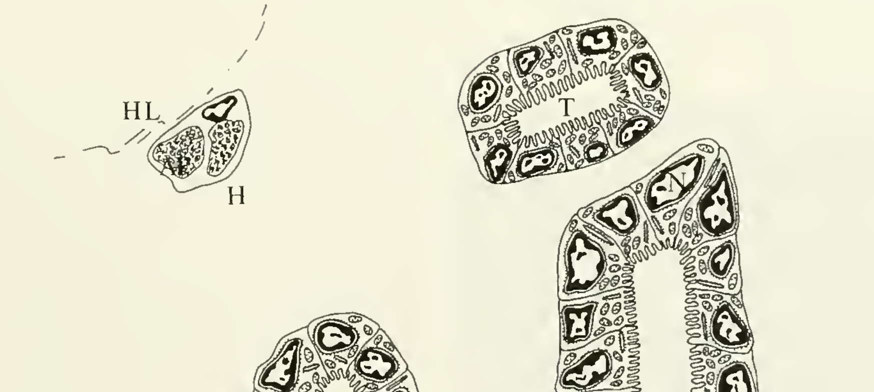

Figure 12. Area 5. Schematic drawing of the tubular area of the outer epithelium (OE). The epithelium is folded and when well developed, it forms tubules (T). Some alimentary pigments (AP) are observed near the hemolymph lacunae (HL). H, hemocyte; M, mitochondria; Mv, microvilli; N, nucleus (not to scale).

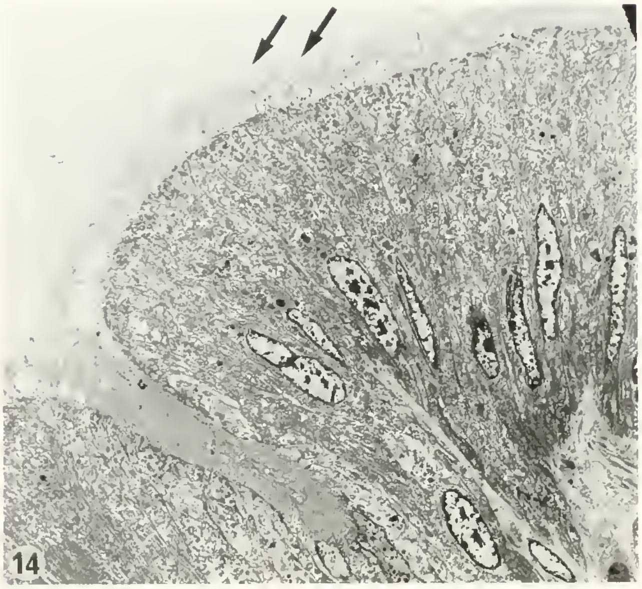

Figure 14. Area 6 of the outer mantle epithelium (TEM). Very high columnar cells are seen; numerous protruding processes (arrows) can be observed within the microvillar border (×2,000).

glandular epithelium called the "periostracal gland" (Beedham 1958), although it does not form a compact body or have a duct. The cells forming the gland seem to be equivalent to the basal cells described in other bivalves (Saleuddin 1974). In some gastropods (*L. stagnalis* and *Biomphalaria pfeifferi*), some different types of gland cells are found which have their cell bodies sunken into the underlying connective tissue at the base of the groove (Zylstra et al. 1978).

In gastropods, the periostracal groove is generally shallow, but it is deeper in *H. tuberculata* and morphologically similar to that in the bivalves: this could be due to the primitive status of this gastropod (Stasek & McWilliams 1973). Periostracal gland and basal cells, described respectively in gastropods and in bivalves (Hillman 1961; Kawaguti & Ikemoto 1962a; Kawaguti & Ikemoto 1962b; Bubel 1973a; Bubel 1973b; Petit et al. 1979), have not been found in *H. tuberculata*. Secretions of material have been observed in the outer surface of the inner fold, whereas it is generally accepted that, in bivalves, the cells lining the inner surface of the outer fold contribute to the periostracum (Bubel 1973b). A group of ciliated cells have been observed in the inner surface of the outer fold of the periostracal groove of *H. tuberculata*. Such cilia have been described in some bivalves (*Pinctada margaritifera*, Jabbour-Zahab et al. 1992), but are generally located at the bottom of the groove. These cilia may help to conduct and aggregate the periostracum material outside the periostracal groove as well as microvilli in the brush border along the inner surface of the outer fold.

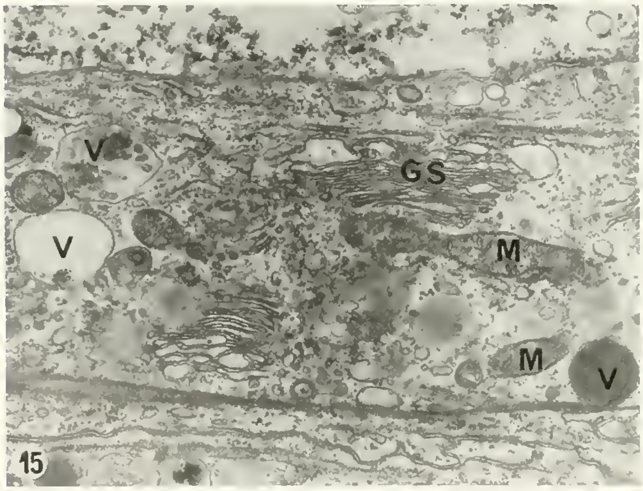

Figure 15. Area 6 of the outer mantle epithelium (TEM). Detail of one very high columnar cell: Inclusions of different content are present in vesicles (V). GS, Golgi stacks; M, mitochondria (×40,000).

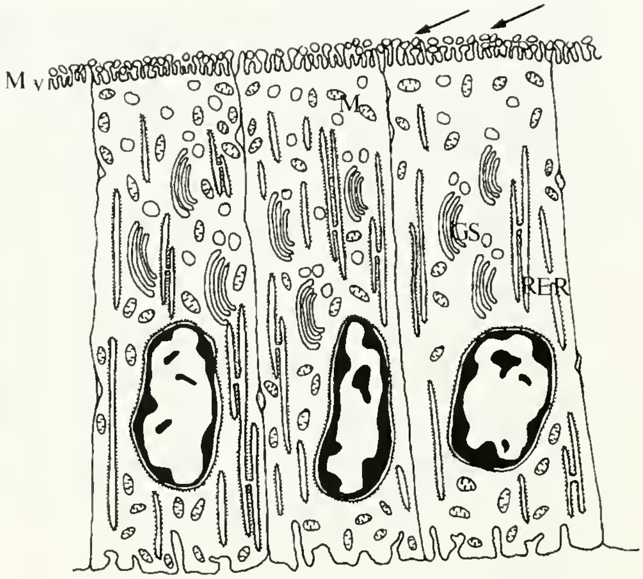

Figure 16. Area 6. Schematic drawing of an active outer epithelium. Palissadic cells are tall and narrow, filled with numerous organites arranged in the longitudinal axis of the cells: Golgi stacks (GS), mitochondria (M), rough endoplasmic reticulum (RER). The microvillar border (Mv) is well developed and some protruding processes (black arrows) are observed (not to scale).

A distinct membrane-like lamellar layer has not yet been observed in any preparation or fixation in *H. tuberculata* periostracal groove, although such a membrane-like layer is reported for other gastropods and bivalves (Saleuddin 1979; Saleuddin & Petit 1983). This could be explained by the fact that animals have not been anesthetized and mantle contractions could tear up the periostracum. Nevertheless, the periostracum has also not been found in juveniles fixed *in toto* with their shell. Anesthetized animals with 3-aminobenzoic acid ethyl ester (MS222) or 7% $MgCl_2$ could be useful to answer this question. Moreover, no periostracal units described in other bivalves or gastropods (Saleuddin 1976; Bielefeld et al. 1993b; Schaefer & Haszprunar 1997) have been observed in cells of *H. tuberculata*. It seems that the secretions and the formation of the periostracum differ from those previously described, and a more detailed study of periostracum formation must be undertaken to determine how the periostracum is secreted.

In *H. tuberculata*, two distinct areas have been identified in the OE. These two areas are morphologically different: both epithelia are folded, but in the outer surface of the outer fold, the epithelium can form tubules, whereas beyond this, the OE folds are quite similar and are almost orientated parallel to the periostracal groove side. In the first area (5), located in the outer surface of the outer fold, cells are cuboidal, and numerous mitochondria have been observed. According to Istin and Masoni (1973), in bivalves, the number of mitochondria underlying the epithelial border of the outer fold indicates a metabolic activity for such cells. This activity is not linked only with calcium movements, but may be linked with matrix components synthesis. In the vicinity of this area, in the connective tissue, and near the hemolymph lacunae, some accumulations of partially digested pigments have been found. These pigments originate from the algae diet, giving red or green color of the prismatic layer, depending on the algae consumed as food. In Haliotidae, these pigments are incorporated into the calcitic layer of shell (Leighton 1961). Thus, this particular area called here the tubular zone (5), can be related to the secretion of the calcitic layer in *H. tuberculata*. This area has already been described by Crofts (1929) as the "glandular acini." This tubular area is always present in juveniles of *H. tuberculata*, but it could be less developed or even absent in adult specimens. A lower development of tubular area in adult specimens could be related to a decrease in shell growth rate compared to juveniles. The presence or the absence of this area may be considered as a cyclic shell secretion, whereas the other area (6) of the OE is likely related to the formation of the aragonitic (nacreous) layer.

This regionalization of the outer mantle epithelium exists in other molluscs: according to Nakahara and Bevelander (1971), in *P. radiata*, the prismatic layer of the shell is derived exclusively from the tall columnar cells lining the outer surface of the outer mantle fold. Although some authors found a distinction between the secretion of the different shell layers (Beedham 1958, *Ostrea edulis*; Kawaguti & Ikemoto 1962b, *Musculus senhousia*; Jabbour-Zahab et al. 1992, *Pinctada maxima*; Schaefer & Haszprunar 1997, *Laevipilina antarctica*), other authors found this distinction not to be effective (Petit et al. 1980 – *Amblema* sp.). In *H. tuberculata*, this regionalization is in accordance with the differences in the organic matrix components of each shell layer and with the presence of alimentary pigments incorporated into the calcitic layer.

In area 6 of the OE in relation with the nacre secretion, two different structures of these epithelia have been found: a tall columnar and a very high, narrow columnar epithelium, depending on the activity of the mantle. These two epithelial structures have been observed, depending on studied specimens. Such morphological differences may be correlated with seasonal and/or individual variations. In the tall columnar epithelium, organelles are not in great number and numerous "empty" cells have been observed, whereas in the very high, narrow columnar epithelium, numerous organelles are found (mitochondria, Golgi complexes, rough endoplasmic reticulum, numerous inclusions of various types). It seems that the last epithelium is much more active than the first. A large number of cytoplasmic vesicles as well as numerous mitochondria were found, indicating an involvement in the mechanisms of shell formation attributed to this epithelium (Wilbur 1964; Istin & Masoni 1973). Moreover, the very high, narrow columnar epithelium is associated with protruding cell processes, which can be related to the secretion of the organic matrix components of the shell (Wilbur 1964; Jabbour-Zahab et al. 1992). In the very high, narrow OE, some distensions of the intercellular spaces have been found: these could act as ion pumps, particularly for calcium (Zylstra et al. 1978; Richardson et al. 1981).

The structure of the mantle margin in *H. tuberculata* is very peculiar and could not be related morphologically to the mantle of other gastropods and bivalves. A spatial organization of the mantle exists in relation with the secretion of the different shell layers: the periostracal groove in relation with the secretion of periostracum, the tubular area (in the outer surface of the outer fold) in relation with the deposition of the prismatic layer, and the OE (beyond the previous area) in relation with the secretion of the nacreous layer. This spatial organization can be related to a temporal variation, because the OE seems to have different structure depending on the stage of secretory activity of the mantle. This temporal variation could be related to the incremental bands deposited annually, monthly, daily, or during another shorter period (Wilbur 1972), because shell formation is incremental rather than continuous.

ACKNOWLEDGMENTS

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DIETARY BIOTIN REQUIREMENT OF JUVENILE ABALONE, *HALIOTIS DISCUS HANNAI* INO

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ABSTRACT The experiment was conducted to quantify the biotin requirement of juvenile abalone, *Haliotis discus hannai* Ino. The possible biotin-synthesizing capacity of intestinal microflora was also examined. Seven purified diets were formulated to provide a series of biotin (0, 0.5, 1.0, 3.0, 6.0, 12.0, 20.0 mg/kg diet). An antibiotic diet was supplemented with tretracycline hydrochloride (4-g/kg diet) in basal diet to suppress possible intestinal bacteria synthesis. To reduce leaching, dietary biotin and other water-soluble vitamins were encapsulated by calcium alginate. Abalone juveniles of similar size (initial mean weight 175.1 ± 3.6 mg; mean shell length 11.66 ± 0.14 mm) were distributed in a flowing-through system using a completely randomized design with eight treatments and three replicates per treatment. They were fed the appropriate diet once every day for a 110-day period. Results of this study clearly showed the necessity of dietary biotin for juvenile abalone as the daily increment in shell length (DISL), visceral pyruvate carboxylase and acetyl-CoA carboxylase activities were significantly influenced by the dietary biotin levels (ANOVA, $P < 0.05$). However, the survival, specific growth rate (SGR) and carcass proximate compositions were not significantly influenced by dietary biotin at the end of the experimental period. The viscera biotin concentration (VBC) of abalone increased as the biotin supplementation level increased ($r = 0.91$). Compared to those of other groups, SGR, DISL, VBC and two carboxylase activities of antibiotic group were obviously depressed. It indicated that the intestinal microflora probably contribute to biotin nutrition for juvenile abalone. Based on measurements of DISL and two carboxylase activities, the optimum biotin requirement was estimated to be 0.42 mg/kg and 0.67–0.70 mg/kg for maximum growth and carboxylase activities, respectively.

KEY WORDS: *Haliotis discus hannai*, biotin, carboxylases, microflora, bio-synthesis, mollusk, nutrition

INTRODUCTION

Biotin is a water-soluble vitamin included in vitamin B complex. As a coenzyme for several carboxylases, such as pyruvate carboxylase (EC 6.4.1.1), acetyl-CoA carboxylase (EC 6.4.1.2), propionyl-CoA carboxylase (EC 6.4.1.3), and methylcrotonyl-CoA carboxylase (EC 6.4.1.4), biotin participates in the reactions of relevant enzymes, and affects the metabolism of amino acids, carbohydrates, nuclear acids and lipids.

Since biotin is one of the most expensive vitamins to add to artificial feed rations, it is necessary to quantify the minimum requirement for the vitamin in order to reduce feed cost. However, several factors have been proven to influence the need for dietary biotin in animals, for example, dietary factors such as the presence of high dietary fat has been shown to obscure the effects of biotin in rats, chicks, brook trout, and rainbow trout (Jacobs et al. 1970; Marson & Donaldson 1972; Poston & McCarteney 1974; Walton et al. 1984). In addition, biotin was found to be synthesized by intestinal bacteria in considerable amounts in some land animals (Victor & Rachel 1945) and freshwater fishes (Sugita et al. 1992). Thus, these factors should be taken into account during estimation of biotin requirements.

Until now, the quantitative requirement of dietary biotin for maximum growth has been studied in only a few species of aquatic animals. Kitamura et al. (1967) reported that 0.01-mg biotin/kg diet was sufficient to support optimum weight gain in rainbow trout fry. The biotin requirement of lake trout appears to be lower than 0.1 mg/kg (Poston 1976). Shiau and Chin (1998, 1999) reported that grass shrimp and tilapia require 2.0–2.4 mg/kg and 0.06 mg/kg of diet, respectively. Günther and Meyer-Buorgdorff (1990) concluded that optimum biotin supply for mirror carp was 2.0–2.5 mg/kg of diet. The deficiency signs of biotin included anorexia, poor food conversion, poor growth, "blue slime" disease, light skin and lower carboxylase activity (Phillips et al. 1950; Ogino et al. 1970; Halver 1972; Poston & McCartney 1974; Robinson & Lovell 1978; Halver 1979).

Abalone is a large algivorous marine mollusk of genus, *Haliotis* (*Gastropoda, Prosobranchia, Archaeogastropoda, Haliotidae*). They are the most commercially important gastropod in aquaculture. At present, limited information has been reported on the essentiality or quantitative requirements of vitamins for abalone. Only the effect of dietary vitamin C on the growth, survival and level of ascorbic acid in the tissues of the abalone *H. tuberculata* and *H. discus hannai* had been investigated (Mai 1998a). For the sake of safety, biotin is generally supplemented in excess to diets for abalone at levels of about 12 mg/kg of diet (Ogino & Kato 1964; Uki et al. 1985; Viana et al. 1993; Mai et al. 1995a; Mai et al. 1995b; Mai 1998a). This dietary level of biotin may not provide maximum efficiency of nutrient utilization and maximum profit margins.

The objective of this study was to quantify the biotin requirements for juvenile abalone, *H. discus hannai* Ino. The possible biotin-synthesizing capacity of intestinal microflora was also investigated.

MATERIALS AND METHODS

Preparation of Vitamin Microsphere

To reduce leaching from diets, dietary biotin, and other water-soluble vitamins were encapsulated with sodium alginate. The method of preparation was modified from Bodmeier and Wang (1993). One hundred milliliters of sodium alginate solution (2%, w/w) containing 2% (w/w) of water-soluble vitamins were mixed with 150 ml of oil containing 6.74 g/L Span80, stirred at 400 rpm for 15 min. The emulsified solution was slowly poured into 1% (w/w) $CaCl_2$ solution, with continuously stirring for 1 min, then filtered below normal pressure. Harvested microcapsules were washed with cyclohexane and absolute alcohol, in turns, to rem

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Corresponding author. Kangsen Mai, Mariculture Research Laboratory, College of Fisheries, Ocean University of Qingdao, Qingdao 266003, Shandong, Peoples Republic of China. E-mail: kmai@ouqd.edu.cn oil and water, dried below normal pressure, then, kept in −20°C until use.

Feed Formulation and Manufacture

The basal diet formulation is given in Table 1. The basal diet contained negligible intrinsic biotin. Dietary treatments were prepared by replacing the dextrin with graded levels of biotin, 0–20 mg biotin/kg diet, in the form of crystalline biotin encapsulated with sodium alginate, was used to prepared seven experiment diets. These diets were designated D0, D0.5, D1, D3, D6, D10 and D20, respectively. The biotin concentrations of the seven diets were determined by HPLC method (Hudson et al. 1984). They contained 0, 0.51, 0.91, 3.04, 6.03, 10.11, and 19.89 mg/kg diet, respectively. A treatment with tetracycline hydrochloride (A. P., Japan) (4 g /kg of diet) was included to ascertain the biotin-synthesizing capacity of intestinal microflora.

Procedures for diet preparation were modified from those described by Mai et al. (1995a, 1995b). Casein, gelatin and some minerals that were in the form of small grains were ground individually using a Pascal Mill and then passed through a mesh with 200-μm pore size. Dry ingredients were weighed on an electronic balance and thoroughly mixed. After adding water (about 120%, v/w) to the mechanically mixed ingredients containing 20% sodium alginate, a paste was made. The paste was shaped into 0.5-mm thick sheets, which were cut into 1-cm^2 flakes. The flakes were dipped into an aqueous solution of $CaCl_2$ (5%, w/v) for 1 min. By this treatment, sodium alginate was converted to an insoluble calcium alginate gel, in which the nutrients were bound (Uki & Watanabe 1992). The surplus solution was drained naturally, then the flakes were sealed in a sample bag and stored at −20°C until use.

TABLE 1.

Composition of the basal diet (g/kg, dry weight basis).[a]

Ingredients	Content
Casein (vitamin-free, Sigma Chemical, St. Louis, MO, USA)	260.0
Gelatin (Sigma Chemical, St. Louis, MO, USA)	65.0
Dextrin (Shanghai Chemical Co., Shanghai, China)	300.0
Carboxymethylcellulose (Sigma Chemical, St. Louis, MO, USA)	50.0
Sodium alginate (Shanghai Chemical Co., Shanghai, China)	200.0
Vitamin mix (biotin-free)[b]	20.0
Choline	5.0
SO/MFO (Food grade)[c]	35.0
Mineral mix[d]	40.0
Filler[e]	25.0

[a] The basal diet contained (dry weight basis): crude protein, 29.67%; crude lipid, 3.68%; gross energy, 18.48 kJ/g.

[b] Vitamin mix, each 1,000 g of diet contained: thiamin HCl, 120 mg; riboflavin, 100 mg; folic acid, 30 mg; PABA, 400 mg; pyridoxine HCl, 40 mg; niacin, 800 mg; Ca pantothenate, 200 mg; inositol, 4,000 mg; ascorbic acid, 4,000 mg; vitamin E, 450 mg; menadione, 80 mg; B_{12}, 0.18 mg; vitamin A, 100 000 IU; vitamin D, 2,000 IU; ethoxyquin, 400 mg. All water-soluble vitamins coated with calcium alginate.

[c] Soybean oil and menhaden fish oil (1:1) with 0.001% ethoxyquin.

[d] Mineral mix, each 1,000 g diet contained: NaCl, 0.4g; $MgSO_4 \cdot 7H_2O$, 6.0g; $NaH_2PO_4 \cdot 2H_2O$, 10.0g; KH_2PO_4, 12.8g; $Ca(H_2PO_4)_2 \cdot H_2O$, 8.0g; Fe citrate, 1.0g; Ca-lactate, 1.4g; $ZnSO_4 \cdot 7H_2O$, 141.2 mg; $MnSO_4 \cdot H_2O$, 64.8 mg; $CuSO_4 \cdot 5H_2O$, 12.4 mg; $CoCl_2 \cdot 6H_2O$, 0.4 mg; KIO_3, 1.2 mg; Na_2SeO_3, 0.4 mg.

[e] Dextrin replaced with biotin supplements.

Leaching

The leaching test of dietary biotin was modified from the method used by Marchetti et al. (1999). Ten-gram aliquots of each feed, stored in nylon bags (mesh size, 149 μm), were immersion in a beaker containing 2 L of sea water maintained at 20.0 ± 1°C and mechanically stirred. Thimerosal was added at 100 ppm to reduce bacterial activity. At the end of allotted time (3, 6, 12 hours, respectively), the remained food was removed from the bags and dried overnight at 60°C in an oven. Dried food was submitted for analysis of total dietary biotin by HPLC. The leaching of dietary supplemented biotin was reflected as retention efficiency (RE), which defined as:

$$\mathrm{RE} = \left[\frac{\text{(Biotin retained in diet after immersion)}}{\text{(Biotin contained in diet before immersion)}}\right] \times 100$$

Animal Rearing

Juvenile abalone (*H. discus hannai* Ino.) used in this experiment was derived from a spawning in June 1999, at Mashan Fisheries Co., Shandong, China. Before the trial, shell lengths were measured with calipers to the nearest 0.02 mm and the animals were weighed to the nearest 0.1 mg using an electronic balance.

Animals were kept in acrylic square cages (35 cm × 28 cm × 20 cm). Each rearing unit was stocked with 40 abalone juveniles. Similar sized juveniles (mean weight 175.1 ± 3.6 mg; mean shell length 11.66 ± 0.14 mm) were assigned to the rearing system using a completely randomized design with eight treatments and three replicates per treatment. The system was flow-through, with water filtered to 30-μm by primary sand filters, then to 10-μm by secondary composite sand filters. The flow rate was about 0.5 L per min per cage. Cages were kept in dim light by screening with black plastic drapes. During the experimental period, water temperature ranged from 18.2 to 22.0°C, salinity 30–34‰, pH 7.6–7.9. Dissolved oxygen was not less than 7.0 mg/l, and there were negligible levels of free ammonia and nitrite (AOAC, 1995).

Prior to initiation of the experiment, the abalone underwent a 1-week conditioning period during which they readily acclimated to environmental conditions. The feeding trial was run for 16 weeks. Abalone were hand-fed with the test diets at a rate equaling 5–10% of abalone wet body weight once daily at 17:00. Every morning, uneaten food and feces were cleaned to maintain the water quality.

Sample Collection and Analysis

At the termination of the feeding trials, to deplete the digestive canal, animals were not fed for 3 d, then all abalone were removed from the rearing system, weighed, measured and counted. Then, 30 abalone from each replicate were quickly frozen (−70°C) for subsequent analysis. Growth was reported as specific growth rate (SGR, %/day) and daily increment in shell length (DISL, μm/d). The formulae are as follows:

$$\mathrm{SGR}\ (\%/\mathrm{d}) = [\,(\ln Wt - \ln Wi)/t] \times 100$$
$$\mathrm{DISL}\ (\mu\mathrm{m/d}) = [\,(\ln SLt - SLi)/t] \times 1000$$

Where, Wt, Wi are final and initial mean weights (mg), respectively; SLt, SLi are final and initial mean shell lengths (mm), respectively; t is the feeding trial period (days).

The frozen samples were finely cut. Then, an aliquot of the whole visceral tissues was hand homogenized with 18 ml of ice-cold phosphate buffer (pH 3.5). The homogenate was centrifuged for 20 min at 3,000 rpm. Then, the biotin was estimated using HPLC method (Hudson et al. 1984). Another aliquot of viscera was homogenized on ice in 5 volumes of Tris-HCl (pH 7.5) buffer. The homogenate was pre-centrifuged (2,000 rpm, 10 min) to reduce the foam and sediment debris. A subsequent centrifugation was carried out at 20,000 rpm for 30 min. The protein concentration of supernatant was assayed following the method of Lowry et al. (1951) using bovine serum albumin as the standard. Then, pyruvate carboxylase (EC 6.4.1.1), and acetyl-CoA carboxylase (EC 6.4.1.2) activities were measured as described by Zempleni et al. (1997). The NaH[^{14}C]O$_3$ (specific radioactivity, 30.1 MBq/mmol; NEN, USA) was used as substrate. Preliminary work with each assay also ensured that enzyme-saturating conditions were achieved. ^{14}C were counted in 5 ml of Ultima Gold XR scintillation fluid (Packard Instrument, Meriden, CT) in a liquid scintillation analyzer Winspectral-1414 (Wallac). Pyruvate carboxylase activity was expressed as units per milligram of protein, where 1 unit equals 1 μmol of H[^{14}C]O$_3$ incorporated into oxaloacetate per minute at 30°C. Similarly, acetyl-CoA carboxylase activity was expressed as 1 unit equaling 1 pmol of H[^{14}C]O$_3$ incorporated into malonyl-CoA per minute at 37°C.

Proximate analyses to determine carcass protein, lipid, and moisture contents were conducted using conventional procedures (AOAC, 1995).

Statistical Analysis

All percentage data were arcsine square-root transformed prior to analysis. Data from each treatment were subject to one-way ANOVA. When overall differences were significant at less than 5% level, Tukeys' test was used to compare the mean values between individual treatments. Statistical analysis was performed using STATISTICA™ package (StatSoft, Inc., USA).

RESULTS

Leaching

The results of the 12-h leaching test of the biotin-supplemented diets are presented in Figure 1. The supplemented biotin contents in all diets decreased with the increasing of the immersion time. After 3 and 6-h of immersion in seawater, the retention efficiencies (RE) were approximately 90.3–96.1% and 76.8–84.2%, respectively; and this value decreased to 42.5–69.2% after 12-h of immersion in all diets. In the first 6-h, there were no differences in the RE among all levels of the biotin supplementation (ANOVA, $P >$ 0.05). After 12-h immersion, the retention efficiencies of all biotin-supplemented diets were significantly different and showed a negative correlation with the biotin supplemental content (r = 0.87). By taking into account the leakage rates in the first 3-h immersion and the amount initially encapsulated, the real amounts of biotin delivered to the abalone were about 0, 0.49, 0.87, 2.88, 5.75, 9.37, and 17.96 mg biotin/kg diet for D0, D0.5, D1, D3, D6, D10, and D20, respectively.

Survival and Growth

Survival, specific growth rate (SGR) and daily increment in shell length (DISL) data are shown in Table 2. During the 110-day experimental period, there were no significant differences in survival (89.2–96.2%.) with any of dietary treatments (ANOVA, $P >$ 0.05). The SGR of the antibiotic group (0.83%/day) was significantly lower than those of biotin treatment groups (1.10–1.28%/day) (P < 0.05). There were no differences observed in SGR among biotin treatment groups during the experimental period (ANOVA, P > 0.05).

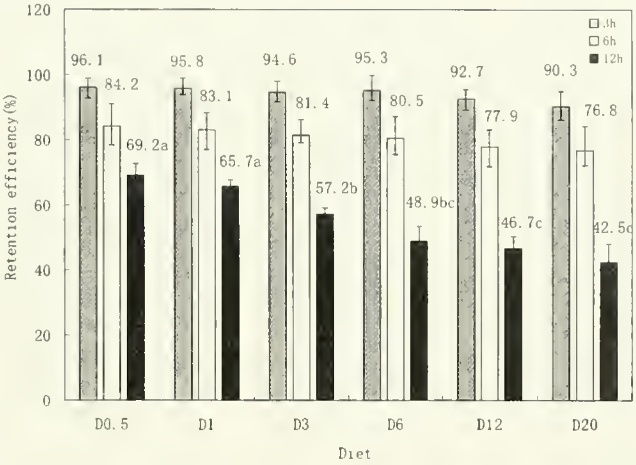

Figure 1. The retention efficiency (RE) of dietary biotin in six biotin supplemented diets at different interval (3, 6 and 12h, respectively) immersed in seawater in triplicate. Error bars are SE. Means with the same letter are not significantly different (P > 0.05). In the first 6h immersion, RE were not significantly different among all diets. After 12h, the values were significantly different and show a negative relationship with the biotin supplemental content (r = 0.87).

Similarly, DISL were significantly higher for dietary biotin treatment groups (60.0–69.1 μm/d) than for the antibiotic group (50.9 μm/d) (P < 0.05). Among all dietary biotin treatments, dietary biotin levels influenced DISL significantly (ANOVA, P < 0.05). DISL increased from 60.0 to 69.1 μm/d with dietary biotin levels increasing to 1mg/kg. There was a decline in DISL observed in abalone fed diets with biotin supplementation higher than 3 mg/kg. Thus, the broken-line regression model (Robbins et al. 1979) was used in this study to express the relationship between DISL and dietary biotin content. The regression equations are shown in Figure 2. As the break point at 0.42 mg/kg gave the least mean square error, the adequate amount of dietary biotin for juvenile abalone is estimated to be 0.42 mg/kg.

Carcass Composition and Biotin Concentration

As seen in Table 3, all the moisture (75.30–77.91%), crude protein (55.87–58.35%), and crude lipid content (5.16–6.37%) of abalone were not affected by dietary treatments (ANOVA, $P >$ 0.05). However, the visceral biotin concentration (VBC) significantly responded to dietary treatments (ANOVA, P < 0.05). The differences among the eight groups were all significant. By addition of 0.4% tetracycline hydrochloride in the basal diet, the VBC was significantly lower than those of other groups were. A linear increase in the VBC was observed when the biotin supplementation level increased (r = 0.91).

Carboxylase Activity

The activities of pyruvate carboxylase (EC 6.4.1.1) and acetyl CoA carboxylase (EC 6.4.1.2) in abalone fed test diets are presented in Table 4. Both enzyme activities showed similar trends

TABLE 2.

Effect of dietary biotin on survival and growth of abalone, *H. discus hannai* Ino. (mean [s.e.], n = 3).

Diet	Dietary Biotin (mg/kg)	Initial Weight (mg)	Initial Shell Length (mm)	Final Weight (mg)	Final Shell Length (mm)	Survival (%)	SGR[1] (%)	DISL[2] (um/d)
D0	0	171.2 (27.5)	11.5 (0.7)	576.9 (18.1)^{bc}	18.1 (0.2)^{ab}	90.0 (7.5)	1.10 (0.15)^{b}	60.0 (5.3)^{b}
D0.5	0.51	173.3 (27.5)	11.6 (0.4)	599.3 (38.9)^{bc}	18.5 (0.2)^{ab}	95.3 (1.4)	1.13 (0.13)^{b}	62.7 (4.0)^{b}
D1	0.91	173.4 (5.0)	11.8 (0.2)	711.8 (29.2)^{d}	19.4 (0.4)^{b}	96.2 (1.4)	1.28 (0.05)^{b}	69.1 (4.0)^{c}
D3	3.04	178.6 (12.5)	11.6 (0.2)	629.0 (85.0)^{cd}	18.7 (0.9)^{ab}	94.2 (5.2)	1.14 (0.08)^{b}	64.5 (6.5)^{bc}
D6	6.03	181.1 (22.5)	11.9 (0.6)	613.5 (43.4)^{bc}	18.7 (0.9)^{ab}	90.8 (3.8)	1.11 (0.08)^{b}	61.8 (5.6)^{b}
D12	10.11	175.0 (5.0)	11.7 (0.3)	599.0 (27.2)^{bc}	18.5 (0.5)^{ab}	92.5 (2.5)	1.12 (0.04)^{b}	61.7 (3.2)^{b}
D20	19.89	177.3 (22.5)	11.7 (0.4)	629.0 (71.6)^{c}	18.0 (0.9)^{ab}	89.2 (7.6)	1.15 (0.17)^{b}	62.0 (1.7)^{b}
Antibiotic[3]	0	170.7 (15.0)	11.5 (0.4)	424.0 (39.4)^{ab}	17.1 (0.3)^{a}	92.5 (2.5)	0.83 (0.01)^{a}	50.9 (2.3)^{a}
ANOVA								
F		0.27	0.46	5.70	4.75	2.52	2.74	3.84
P		0.956	0.851	0.019	0.005	0.059	0.044	0.012

[1] Specific growth rate.

[2] Daily increment in shell length.

[3] Supplemented with 0.4% tetracycline in basal diet.

[a–d] Means in the each column sharing the same letter are not significantly different based on Tukeys' test ($P > 0.05$).

dietary treatments. Compared to the basal group, two carboxylase activities in viscera were significantly depressed when animals were fed the antibiotic diet (93.8 U/mg protein and 7.31 U/mg protein for the activity of pyruvate carboxylase and acetyl-CoA carboxylase, respectively). Apart from the antibiotic diet, the activities of pyruvate carboxylase activity (155.9 U/mg protein) and acetyl-CoA carboxylase (8.33 U/mg protein) of basal group were the lowest among the biotin treatment. The activities of both enzymes generally increased with increasing dietary biotin up to 3-mg/kg diet then leveled off. The differences between the lowest group and the groups fed diets with biotin higher than 1 mg/kg diet were significant ($P < 0.05$). It is obvious that the relationships between both enzyme activities and dietary biotin levels were in accord with the broken-line regression model (Robbins et al. 1979). Based on pyruvate carboxylase and acetyl-CoA carboxylase activities, the regression equations were $Y = 8.97 + 0.03 \times (X - 0.70)$ ($r^2 = 0.93$) and $Y = 171.1 + 0.38 \times (X - 0.67)$ ($r^2 = 0.85$), respectively. Therefore, the biotin requirements were estimated to be 0.67–0.70 mg/kg that based on two carboxylase activities.

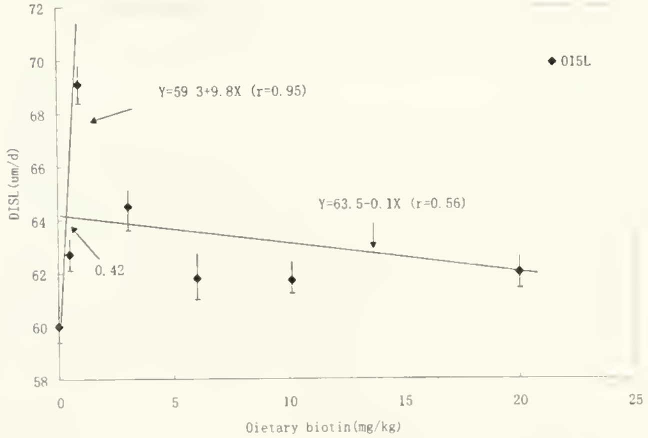

Figure 2. The effect of dietary biotin on daily increment in shell length (DISL) of abalone. Each point represents the mean of three groups o abalone (n = 3), with thirty abalones per group. Based on the broken-line model, the biotin requirement is estimated to be 0.42 mg/kg.

DISCUSSION

Abalone are known to be slow feeders or nibblers. They can take several hours before consuming one feed flake. Thus, it is necessary to reduce leaching to precisely quantify the requirement of water-soluble micronutrients including water-soluble vitamins. Microencapsulation is one of the most potential methods to reduce leaching by sheltering encapsulated materials from outer environment (Louis 1970; Gupta & Rao 1985; Shun et al. 1988). According to Marchetti et al. (1999), the retention efficiencies of lipid-walled vitamin B_1 in pelleted food were 87.5% and 72.5% for 1 and 2-h immersion, respectively. In the present study, the RE of biotin was up to 90.3–96.1% after 3-h immersion. It is evident that leaching out of biotin from the experimental diets was reduced in a great degree. A previous study indicated that the guts of most

TABLE 3.

The effects of dietary biotin on carcass composition and VBC[1] of abalone, *H. discus hannai* Ino. (mean [s.e.], *n* = 3.

Diet	Protein[2] (%)	Lipid[3] (%)	Moister (%)	VBC (ng/g)
D0	56.30 (0.14)	5.74 (0.78)	75.30 (3.85)	202.2 (17.6)^{b}
D0.5	56.88 (0.10)	6.37 (0.38)	75.51 (0.81)	292.3 (21.9)^{c}
D1	56.73 (1.60)	5.64 (0.51)	75.73 (0.28)	369.4 (27.4)^{d}
D3	58.16 (0.59)	6.15 (0.64)	77.91 (1.11)	555.2 (40.1)^{e}
D6	58.35 (1.92)	5.79 (0.25)	76.03 (0.42)	936.2 (22.2)^{f}
D12	56.39 (0.83)	5.16 (0.44)	76.60 (1.22)	1068.4 (31.3)^{g}
D20	56.42 (0.67)	5.59 (0.28)	77.31 (2.77)	1208.7 (59.2)^{h}
Antibiotic[4]	55.87 (0.97)	6.04 (0.04)	76.50 (0.47)	75.8 (7.2)^{a}
ANOVA				
F	2.21	1.22	0.75	566.23
P	0.089	0.135	0.636	0.000

[1] Viscera biotin concentration.

[2] Dry weight basis.

[3] Dry weight basis.

[4] Supplemented 0.4% tetracycline in basal diet.

[a–h] Means in the each column sharing the same letter are not significantly different based on Tukeys' test ($P > 0.05$).

TABLE 4.

Effect of dietary biotin on carboxylase activities in viscera of abalone *H. discus hannai* (mean [s.e.], $n = 3$).

Diet	Pyruvate Carboxylase[1] (U/mg Protein)	Acetyl-CoA Carboxylase[2] (U/mg Protein)
D0	155.9 (4.07)^{b}	8.33 (0.19)^{b}
D0.5	167.5 (6.63)^{bc}	8.80 (0.14)^{bc}
D1	173.2 (5.59)^{c}	9.36 (0.21)^{c}
D3	175.0 (7.72)^{c}	9.45 (0.33)^{c}
D6	171.8 (8.33)^{c}	9.20 (0.29)^{c}
D12	174.7 (9.25)^{c}	9.32 (0.14)^{c}
D20	178.3 (5.31)^{c}	9.40 (0.24)^{c}
Antibiotic[3]	93.8 (6.22)^{a}	7.31 (0.21)^{a}
ANOVA		
F	93.88	41.12
P	0.000	0.000

[1] Per unit equal 1 μmol of $H[^{14}C]O_3$ incorporated into oxaloacetate per minute at 30°C.

[2] Per unit equal 1 pmol of $H[^{14}C]O_3$ incorporated into malonyl-CoA per minute at 37°C.

[3] Supplemented with 0.4% tetracycline in basal diet.

[a–c] Means in the each column sharing the same letter are not significantly different based on Tukeys' test ($P > 0.05$).

abalone were full of food after 2 to 3-h feeding with premium quality diets (Mai et al. 1998b). There were no significant differences observed in retention efficiency when all diets immersed in seawater for 6-h (ANOVA, $P > 0.05$). Thus, in this study, the leaching may not influence the result of biotin requirement for abalone by encapsulating biotin and other water-soluble vitamins. On the other hand, wall-forming material calcium alginate is apt to be digested by abalone as high level of alginase appears in the digestive tract of abalone (Oshima 1931; Nakado & Sweeny 1967; McLean 1970; Toshio 1985; Hugo & Maria 1998). In comparison to those reported by other authors (Uki et al. 1985; Uki & Watanabe 1992; Mai et al. 1995a; Mai et al.1995b), the satisfactory abalone growth indicates encapsulated vitamins can be efficiently utilized by abalone and supports our conclusions as follows.

Until now, the essentiality of dietary biotin for any mollusk species was unknown. Results of this study clearly show that dietary biotin is necessary for juvenile abalone as the shell growth (DISL) was significantly influenced by dietary biotin. The shell growth of abalone fed lower levels of dietary biotin was depressed implying that biotin probably affects the course of biomineralization of shell. This is in accord with the findings of Bain et al. (1988) that biotin deficiency affects bone growth in broiler chick. During the 110-day experimental period, dietary biotin did not significantly affect SGR. However, there still was an obvious trend that lower biotin levels reduced SGR. As we know, biotin is a type of micronutrient in diets and the signs of biotin deficiency are usually produced under specific conditions. Thus, a 110-day experimental period is probably not long enough to significantly affect SGR in abalone. In juvenile animals, body length growth is usually faster than the body weight gain. This agrees with DISL being a more sensitive parameter to dietary biotin than SGR, and DISL may be a responsive criterion for estimating dietary biotin requirement for other mollusk juveniles. Based on the daily increment in shell length (DISL), the optimum biotin requirement for the maximum growth is estimated to be 0.42 mg/kg diet by the broken-line regression analysis. Compared with other reports, the optimum requirement for juvenile abalone is higher than that of brook trout (0.05 and 0.25 mg/kg) (Poston & McCartney 1974), lake trout (0.1 mg/kg) (Poston 1976), rainbow trout (0.05–0.14 mg/kg) (Woodward & Frigg 1989; Castledine et al. 1978), common carp (0.02–0.03 mg/kg) (Ogino et al. 1970), tilapia (0.06 mg/kg) (Shiau & Chin 1999), but less than the requirements reported for the mirror carp (2.0–2.5 mg/kg) (Günther & Meyer-Buorgdorff 1990) and grass shrimp (2.0–2.4 mg/kg) (Shiau & Chin 1998). The different biotin requirements probably attribute to the differences of experimental procedures and species.

The presence of high dietary fat has been shown to obscure effects of biotin in rats, chicks, brook trout, and rainbow trout (Jacobs et al. 1970; Marson & Donaldson 1972; Poston & McCarteney 1974; Walton et al. 1984). In the present study, 3.68% of lipid was measured in the basal diet. According to Mai et al. (1995a), optimum dietary lipid content was 3–5% to maintain maximum weight gain for abalone. Therefore, 3.68% of lipid is just sufficient to meet requirements of the abalone and lipid effects on biotin requirements can be omitted here.

As a coenzyme for several carboxylases, biotin affects the metabolism of amino acids, carbohydrates, and lipids. Thus, some studies have demonstrated that the proximate composition of animals usually respond to dietary biotin treatment. Poston (1970) reported the carcass fat content of lake trout was depressed when animals were fed biotin-free diets. The crude ash, fat and protein contents of mirror carp were significantly affected by dietary biotin (Günther & Meyer-Buorgdorff 1990). Similar results were also obtained with common carp (Ogino 1970) and tilapia (Shiau & Chin 1999). However, in the current study, the crude protein and lipid contents in the soft body tissues, which consist of mantle, foot muscle, and viscera that include all inner organs, of juvenile abalone remained independent of biotin supplementation. This effect may be manifested by a longer experimental duration. It is also generally believed that carboxylase activities are depressed in biotin deficient animals such as mammals, fishes and shrimp (Deodhar & Mistry 1969; Arinze & Mistry 1971; Poston & McCarteney 1974; Castledine et al. 1978; Walton et al. 1984; Shiau & Chin 1998, 1999). In the present study, the pyruvate carboxylase and acetyl-CoA carboxylase activities of abalone visceral tissues positively responded to dietary biotin levels. This implies that abalone did experience biotin deficiency, which would lead to depressed enzyme activities. Thus, both carboxylase activities are useful criteria in estimating dietary biotin requirements for abalone. Based on two carboxylase activities, the optimum biotin requirement for the maximum enzyme activities is estimated to be 0.67–0.70 mg/kg diet by the broken-line regression analysis. In the present study, the recommended biotin requirement for maximum carboxylase activities is higher than that for maximum growth (0.42 mg/kg diet). This means that the biotin level resulting in maximum enzyme activities and the level resulting in maximum growth are not necessarily the same.

In some species of land animals, such as calves and cows, biotin has been found synthesized in considerable amounts by intestinal bacteria (Victor & Rachel 1945). In the present study, when tetracycline was incorporated into basal diet as an antibiotic group, a deficiency developed. The SGR DISL of antibiotic group were significantly lower than those of the basal group ($P < 0.05$). Similarly, the visceral biotin concentration (VBC) and the carboxylase activities were also obviously depressed by addition of tetracycline. Sugita et al. (1992) examined the biotin-producing ability of intestinal microflora in freshwater fishes and proved requirement of freshwater fishes for biotin was significantly influenced by intestinal microflora. Thus, we believe that the poor growth of the abalone in the treatment with antibiotics could attribute to the suppression of intestinal microflora that can probably synthesize biotin. Further investigation is needed to provide direct evidence.

Another interesting phenomenon is that VBC increased from 292.3 to 1208.7 ng/g with increasing biotin supplemental levels (r = 0.91). Theses results indicate that biotin can be stored in abalone viscera in amounts corresponding to the dietary supply. Therefore, the VBC can be used to monitor biotin status in abalone. To our knowledge, the toxicity of biotin in animals is still uncertain, however, depressed growth was observed when abalone were fed high levels of dietary biotin and this would require further studies.

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IDENTIFICATION OF SOUTHERN HEMISPHERE ABALONE (*HALIOTIS*) SPECIES BY PCR-RFLP ANALYSIS OF MITOCHONDRIAL DNA

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ABSTRACT Illegal fishing and species-substitution of abalone (genus *Haliotis*), a highly valuable marine gastropod, are of worldwide concern. A mitochondrial DNA PCR-RFLP analysis of fragments of the cytochrome oxidase I (mtCOI) and II (mtCOII) genes was developed for the identification of 11 Southern Hemisphere species of abalone. These included five temperate and one tropical species from Australian waters, three temperate species from New Zealand and two temperate species from South Africa. All species, with the exception of the *Haliotis rubra*/*H. conicopora* complex, can be unequivocally identified using the combined profiles from four individual restriction enzyme digests (*DdeI*, *HhaI*, *HinFI* and *HpaII*) on a 193 base pair fragment of mtCOI. Six species each displayed a unique profile for a single restriction enzyme. A one hundred and fifty-nine base pair fragment of mtCOII allowed individual identification of six of the species using the combined profiles from five individual restriction enzyme digests (*DdeI*, *EcoRV*, *HhaI*, *HpaII*, and *RsaI*). These primers failed to amplify in *H. iris*. Again *H. rubra* and *H. conicopora* could not be separated, and neither could *H. australis* and *H. spadicea*. No DNA sequence variation in either fragment was observed between *H. rubra* and *H. conicopora*; the latter may be a subspecies of *H. rubra*. The use of both fragments and a minimum of two restriction enzymes is recommended for species differentiation. DNA was successfully extracted, PCR amplified and identified from canned tissue and mucous samples of *H. rubra*. A conformational mutation in the mtCOI fragment was observed in *H. midae*, but in no other species nor in the mtCOII fragment.

KEY WORDS: *Haliotis*, abalone, mitochondrial DNA, identification

INTRODUCTION

Abalone, genus *Haliotis* Linnaeus, are a highly valuable commercial marine univalve mollusk. There are over 55 recognized species worldwide (Geiger 1998), of which nearly half are exploited by commercial or recreational divers. Abalone generally inhabit rocky reefs to depths of 65 m, but more are usually found in shallower waters to 30 m. The foot muscle of abalone attracts high prices in Asian markets, with species differential. Once the distinguishing shell and mantle tissue have been removed, it is very difficult to differentiate the commercial product of one species from another. The high price, market demand, ease of harvest and similarity of processed product between species makes abalone very suitable targets for illegal marketing and both highly organized and small scale poaching.

Abalone poaching and species-substitution of abalone products is of concern to many countries, including the USA (Daniels & Floren 1998), Mexico (Ponce-Díaz et al. 1998), South Africa (Sweijd et al. 1998) and Australia. The value of the illegal trade is difficult to quantify. Conservative estimates in Australia alone are over $US25M annually. The legal Australian abalone fisheries, dominated by *Haliotis rubra*, account for about half the annual world abalone harvest of ca. 10,500 mt (FAO 2000) and is worth around $US80M per year. The South African abalone fishery (*H. midae* ca. 500 mt/year) is worth approximately $US15M with legal sales of confiscated (poached) abalone from just one area fetching over $US1m (Sweijd et al. 1998). In New Zealand the main commercial species is *H. iris* and the illegal harvest is estimated at about 33% of the annual commercial catch of 1,300 mt (Roberts et al. 1999). The high but unknown level of illegal harvesting of abalone creates major problems for fishery managers endeavoring to maintain viable and economic fisheries.

Whilst *H. rubra* is the dominant commercial species within temperate Australian waters, both *H. laevigata* and *H. roei* are subject to significant levels of commercial fishing under independent quota systems, and a *H. scalaris* fishery is under consideration. A problem for fisheries enforcement is the overlapping ranges of these and non-commercial species. Such species richness is common with abalone (Geiger 1999), and after removal of the characteristic shell and mantle species identification of the foot muscle is obscure. The need exists for a definitive means of identifying tissue and by-products (e.g., mucous in instances of suspected poaching when tissue has been disposed) of individual abalone species.

Identification of plant and animal species when morphological characters have been removed is possible using either protein or DNA-based methods (Palumbi & Cipriano 1998; Toro 1998; Johannesson & Stenlid 1999; Hare et al. 2000; Sweijd et al. 2000). The protein based methods are very dependent on tissue quality; generally requiring fresh or frozen material. Often identification for commercial needs may require analysis of processed (dried or canned) tissue or degraded tissue. DNA-based methods are relatively independent of tissue quality, and those that rely on amplification of small DNA fragments are less likely to be affected by degradation (Mackie et al. 1999). A number of techniques are available for species identification including: random amplification of polymorphic DNA (RAPD) (Martinez & Malmheden Yman 1998), restriction fragment length polymorphism (RFLP) analysis (Innes et al. 1998; Wolf et al. 2000), direct DNA sequencing (Quinteiro et al. 1998) and single-stranded conformation poly

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Corresponding author. Nicholas G. Elliott. CSIRO Marine Research, GPO Box 1538, Hobart, Tasmania 7001, Australia. E-mail: nick.elliott@marine.csiro.au morphism (SSCP) (Mackie et al. 1999) of PCR (polymerase chain reaction) amplified fragments.

A PCR-RFLP analysis of a 1,300 base-pair (bp) fragment of the nuclear lysin gene was devised for identification of two South African abalone species, *Haliotis midae* and *H. spadicea* (Sweijd et al. 1998). Generic PCR primers that amplify across the intron differentiated between species based on the size of the intron. Preliminary analyses found that the size of the lysin intron varied greatly between other *Haliotis* species (generally 500 to 1,100 bp), but the intron in the Australian greenlip abalone *H. laevigata* was over 4,000 bp (unpublished data). Products of such size are not ideal for species identification tests with the likelihood of unreliable PCR products due to tissue and DNA degradation.

To differentiate between the more common Southern Hemisphere abalone species a PCR-RFLP method was developed using short fragments (less than 200 bp) of the mitochondrial DNA (mtDNA) molecule. To satisfy potential legal scenarios in Australia and South Africa, 11 species were included. Within species variation and potential non-*Haliotis* amplification of our designed primers were examined in addition to testing the primers with canned abalone tissue and abalone mucous samples.

MATERIALS AND METHODS

Sample Collection and DNA Extraction

Whole individuals (live or frozen) or alcohol preserved tissues were obtained for 11 purported *Haliotis* species: *Haliotis asininal* Linnaeus, Queensland, Australia (30 individuals); *Haliotis australis* Gmelin, New Zealand (10); *Haliotis conicopora* Péron, Western Australia, Australia (11); *Haliotis iris* Gmelin, New Zealand (10); *Haliotis laevigata* Donovan, Tasmania & Victoria, Australia (62); *Haliotis midae* Linnaeus, South Africa (10); *Haliotis roei* Gray, Western Australia, Australia (10); *Haliotis rubra* Leach, Tasmania, Victoria & New South Wales, Australia (50); *Haliotis scalaris* Leach, Tasmania & Western Australia, Australia (22); *Haliotis spadicea* Donovan, South Africa (10); *Haliotis virginea* Gmelin, New Zealand (10).

Total genomic DNA was extracted from ca. 25 mg of foot muscle or gill tissues using a modified CTAB (hexadecyltrimethlammoniumbromide) protocol (Grewe et al. 1993). Tissue was incubated overnight at 50°C instead of 30 to 60 min at 60°C.

To verify the use of our PCR primers on processed product, DNA was extracted from commercially canned *H. rubra*. Approximately 0.5 g tissue was digested for 30 min at 65°C in 5 mL digestion buffer (100 mM Tris, 50 mM EDTA, 400 mM NaCl, 1% SDS). 50 µL proteinase K (10 mg/mL) was then added and the solution was incubated overnight at 55°C. 150 µL NaCl (5 M) and 520 µL of 10% CTAB were added and the solution incubated at 65°C for 1 h with regular mixing. Samples were then extracted once with equal volumes of chloroform/isoamyl alcohol (24:1) and precipitated with 2 volumes of 100% ethanol. Precipitated DNA was washed twice with 70% ethanol, once with 100% ethanol, air-dried and re-suspended in 200 µL TE.

PCR amplification was also tested using DNA extracted from *H. rubra* mucous. Two mucous samples were obtained by placing individual freshly captured *H. rubra* in separate plastic bags for approximately 2 h, removing the abalone and placing the bag and fluid contents at 4°C. Tissue samples were taken from the individual abalone as positive controls for DNA extraction. DNA was extracted from mucous swabs taken from the sides of the bags and from the control tissue samples using the modified CTAB protocol described above. In addition, a 600 µL sample of fluid (mixture of seawater and mucous) from the bottom of each plastic bag was taken, incubated overnight in 20 µL proteinase K (10 mg/mL) and 5% SDS, and then genomic DNA extracted using the same modified CTAB protocol.

Genus specificity of the PCR amplification was tested on total genomic DNA extracts (using above CTAB protocol) from a variety of marine organisms. These consisted of an alga (unidentified red alga), an anemone (unidentified), a crustacean (Antarctic krill *Euphausia superba*), mollusks (unidentified chiton and Pacific oyster *Crassostrea gigas*) and teleosts (bigeye tuna *Thunnus obesus*, southern bluefin tuna *T. maccoyii*, pink ling *Genypterus blacodes*, Patagonian toothfish *Dissostichus eleginoides*, school shark *Galeorhinus galeus* and gummy shark *Musteleus antarcticus*).

PCR Primers and Amplification

Generic PCR primers were designed for the mitochondrial cytochrome *c* oxidase subunit I gene (mtCOI) by alignment of either our own unpublished or published *Haliotis* sequences (Metz et al. 1998). DNA sequences used for the design of the mitochondrial cytochrome *c* oxidase subunit II gene (mtCOII) were either our own or other unpublished sequences (Sandy Degnan, University of Queensland).

The primers designed to amplify a 193 bp fragment of the mtCOI gene were designated HALCO1-NG1 (5′-CIGACATRGCITTYCCICGACT-3′) and HALCO1-NG2 (5′- CCGGCTARGTGIAGIGARAAAAT-3′). Those designed for a 159 bp fragment of the mtCOII gene were designated HALCO2GENA (5′-CAATYTGAACYATTCTMCCAGC-3′) and HALCO2GENB (5′-CCTTAAARTCTGAGTATTCGTAGCC-3′). (Degenerate nucleotide IUB codes: I, Inosine = A, C, G or T; M, aMino = A or C; R, puRine = A or G; Y, pyrimidine = C or T).

PCR reactions consisted of 50 to 100 ng of total genomic DNA, 2.5 mM $MgCl_2$, 200 µM each dNTP, 10 pmoles of each primer, and 0.55 U *Taq* DNA polymerase (Biotech) in a buffer supplied by the manufacturer. PCR amplifications were carried out in a 50 µL final volume using a Perkin Elmer GeneAmp® System 9600 with hotlid. The cycling parameters were as follows: denaturation at 95°C for 3 min, 10 initial amplification cycles (94°C for 30 s, 60–55°C for 30 s, 72°C for 1 min, with a decrease in the annealing temperature of 0.5°C per cycle), a further 25 amplification cycles (94°C for 30 s, 55°C for 30 s, 72°C for 1 min) and final extension at 72°C for 5 min. Negative controls, without DNA template, were prepared for each series of amplifications to exclude the possibility that PCR reagents and buffers were contaminated with template DNA. Amplification products were examined by electrophoresis through a 2% agarose gel (GIBCOBRL) made up in 1 X TBE. Gels were stained in ethidium bromide at a concentration of 0.5 µg/mL and visualized under UV light. A one-hundred bp ladder (GIBCOBRL) was run concurrently to facilitate sizing of amplification products.

DNA Sequencing

PCR products were sequenced to confirm variation in restriction fragments and sizes, and to improve PCR primer design. PCR products were purified using Wizard™ PCR purification columns (Promega) according to manufacturers instructions, and sequenced using an ABI Prism™ BigDye™ Terminator Cycle Sequencing Ready Reaction Kit (Perkin Elmer). Cycle sequencing reactions were electrophoresed on an ABI377 automated DNA sequencer (Perkin Elmer) and analyzed using ABI Prism™ Sequencing Analysis Version 3.3 (Perkin Elmer).

RFLP Analysis

For each individual of the 11 species, four separate restriction digestions of the mtCOI fragment were performed using the four enzymes *DdeI, HhaI, HinFI* and *HpaII* (New England Biolabs, Genesearch). For the mtCOII fragments five separate restriction digestions were performed for each species individual using the enzymes *DdeI, EcoRV, HhaI, HpaII* and *RsaI* (New England Biolabs, Genesearch). Restriction digestions were carried out in a 15 μL total volume consisting of 5 μL of PCR product, 1.5 μL digestion buffer supplied by the manufacturer, 0.5 μL enzyme, and 8 μL ddH$_2$0 for all enzymes except *HhaI*. Digestions for *HhaI* were carried out in a 15 μL total volume consisting of 5 μL of PCR product, 1.5 μL digestion buffer supplied by the manufacturer, 0.5 μL enzyme, 1.5 μL 10 X Bovine serum albumin (BSA) and 6.5 μL ddH$_2$0.

Mitochondrial haplotypes were scored by electrophoresis of 10 μL of digested PCR product in a 3% agarose gel made up in 1 X TBE at 100V for 3 h, stained in ethidium bromide (0.5μg/mL) and visualized under UV light. Electrophoresis of restriction digestions was also performed on 12% polyacrylamide (Austral Scientific) gels made up in 1 X TBE and run for 2 h at 100 V.

RESULTS

DNA Extraction and PCR Amplification

DNA extractions from fresh, alcohol preserved and canned tissue, resulted in high yields of high molecular weight total genomic DNA. Amplification of these extracts consistently produced high quality PCR products.

Extractions from mucous scrapings and fluid samples from plastic bags produced a small amount of high molecular weight genomic DNA. PCR amplification of these extracts failed at times to yield a product when undiluted, however when diluted 10 fold, produced a strong PCR product in all samples (Fig. 1).

PCR amplification of non-*Haliotis* DNA with the designed primers was only observed in the tuna samples. Both tuna species amplified (160 bp fragment) with the mtCOI primers. Sequencing of the tuna mtCOI products confirmed that the observed product was not contamination from abalone DNA. While nucleotide differences and RFLP cut site differences existed to separate these teleost products from abalone products, high levels of nucleotide sequence conservation suggests that the amplified product was part of the tuna COI gene.

Restrictian Digests mtCOI

The expected 193 bp fragment was generated in each abalone species following PCR amplification with the HALCOI-NG1/HALCOI-NG2 primers. Comparison of the DNA sequences indicated suitable restriction sites for discrimination between species using four restriction enzymes (Fig. 2).

Intraspecies restriction digest polymorphisms were observed in four species, but in each case for a single individual for only one enzyme (Table 1). Two of the observed polymorphisms were the result of a loss of a restriction site and two the result of a gain. All individuals showing a different restriction fragment profile for the species were sequenced to confirm the profile. All other digestions returned a single restriction pattern in all individuals examined for each species.

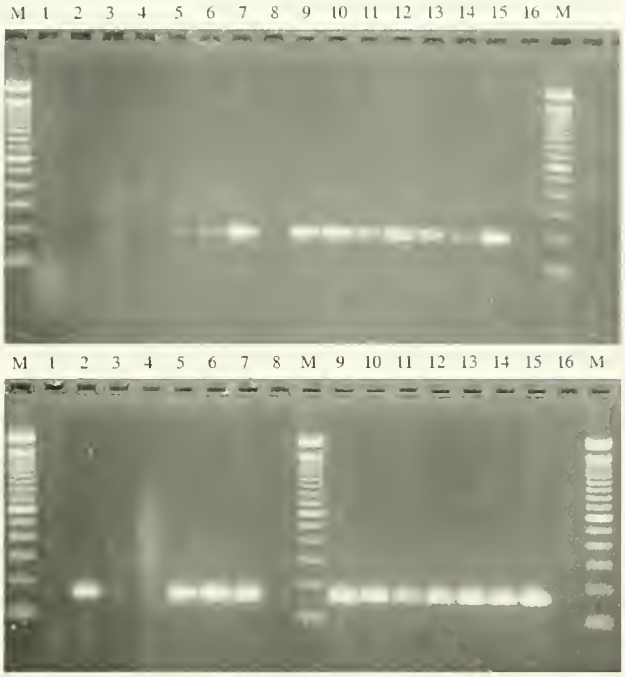
M 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 M
M 1 2 3 4 5 6 7 8 M 9 10 11 12 13 14 15 16 M

Figure 1. Electrophoretic analysis of undiluted (lanes 1 to 6) and diluted (x10, lanes 9 to 14) 193 bp mtCOI (upper image) and 159 bp mtCOII (lower image) PCR fragments for *H. rubra* tissue and mucous samples. M = 100 bp ladder. Samples in lanes are as follows: 1, 2, 9 and 10 muscle tissue; 3, 4, 11, and 12 fluid sample from plastic bag; 5, 6, 13, and 14 mucous swab from plastic bag; 7 and 15 positive *H. rubra* DNA (x20 dilution) control; 8 and 16 negative H$_2$O control.

At this 193 bp fragment, six of the eleven species had a unique species-specific restriction pattern for at least one enzyme, and so could be individually identified (Table 1). With the exception of the *H. rubra* and *H. conicopora* pairing, all species are discernible from each other using the four restriction enzymes, regardless of all but one observed polymorphism. The exception polymorphism was a single *H. rubra* individual that had gained a *DdeI* cut site, and therefore had a profile similar to *H. scalaris*. The restriction profiles for the canned tissue, mucous and fluid samples all matched that expected for *H. rubra*.

One purported *H. scalaris* individual returned a different profile at three enzymes to all other *H. scalaris* individuals. This particular individual displayed the expected cut pattern for *H. laevigata* for all four enzymes; three of which are diagnostic between the two species for all other specimens analyzed. Laboratory contamination was ruled out and the results confirmed with repeated tissue sampling, DNA extraction and PCR amplification for this one individual.

The observed fragment lengths produced in this study were all examined on agarose and (non-denaturing) polyacrylamide gels and confirmed by sequence analysis. A fragment mobility change was observed in the mtCOI fragment for *H.midae* when run on a polyacrylamide gel (Fig. 3). This assumed conformation-induced mutation was only observed in *H. midae*.

Restriction Digests mtCOII

The expected 159 bp fragment was generated in each abalone species following PCR amplification with the HALCOIGI N[illegible]

```
                  1                                                  50                                                     100
H.rubra           CTGACATGGC TTTTCCTCGA CTAAATAATA TAAGATTCTG ACTACTCCCA CCCTCACTAA CCCTTCTATT AACATCGGGT GCTGTAGAAA GTGGTGCCGG
                                                      HinFI                                                                HpaII
H.conicopora      .......... .......... .......... .......... .......... .......... .......... .......... .......... ..........
                                                      HinFI                                                                HpaII
H.laevigata       .C........ ...C...... .......... .......... G..T...... ..T....... .T..GT.... G.....A... ..C....... .......T..
                                                      HinFI
H.scalaris        .C........ C..C...... ........C. .G........ ...T...... ..T....... .T..G..... ......A... .......... ..........
                                                      HinFI                                                                HpaII
H.asinina         .......A.. C..C..A... .....C..C. .G........ .........C ..A..C..C. .T..A..TC. ......C..G .....C..G. .......A..
                                                      HinFI                                     HpaII
H.roei            .......... C..C...... ..T..C.... .......... ...C...... ..T....... .T..GT.... ......A... .......... ..........
                                                      HinFI  HinFI                                                         HpaII
H.midae           .A.....A.. A..C...... ..T.....C. .......... ...T..T..C ..A..TT.G. ....A..T.. ......A..G .......... .......A..
                                                      HinFI
H.spadicea        .C........ ...C...... ..C.....C. .G........ ...T...... ..T...T... .TT.A..C.. .......... .......... .......A..
                                     HinFI            HinFI
H.australis       .A.....A.. C.....C... ........C. .......T.. G..T..T..C ..A..T..C. .......TC. G........C .......... .C........
                                                                                                       HhaI                HpaII
H.iris            .A..T..... .......... ..C..C..C. .......T.. ......T..C ..A..C.... .T.....CC. ......A..A ..C....... .C.....A..
                                     HinFI
H.virginea        .G........ G..N..G... ..T..C.... .......T.. ...T.....T ..G..CT... .T..A..TC. C.....A..G ..C....... ....C..A..
                                                                                                                        HhaI
HALCO1-NG1        CIGACATRGC ITTYCCICGA CT-------- ---------- ---------- ---------- ---------- ---------- ---------- ----------
HALCO1-NG2        ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ----------

                  101                                               150                                                    200
H.rubra           GACAGGATGA ACAGTCTACC CCCCACTATC CAGCAACCTA GCCCATGCCG GCGCATCAGT AGACTTGGCA ATTTTTTCAC TTCACCTAGC CGG
                                                                     HpaII HhaI                                    HpaII
H.conicopora      .......... .......... .......... .......... .......... .......... .......... .......... .......... ...
                                                                     HpaII HhaI                                    HpaII
H.laevigata       .......... .......... .......... ...T...... .......... .T........ G...C..... .......... .......... ...
                                                                     HpaII                                         HpaII
H.scalaris        .......... .......... .......... ...T...... .......... .......... ......A... .......... .......... ...
                                                                     HpaII HhaI        DdeI                        HpaII
H.asinina         T......... ..C..A.... .G..C..... G......T.. .....C.... .T........ ...TC.C..T .....C..C. .A...T.... ...
                                                         DdeI        HpaII                                    DdeI HpaII
H.roei            ......G... .......... .......... T..T.....C .......... .A........ ......A... .....C.... .......... T..
                                                                     HpaII             DdeI
H.midae           A....C.... ........T. .A.....T.. ...T...... ..A....... .......... ...T..A... ........C. .C........ ...
                                                                     HpaII HhaI                                    HpaII
H.spadicea        A....C..G. ........T. .G.....T.. T..G.....C ..A..C..T. .......T.. ...T...... .....C.... .C..TT.... ...
                                                                         HhaI                                      HpaII
H.australis       A......... .......... .A..TT.... T..T.....T ..T.....A. .G........ ...C..A... .....C.... .......... ...
                                                                                                                   HpaII
H.iris            ......G... .....T.... .T..TT.G.. T..T.....T ........A. .......C.. ...C..T... .....C..C. .A...T.... ...
                                                                         HhaI                                      HpaII
H.virginea        A....C.... .......... .T..C..... ...T.....T ..T..C..G. .......C.. ....C.T... .....N..C. .C........ ...
                                                                         HhaI                                      HpaII
HALCO1-NG1        ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---
HALCO1-NG2        ---------- ---------- ---------- ---------- ---------- ---------- ---------- TAAAARAGIG AIGTGRATCG GCC
```

Figure 2. Sequence alignment of the 193 bp mtCOI fragment for eleven abalone species. Primer sequences and cut sites for the four restriction enzymes *DdeI*, *HhaI*, *HinFI*, and *HpaII* are included. (N = sequence data unclear whether C or T).

TABLE 1.

Expected restriction fragment lengths for eleven *Haliotis* species for the 193 bp mtCOI fragment when cut with restriction enzymes *DdeI*, *HhaI*, *HinFI* and *HpaII*. The number in parenthesis represents the total number of individuals examined for each species that displays the given restriction pattern. Unique restriction profiles are shown in bold. *H. laevigata* numbers include the misidentified *H. scalaris* individual.

	COI Restriction Digestion Patterns			
	DdeI	*HhaI*	*HinFI*	*HpaII*
H. asinina	**7,50,137** (29) 7,187 (1)	193 (30)	34,159 (30)	3,42,72,76 (30)
H. australis	193 (10)	**79,114** (10)	193 (10)	**3,93,97** (10)
H. conicopora	193 (11)	42,151 (11)	34,159 (11)	3,42,51,97 (11)
H. iris	193 (10)	42,151 (10)	**19,174** (10)	3,190 (10)
H. laevigata	193 (63)	193 (62) 94,99 (1)	34,159 (62) 193 (1)	3,42,148 (63)
H. midae	193 (10)	42,151 (10)	34,159 (10)	3,42,148 (10)
H. roei	29,164 (10)	193 (10)	**6,34,97** (10)	45,51,97 (10)
H. rubra	193 (49) 29,164 (1)	42,151 (50)	34,159 (49) 193 (1)	3,42,51,97 (50)
H. scalaris	29,164 (21)	42,151 (21)	34,159 (21)	3,42,51,97 (21)
H. spadicea	193 (10)	42,151 (10)	**15,19,159** (10)	3,190 (10)
H. virginea	193 (10)	**42,57,94** (9) 42,151 (1)	193 (10)	3,190 (10)

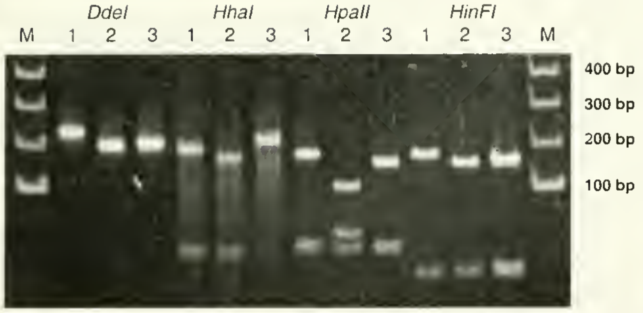

Figure 3. RFLP patterns on a non-denaturing polyacrylamide gel of the 193 bp mtCOI fragment for three abalone species produced with four restriction enzymes. Species 1 – *H. midae*, species 2 – *H. rubra*, species 3 – *H. laevigata*, M – 100 bp DNA ladder. Reduced mobilty in *H. midae* fragments suspected to be due to a conformational mutation.

HALCO2GENB primers, except *H. iris* that failed to amplify for all ten individuals examined. Comparison of the DNA sequences for the other species indicated suitable restriction sites for discrimination between species using five restriction enzymes (Fig. 4).

All restriction digestions for the five enzymes resulted in a single restriction pattern for each species, except for two enzymes for *H. rubra* (Table 2). The two polymorphisms were each observed in two different individuals, all were sequenced to confirm the observed RFLP. None of these four individuals was responsible for the polymorphisms observed at the mtCOI fragment, and the individual *H. rubra* with a mtCOI profile similar to *H. scalaris* was clearly identified as *H. rubra* at this fragment. The restriction profiles for the canned tissue, mucous and fluid samples all matched that expected for *H. rubra*.

As with the mtCOI RFLP analysis, an unusual species profile was observed for three enzymes with a single *H. scalaris* individual (the same individual), and again all three profiles match that recorded for *H. laevigata*. Sequence data showed a 100% similarity to *H. laevigata* across the 159 bp fragment, while three other *H. scalaris* samples each differed at 9 nucleotides from the *H. laevigata* sequence.

At this 159 bp fragment, three of the ten species (excluding *H. iris* that did not amplify) had a unique restriction pattern for the enzyme *DdeI* and so could be individually identified (Table 2). In addition to the *H. rubra/H. conicopora* complex it was not possible to separate *H. australis* and *H. spadicea* using the five enzymes on this fragment. All remaining species combinations were separable from each other using one to five of the enzymes (Table 2).

Fragment mobilities on polyacrylamide gels were all consistent with known fragment lengths; no conformation induced mutations were observed in the mtCOII fragment.

DISCUSSION

The ability to identify abalone species from tissue samples and/or mucous is important to the continued survival of significant abalone fisheries. The tests described in this article will provide one more tool in the fight against illegal fishing, which has the potential, along with commercial over-fishing and environmental variables (Davis et al. 1998, Shepherd et al. 1998), to lead to the decline and collapse of fisheries. The methods are straightforward

```
                1                                                   50                                                    100
H.rubra         CAATTTGAAC CATTCTACCA GCCATTATCC TTATTTTCCT CGCCCTACCA TCCTTGCGCC TCCTTTACCT ACTAGACGAA GTCGGTATAT CGTGCCTTCT
                                                                            HhaI
H.conicopora    .......... .......... .......... .......... .......... .......... .......... .......... .......... ..........
                                                                            HhaI
H.laevigata     .......... ......C... .......... .C..C..... .......... .......... .......... CT........ ..A..G.... .C........
                                                                            HhaI              DdeI          EcoRV
H.scalaris      .......... C.....C... .......... .C..C..... A.....G... .....A.... .......... CT........ ..A..A.... .C........
                                                                                              DdeI
H.asinina       .......... T......... .....C.... .G..C...T. A.....T... ..TC.C..A. .T..A..... .......... ..T..G.... .T.....CT.
                                                        DdeI                                                EcoRV
H.roei          .......... T.....C... .......... .C..C..... A.....C... .....A.... .T........ .......... ..A....... .C.....C..
H.midae         .......... C.....C... ..T....... .A..C..... ......C... ..TC.A..A. ....C..... C......... ..G....... .A..T..CT.
H.spadicea      .......... 1.....C... ..T....... ..G.C..... ......T... .....A..A. .......T.. ......T... ..A.....G. .A..T...T.
H.australis     .......... 1.....2... .....C..TT .A........ A.....G..G ...C.A..A. .......... C.....T... .......... .A.....CT.
H.virginia      .......... ..C....... .....C.... .A..C...T. A.....C..C ..AC.T..A. .T........ T.....T..G .....A.... .A.....A..
                                                        DdeI
HALCO2GENA      CAATYTGAAC YATTCTMCCA GC-------- ---------- ---------- ---------- ---------- ---------- ---------- ----------
HALCO2GENB      ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ----------

                101                                                150       159
H.rubra         AACAATCAAG GCAACTGGTA ACCAGTGATA CTGAGGCTAC GAATACTCAG ACTTTAAGG
                                                 DdeI            DdeI
H.conicopora    .......... .......... .......... .......... .......... .........
                                                 DdeI            DdeI
H.laevigata     ......T... .....G.... .T.....G.. .......... .......... .........
                                             RsaI DdeI           DdeI
H.scalaris      ...G...... .....A.... .T..A..G.. ...G...... .......... .........
                                             RsaI                DdeI
H.asinina       .........A .....C..A. ....A..... ...G...... .......... .........
                               HpaII                             DdeI
H.roei          ......T... .....C.... .T..A..G.. ...G...... .......... .........
                               HpaII         RsaI                DdeI
H.midae         ...G..T... .......... ....A..... .......... .......... .........
                                                 DdeI            DdeI
H.spadicea      ......T..A .....C..C. ....A..... .......... .......... .........
                               HpaII             DdeI            DdeI
H.australis     ......T..A .....C..A. ....A..... .......... .......... .........
                               HpaII             DdeI            DdeI
H.virginia      .........A .....C..C. ....A..... T......... .......... .........
                               HpaII                             DdeI
HALCO2GENA      ---------- ---------- ---------- ---------- ---------- ---------
HALCO2GENB      ---------- ---------- ---------- ----CCGATG CTTATGAGTC TRAAATTCC
```

Figure 4. Sequence alignment of the 159 bp mtCOII fragment for ten abalone species. This fragment did not amplify for *H. iris*. Primer sequences and cut sites for the five restriction enzymes *DdeI*, *EcoRV*, *HhaI*, *HpaII*, and *RsaI* are included. (1 = sequence data unclear whether C or T; 2 = sequence data unclear whether C or A).

TABLE 2.

Expected restriction fragment lengths for 10 *Haliotis* species for the 159 bp mtCOII fragment when cut with restriction enzymes *DdeI*, *EcoRV*, *HhaI*, *HpaII*, and *RsaI*. *H. iris* did not amplify with these primers. The number in parenthesis represents the total number of individuals examined for each species that displays the given restriction pattern. Unique restriction profiles are shown in bold. *H. laevigata* numbers include the misidentified *H. scalaris* individual.

	COII Restriction Digestion Patterns				
	DdeI	*EcoRV*	*HhaI*	*HpaII*	*RsaI*
H. asinina	13,38,108 (30)	71,88 (30)	159 (30)	44,115 (30)	159 (30)
H. australis	13,15,131 (10)	159 (10)	159 (10)	44,115 (10)	159 (10)
H. conicopora	13,15,131 (11)	159 (11)	58,101 (11)	159 (11)	159 (11)
H. iris					
H. laevigata	**13,15,60,71** (63)	71,88 (63)	58,101 (63)	159 (63)	30,129 (63)
H. midae	13,15,131 (10)	159 (10)	159 (10)	159 (10)	159 (10)
H. roei	**13,146** (10)	159 (10)	159 (10)	44,115 (10)	30,129 (10)
H. rubra	13,15,131 (50)	159 (50)	58,101 (48)	159 (48)	159 (50)
			159 (2)	44,115 (2)	
H. scalaris	**13,71,75** (21)	159 (21)	159 (21)	159 (21)	30,129 (21)
H. spadicea	13,15,131 (10)	159 (10)	159 (10)	44,115 (10)	159 (10)
H. virginea	13,38,108 (10)	159 (10)	159 (10)	44,115 (10)	159 (10)

and suitable for use in any laboratory with basic DNA analytical equipment. The PCR-RFLP tests utilize short DNA fragments that can be amplified from processed products and slightly degraded material, and therefore are of potential forensic use.

Care has been taken in this study to include examination of intraspecies variation as well as possible non-*Haliotis* amplification with our PCR primers. Samples from different geographic locations were examined for the two main Australian commercial species (*H. rubra*, five locations and *H. laevigata*, three locations). While not exhaustive, the results suggest that what limited intraspecies variation exists can be accounted for using the two fragments and multiple restriction enzymes. Both PCR primer sets devised for the test are relatively degenerate and so cross genus amplification was not unexpected. However, of the groups we have examined only DNA from the tunas (*Thunnus* spp.) amplified, and it was possible to easily differentiate these from *Haliotis* species.

The restriction patterns produced by *DdeI* for the mtCOII fragment would discriminate three of the species, while five other species would be differentiated by a single restriction pattern at the mtCOI fragment. Such species-specific patterns are useful, however as rare polymorphisms may exist it would be wise to confirm identification with multiple enzymes and/or both short fragments. None of the rare polymorphisms observed occurred in more than a single individual, and no individual displayed more than one variation. With the exception of the *H. rubra/H. conicopora* pairing, all other combinations of the 11 abalone species can be differentiated from each other using two or more of the restriction profiles shown in this study. We therefore recommend using both fragments and at least two of the restriction enzymes included here to differentiate species.

The ability of our test to differentiate between species was inadvertently put to the test during the intraspecies examinations. Of 20 purported *H. scalaris* individuals, one was found to display a different restriction profile at six of the nine profiles examined. The combined profile of this individual matched completely the expected profile for *H. laevigata*; and was confirmed by DNA sequence analyses. Although occupying different microhabitats, these two species have overlapping distributions and co-occur in the same area (Shepherd 1973). Shell and mantle morphology did not separate the aberrant individual from other *H. scalaris* individuals. This individual is either a *H. laevigata* and morphological characters between the two species are more plastic than currently recognized, or a hybrid between the two species.

Naturally occurring hybrids between abalone species with overlapping ranges, although relatively rare, have been reported (e.g., Talmadge 1977; Sasaki et al. 1980; Arai et al. 1982; Messier & Stewart 1994). The two Australian species *H. rubra* and *H. laevigata*, also show evidence of backcrossing and introgression (Brown 1995). There are no records of hybrids between *H. laevigata* and *H. scalaris*, but *H. laevigata* is more closely related to *H. scalaris* than to *H. rubra* (Brown & Murray 1992), and hybrids would not be unexpected. Allozyme analysis of the aberrant individual could not confirm nor refute its putative hybrid status as there are no known diagnostic loci between the two species (Brown 1991).

The possible existence of hybrids, albeit at low frequencies, does not minimize the validity of our mtDNA-based test for abalone. However, the possibility of hybridization and backcrossing between species does question the legal "species identity" of an individual. If hybrids were infertile and only F1 hybrids were possible, then a single diagnostic nuclear DNA marker would confirm the individual as a hybrid, and the mtDNA marker would confirm the maternal species. Such individuals could be legally classed as hybrids. However at least some abalone hybrids appear to be fertile and backcrossing occurs (Brown 1995), and identifying the "nuclear lineage" of a potential backcross offspring would require multiple nuclear DNA markers. Even then it could never be proved that an individual was not the offspring of a backcrossing event, except based on probability. A suite of nuclear DNA markers could never disprove a claim of backcrossing, although making it improbable. On the other hand using a mtDNA-based test, the maternal lineage of the individual can always be validated. We suggest that for legal purposes where hybrid backcrossing may exist between abalone species that the genetic "species identity" of an individual be classified as its maternal lineage, which can be confirmed from its mtDNA. Hybrid individuals (those with mtDNA of one species and nuclear DNA wholly or partly of another species) while biologically acknowledged should not be legally recognized as the existence of backcross hybrids cannot be disproved except by probability based on a large number of diagnostic nuclear DNA markers. Mitochondrial DNA in abalone as in most organisms appears to be only maternally inherited (Conod 2000). The aberrant individual in our study therefore is classed as *H. laevigata*.

The advantage of the tests described here to previous studies (Sweijd et al. 1998) for abalone is the smaller size of the DNA fragment; an advantage when examining processed or slightly degraded material (Mackie et al. 1999). The lysin gene protocol described by Sweijd et al. (1998) did aim for fragments less than 300 bp, but the presence of an intron increased this at least three times, and for *H. laevigata* by about ten fold (unpublished data). The authors did however successfully use PCR primers for a smaller 146 bp fragment to discriminate between canned *H. midae* and *H. rubra* products.

PCR inhibition was observed when testing our primers on the mucous samples of *H. rubra*. Dilution (10 fold) to a lower concentration did not have the same inhibitory effect. Similar PCR inhibition due to high levels of polysaccharides is common in plant tissue extracts (Fang et al. 1992), and inhibition due to mucopolysaccharides in the abalone mucous may have caused the observed PCR failure.

No DNA sequence variation was observed between *H. rubra* and *H. conicopora* in either short mtDNA fragment examined in this study. In an assessment of all recent taxa in the family Haliotidae, Geiger (1998) concluded that there was some justification for sub-species recognition of *conicopora* under *H. rubra*. Allozyme data suggested conspecifity but shell and geographic distributions indicated distinct taxa. Fifteen of 22 DNA microsatellite primers developed for use in *H. rubra* amplified a similar product in *H. conicopora* (Evans et al. 2001). This compares to the conservation of only 12 of the 22 markers in other temperate Australian species (*H laevigata, H.scalaris* and *H. roei*). Our short DNA sequences lend some support to the possibility of sub-species status for *conicopora*, however further research is required to resolve the issue.

The altered mobility of the *H. midae* mtCOI fragment run on polacrylamide gels is most likely due to a conformation change. Conformational mutations attributed to sequence-specific variations are restricted to polyacrylamide gels and not seen on agarose gels (Singh et al. 1987). The location and conservation of this conformation variant requires further investigation. Its presence, however, raises a note of caution when using RFLPs as mobility variation of fragments seen on polyacrylamide gels may be misleading as they can be length or conformation polymorphisms. It is therefore recommended that species differentiation using the RFLP tests described here be run only on agarose gels.

The test described here fulfills the aim of our study to provide a relatively straightforward and cost-effective means for identifying several abalone species of commercial importance to Australia. Costs for any DNA-based analyses are not insignificant, but the PCR-RFLP technique is generally considered more cost-effective for routine species identification than alternatives such as direct DNA sequencing of the PCR product (e.g., Asensio et al. 2000). The opportunity to sequence a PCR product is of course still available for differentiation of individuals if problems arise following PCR-RFLP analysis.

To increase the potential value of this study to the sustainability and protection of abalone fisheries worldwide, additional species, particularly from Northern Hemisphere waters, need to be incorporated either into this test or a modified one, so that a single test is available for discrimination of all abalone species.

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DISTRIBUTION AND ABUNDANCE OF *STROMBUS COSTATUS* (GMELIN, 1791) LARVAE AND ADULTS AT THE BIOSPHERE RESERVE: BANCO CHINCHORRO, QUINTANA ROO, MEXICO

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ABSTRACT In order to study the distribution and abundance of *Strombus costatus*, larvae and adults, samples were collected bimonthly from August 1997 to July 1998 at six sites at Banco Chinchorro. To collect larvae, replicate surface plankton tows were made with a conical net. Larval density ranged from 0.0018 ± 0.026 veligers/10 m^3 to 4.77 ± 3.50 veligers/10 m^3. Presence of all larval stages suggested a complete development of veligers from egg stage to metamorphic competence in the reef lagoon; 8.52% of the larvae corresponded to stage II veligers, 18% to stage III and 72.65% to stage IV. At the bottom, all conch found within three 100-m^2 replicate areas were counted, shell lengths were measured and egg masses recorded. Conch density varied from 0.0025 to 0.22 conch/m^2 with a dominance of juveniles. The total population in Banco Chinchorro was estimated at 1.3×10^6 conch, but only 8% were estimated to be of legal size. Larvae and adults were more abundant in Cayo Centro, the principal distribution site. Egg masses were abundant from May to October with a greater abundance in May. Banco Chinchorro is an important source of *S. costatus* veligers and sustains an adult and juvenile population within the reef lagoon. Nevertheless, this *S. costatus* population is not large enough to support a commercial fishery. It is necessary to protect the reproduction sites to maintain larval supply downstream.

KEY WORDS: Caribbean, distribution, larvae, *Strombus costatus*

INTRODUCTION

In 1997, Mexico harvested 140,021 tons of mollusks, with a total value of US$39.79 million. This harvest represents 10.06% of the country's total fish and invertebrate catch and corresponds to 4.15% of the total economic value (SEMARNAP, 1998).

In the Yucatan Peninsula, the mollusk fishery is multispecific; catches principally consist of queen conch (*Strombus gigas* Linnaeus, 1758), and on a minor scale, milk conch (*Strombus costatus* Gmelin, 1791), and the fighting conch (*S. pugilis* Linnaeus, 1758). Other gastropods are also caught, such as the red conch, (*Pleuroploca gigantea*, Kiener, 1840) and the black conch, (*Xancus angulata*, Lightfoot, 1786) (Sosa–Cordero et al. 1993; Perez 1997).

The milk conch (*S. costatus*) is widespread in the Caribbean, but it is commercially caught only off the Yucatan (Stoner 1997) where it has the same market value and demand as the queen conch (Aldana-Aranda & Patiño-Suárez 1998). In the South of Quintana Roo fishing regulations only allow harvesting mollusks in Banco Chinchorro. The queen conch is the targeted species and the milk conch are not harvested.

The decline of *S. gigas*, between 1985 and 1990 in the Yucatan Peninsula, led to the closure of the conch fishery off Yucatan State and a ban in some areas off Campeche State and northern Quintana Roo State. In addition, a system of capture quotas was established in sourthern Quintana Roo.

With the descend of the queen conch fishery, alternative sources such as milk conch could be caught to meet the growing demand for conch meat earmarked for the local markets and the Costa Maya's tourism industry in southern Quintana Roo. The potential for the non-exploited resource milk conch, *S. costatus*, needs to be evaluated.

There are only a few studies on milk conch in the Caribbean. Percharde (1968) studied the distribution of the genus *Strombus* in Trinidad and Tobago. Brownell (1977) reported that *S. gigas* was the most important fishery resource in Los Roques, Venezuela, but that other mollusks such as *S. costatus* and *S. pugilis* also contributed to the fishery. Appeldoorn (1985) studied the growth, mortality and dispersion of laboratory reared *S. gigas* and *S. costatus* in Puerto Rico, and Berg et al. (1989) described the abundance and distribution of *S. costatus* in Bermuda. In Mexico, Aldana-Aranda et al. (1989) studied the effect of temperature and algal food on larval growth of milk conch. Recently, Aldana-Aranda and Patiño-Suárez (1998) reviewed algal diets used in larviculture, of several Strombid species, including the milk conch, and Shawl et al. (in press) reared *S. costatus* juveniles raised from egg masses laid in captivity.

There are no studies related to the milk conch at Banco Chinchorro. Consequently, the objective of this work is to describe the distribution and abundance of *S. costatus* adults, determine their reproduction sites, and evaluate larval abundance. This study tests the following hypotheses: (1) the lagoon reef is an important site for reproduction and distribution of milk conch; and (2) there is complete larval development (egg to metamorphosis competence) of this specie in Banco Chinchorro.

MATERIALS AND METHODS

Study Area

Biosphere Reserve Banco Chinchorro is a false atoll on Quintana Roo's South coast, within the Mexican Exclusive Economic Zone (18°23'; 18°47'N, 87°14'; 87°27'W) (Jordán and Martín 1987) (Fig. 1). Chinchorro is 46 km long and 19 km at its maximum width, with a reef lagoon area of 560 km^2. The reef lagoon has an extensive sand bottom with patches of sea grass. In the North these species are most abundant, *Thalassia testudinum* Banks ex Köning, *Halodule wrigthii* Ascherson, and *Syringodium filiforme* Kützing. Reef patches are common in the South region of the lagoon.

Banco Chinchorro has four keys; in the north there are two small keys known as Cayo Norte. In the central area, there is Cayo Centro, which is the largest, and in the southern area, Cayo Lobos which is the smallest (Fig. 1).

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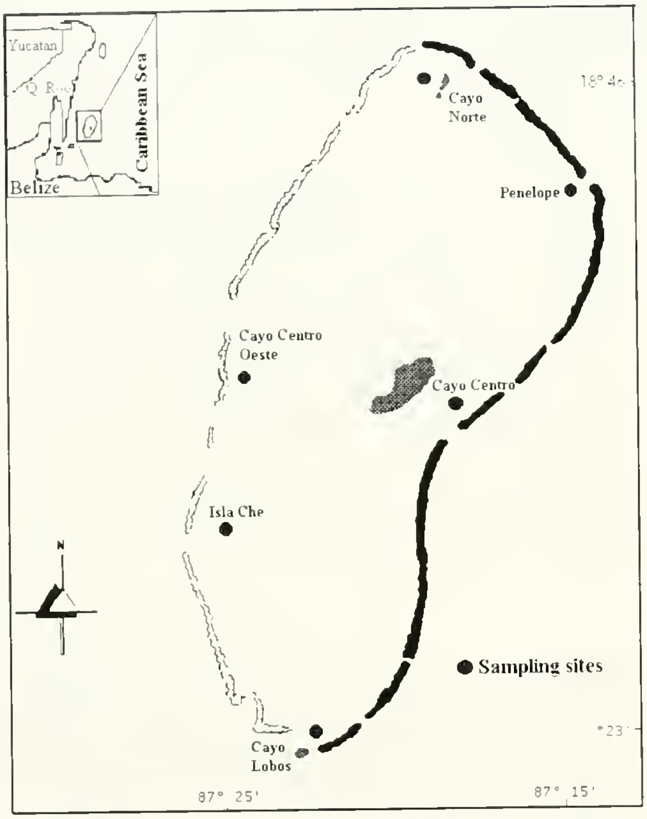

Figure 1. Map of Banco Chinchorro showing six sampling sites within the reef lagoon.

The dry season extends from March to June, while the rainy season is from July to October, and the cold season, characterized by strong northerly winds ("nortes"), is from November to February.

Sampling Methods

Samples were collected bimonthly from August 1997 to July 1998, within the reef lagoon, in six traditional queen conch fishing sites: Cayo Lobos (18°23′45. 0′N, 87°21′ 20.9′W), Isla Che (18°30′ 12.3′N, 87°26′13. 1′W), Cayo Centro (18°33′ 32.7′N, 87°18′ 24.5′W), Cayo Centro Oeste (18°33′24. 1′N, 87°24′; 56.6′W), Penelope (18°42′ 47.6′N, 87°14′55.5′W), and Cayo Norte (18°45′28. 1′N, 87°47′01.1′W) (Fig. 1). At each site, temperature (°C) and dissolved oxygen (mg/l) were measured at surface level using an oxygen meter (YSI model 58). Salinity was measured with a thermoconductivity meter (OHALUS model 50).

Replicate surface plankton tows were made at each site using a conical net, 0.50-m dia, with a 202-μm mesh size. Plankton tows were conducted for 15 min at a velocity of −1.0 m/min. Water volume was measured with a flow meter (General Oceanic model 2031H) attached to the net. Plankton were fixed with a mixture of neutralized 5% sea water-formalin (Stoner & Davis 1997a). Tows were conducted diurnally and additional night collections were made at Cayo Centro and Cayo Lobos.

Gastropod larvae were sorted from other plankton using a dissecting microscope (×20). Identification and developmental stages (I to IV) were assigned following the descriptions of Davis et al. (1993), and larvae were counted and measured for maximum shell length. Total larval abundance was standardized to larvae/10 m^3.

To determine the abundance of juvenile and adult conch, bimonthly samples were conducted at each site. All conch found in each of the three circular unit replicates (100 m^2) were counted. The shell length and lip width were measured to the nearest mm using calipers, after measurements all conch were returned to the sea bottom. Egg masses within the unit samples were counted.

Conch abundance was compared among months and sites sampled using a two-way Analysis of Variance (ANOVA), on Log (x + 1) transformed data. The procedure considered independence between sites and months.

RESULTS

Maximum mean temperatures were recorded during August and October, 28.9 ± 0.55 and 29.3 ± 0.28°C ($n = 6$), respectively, whereas lowest temperature occurred in December (26.3 ± 0.94 $n = 6$). Dissolved oxygen varied between 5.87 ± 0.37 mg/l in July and 7.01 ± 0.56 mg/l ($n = 6$) in August. Salinity ranged from 35.9 ± 0.12% in October to 37.0 ± 0.9‰ ($n = 6$) in March.

A total of 245 larvae were collected over the 12 months. Most larvae were collected in July during night tows (158 veligers at Cayo Lobos and 75 veligers at Cayo Centro). Diurnal tows contained a very low number of veligers. Six larvae were collected in October; one at Isla Che, one at Cayo Centro, and four at Centro Oeste. In May, six larvae were collected at Penelope. No veligers were found in August, December, and March. Larvae were categorized into the following stages, 8.53% of larvae were stage II (451–750 μm), 18.77% corresponded to stage III (751–950 μm) and 72.65% were stage IV at Cayo Centro in October, to 4.77 ± 3.50 veligers/10 m^3 at Cayo Lobos in July (Table 1).

A total of 280 juvenile and adult conch were collected during the sampling period. Conch abundance was significantly different between sites ($P = 0.0036$), Cayo Centro had the highest density and Isla Che had the lowest (Tables 2 and 3). A total 184 individuals were found at Cayo Centro; 89 conch were collected in

TABLE 1.

Total number of larvae and density (veligers/10 m^3) of *Strombus costatus* (all stages) collected at Banco Chinchorro, August 1997 through July 1998, No veligers were found in August, December, and March.

	Cayo Lobos		Isla Che		Cayo Centro		C. Centro Oeste		Penelope	
	Counts	Density	Counts	Density	Counts	Density	Counts	Density	Counts	Density
October			1	0.018 ± 0.03	1	0.026 ± 0.03	4	0.11 ± 0.15		
May									6	0.17 ± 0.24
July‡	158	4.77 ± 3.50			75	1.62 ± 1.07				

‡ Night collections

TABLE 2.

Milk conch adults density (no. conch/m^2) at Banco Chinchorro.

	Cayo Lobos	Isla Che	Cayo Centro	C. Centro Oeste	Cayo Norte
August	0.0075	0	0.0075	0.0025	0.0100
October	0.0050	0.0025	0.0825	0.0025	0.0200
December	0.0125	0.0025	0.0025	0	0
March	0.0725	0	0.2225	0.0125	0.0125
May	0	0	0.1400	0	0.0025
July	0.0125	0.0025	0.0050	0.0050	0.0550

March, 56 in May, 33 in October, 3 in August, 2 in July, and 1 in December. Juvenile and adult density varied from 0.0025 conch/m^2 to 0.222 conch/m^2. Despite significant differences between sites (Table 3), a uniform conch distribution was used to calculate the population size at Banco Chinchorro. Based on the lowest conch density (0.0025 ind/m^2), and a lagoon area of 560 km^2 for Banco Chinchorro, a total of 1.3×10^6 conch were estimated. However, only 100,000 conch were estimated to be harvest size (8%). This low population size does not appear sufficient to support a commercial fishery.

The highest numbers of conch found in Banco Chinchorro were collected in March, with a total of 128 organisms. Fifty-seven conch were collected in May, 45 in October, 32 in July, 11 in August, and 7 in December. The size-frequency distribution varied from 30 to 180 mm shell length with 92% of total sample of conch in the range of 81 to 160 mm shell length (Fig. 2).

Reproductive activity, copulation, and the presence of egg masses in medium sands, were observed from March to December. A total of 113 egg masses were counted with the majority at Cayo Centro (39 egg masses in October, 58 in May, and 1 in December). Fifteen egg masses were counted at Cayo Lobos in March.

DISCUSSION

Temperature has a direct influence on the beginning and cessation of conch reproductive activity, egg-hatching time, and on the duration of the larval phase (Berg et al. 1989; Appeldoorn et al. 1983; Stoner et al. 1992; Stoner & Davis 1997a; Pechenik 1999).

At Banco Chinchorro, egg masses of *S. costatus* were first observed in March and were found until December when bottom water temperature decreased to 26.3°C. In Banco Chinchorro, the reproductive season of *S. costatus* is during a ten-month period, with a peak in copulation and egg laying in May. This period is longer than in Trinidad and Tobago where mating occurs from November until June (Perchard 1968) and longer than in Venezuela, where reproduction occurs from November to May (Brownell 1977). In comparison, the reproductive season for queen conch, *S. gigas*, has great variation. It varies from 5 to 12 months, with the shortest season duration in Bermuda and Florida and the longest at Banco Chinchorro (Cruz 1984; Corral & Ogawa 1987; Stoner et al. 1992). A difference in reproductive cycles of *S. gigas* and *S. costatus* as a function of temperature was mentioned by Brownell (1977). He observed that *S. costatus* reproductive season began when the season for *S. gigas* usually ended. Brownell (1977) associated this with a one-centigrade degree decline in mean water temperature. However, in this study *S. costatus* reproduction season was from March to December and coincided with *S. gigas* reproduction period at Banco Chinchorro.

In Banco Chinchorro, the number of *S. costatus* egg masses exceeded that of *S. gigas* (113 vs. 19 egg masses) for the same study period (de Jesús-Navarrete 1999). This could be an effect of fishing activities, because only *S. gigas* is collected not *S. costatus*. Females constitute a high percentage (–65%) of the *S. gigas* catch, (pers. obs.) and that might also explain why there were higher number of *S. costatus* egg masses, juvenile and adults than *S. gigas*, in some areas, like Cayo Centro.

Fishing practices are known to modify the structure of populations by reducing the overall biomass (Alcalá, 1988; Roberts, 1995), decreasing age and size at sexual maturity (Harmelin et al. 1995), and altering sex ratios and genetic structure (Ryman et al. 1995). The displacement of target species due to the effect of fishing pressure and a consequently higher abundance of non-target species was mentioned by Seijo et al. (1997). This may be happening in Banco Chinchorro, as the *S. gigas* population declines there may be an increase in the *S. costatus* density, due to habitat and food resources becoming available.

A greater quantity of *S. costatus* larvae was found in July when mean water temperature was at its maximum. It is possible that this is related to higher egg mass production, and increase in food availability for the larvae (Davis 1998). Local water circulation might also influence number of larvae retained inside of Banco Chinchorro. Larvae were collected from nearly all stages (II, III and IV) indicating a complete larval growth process within the reef lagoon. Although the current circulation pattern of Banco Chinchorro is unknown, it is probably influenced by trade winds, and

TABLE 3.

Results of two way ANOVA of *S. costatus* abundance in Banco Chinchorro Quintana Roo, Mexico.

Source of Variation	Sum of Squares	D.F.	Mean Square	F-ratio	p-level
Sites	20.5434	5	4.1086	4.7170	0.00364
Months	8.24020	5	1.6480	1.8229	0.1310
Residual	21.7742	25	0.8709		
Total	50.5578	35			

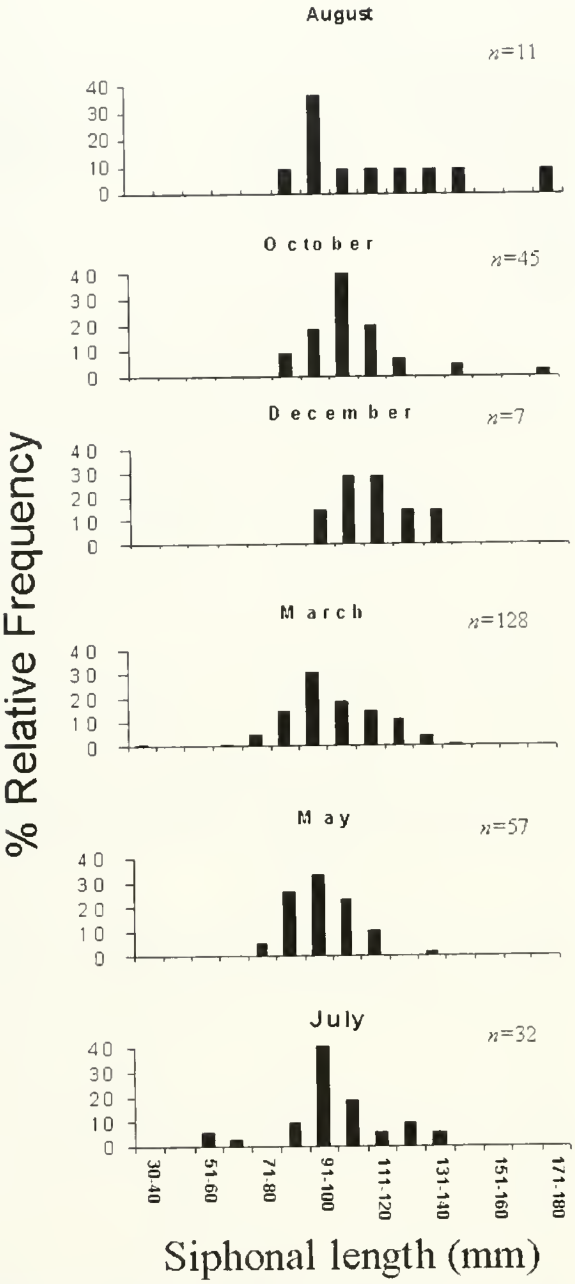

Figure 2. Relative frequency (%) and size distribution of juvenile and adults of *Strombus costatus* at Chinchorro Bank.

this circulation would transport larvae toward the interior of the reef lagoon causing local retention. In Florida, Stoner et al. (1997) found that larvae of *S. costatus* were very abundant in early stages and rare in advanced development stages. They noted that the abundance of later larval stages was associated with water flow produced by the wind, which suggests local recruitment from spawning populations that inhabit outer reefs. The same effect could occur in Banco Chinchorro. Evaluating populations outside of the reef lagoon might explain why 68% of the larvae were collected at Cayo Lobos, the southern site of Chinchorro. The high number of larvae may indicate a contribution from deep-water *S. costatus* populations or populations external to Banco Chinchorro.

The density of *S. costatus* larvae found in this study (4.77 ± 3.50 veligers/10 m^3) is higher than the values reported from other sites in the Caribbean. In Florida, densities of 0.04 to 1.40 veligers/10 m^3 have been found with maximum abundance peaks in June (Stoner et al. 1997). In the Bahamas, Stoner and Smith (1998) reported *S. costatus* densities ranging from 0.026 to 0.069 veligers/10 m^3. In Banco Chinchorro, the higher percentage of *S. costatus* larvae in night tows (95p), may be due to lack of intense surges and wind action that were prevalent during the day. Stoner and Davis (1997b) documented vertical movement of *S. gigas* larvae toward deeper zones due to wind and surge effect, and Stoner and Smith (1998) noted that wind and surge also produce horizontal transportation of larvae.

Density of *S. costatus* adults and juveniles found in Banco Chinchorro is higher (0.22 conchs/m^2) than other values reported in the Caribbean. In Bermuda, *S. costatus* density was highest in the basins, (0.00299 conchs/m^2), than on the platform, (1.9×10^{-4} conchs/m^2) (Berg et al. 1989). In Puerto Rico, the density of *S. costatus* varied from 0.18×10^{-4} conchs/m^2 in reef patches to 0.0032 conchs/m^2 in coarse sands to 0.0049 conchs/m^2 in rubble areas (Appeldoorn 1985). Although the densities found in Banco Chinchorro exceed these values, 92% of the population consisted of individuals smaller than 170-mm shell length, which cannot support commercial harvest. In Yucatan State, *S. costatus* shell sizes varied from 65 to 225 mm shell length, and legal fishing size (180 mm) represented over 65% of the relative abundance of conch catches. (Perez 1997). In Banco Chinchorro, the large size was 180-mm shell length and this represented only 8% or an estimated population of 100,000 conch. Therefore commercial catch of this species is not recommended.

Banco Chinchorro was designated as a Biosphere reserve in 1996. The main goals were to conserve biodiversity, protection, and enhance overexploited species, such as queen conch (*S. gigas*) and spiny lobster (*Panulirus argus*). It is now clear that other species should be considered in the management plan, including the milk conch, *S. costatus*.

Many marine reserves like Banco Chinchorro, have small nucleus areas to protect fishing species. At Banco Chinchorro, only 3.2% of the total lagoon area comprises of protected zones. It is likely that these areas do not contain the full habitats needed to protect or enhance species during their entire life cycle. Therefore, the role of these nucleus areas in protecting species and connecting different habitats together cannot be totally ascertained (Appeldoorn & Lindeman, in press). In the establishment of management plans, data on distribution and abundance of adults and larvae, reproductive sites, egg mass numbers, and recruitment dynamics at a regional level are required. For example, the data from this study suggests that critical areas like Cayo Centro with its high distribtuion of milk conch adult, juveniles, egg masses and larvae needs to become a conch protected area within the Biosphere Reserve Banco Chinchorro.

In general, marine reserves help to protect some fishing species, and can be effective in the recovery of commercial exploited species (Alcalá 1988; Roberts & Polunin 1991; Roberts 1995; Allison et al. 1998). However, to assure conservaton of the species it is essential to design reserves with an understanding of the ontogenetic requirements of target and non-target species. Reserves need to be placed in strategic locations to protect larval production sites and settlement sites. This information on source sites and sink sites will support metapopulation dynamics, and help in optimize fishery benefits for many marine invertebrates species like *S. costatus* that have a pelagic larvae. In the Bahamas, a high density of *S. gigas* larvae and adults have been reported in a protected area in Bahamas (Stoner & Ray 1996). It is possible that both *S. gigas* and *S. costatus* populations in Banco Chinchorro can be maintained for conservation and fisheries if reserve areas are expanded and management regulations to be practiced.

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VELAR CHARACTERISTICS AND FEEDING CAPACITY OF ENCAPSULATED AND PELAGIC LARVAE OF *CREPIDULA FECUNDA* GALLARDO, 1979 (GASTROPODA, CALYPTRAEIDAE)

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ABSTRACT Veligers of *Crepidula fecunda* develop the capacity for ingesting particulate material during early stages of their development within the egg capsules. The potential feeding rate of encapsulated larvae is low compared with that of hatched larvae and pelagic larvae of other mollusks. Once the veligers emerge from the capsules, they increase the capacity for particle retention. This situation could result from the increase in the velar area, length of the ciliated border, length of the preoral cilia, potential filtering area and the width of the feeding canal, or to some combination of these, compared with encapsulated larvae of the same shell length. Extra-capsular development of the velum is an adaptive feature of the species which promotes efficient feeding and active locomotion during the pelagic phase, in preparation for settlement and metamorphosis on inshore rocky substrates.

KEY WORDS: *Crepidula fecunda*, veliger larvae, velar morphology, particle clearance rate

INTRODUCTION

The presence of a lobed velum is a common characteristic in veliger larvae of the Mollusca (Strathmann & Leise 1979), where in free living species the velar lobes carry out functions of swimming and feeding (Hadfield & Iaea 1989). Veligers have external borders on the velar lobes that include preoral and postoral ciliary bands (Strathmann et al. 1972; Strathmann & Leise 1979). The pre-oral band is formed of long cilia that produce water currents used in locomotion and feeding. The postoral band consists of shorter cilia that beat toward the preoral band. The combined activity of these ciliary bands promotes capture and retention of food particles. Between the two ciliary bands lies the food canal covered with very small cilia that transport food particles to the larval mouth.

In suspension-feeding mollusks the particle clearance rate (CR) may be used to calculate ingestion rate when food concentration is known (Sprung1984). Clearance rate in veliger larvae is partially determined by the length of the velar margin and the length of the preoral cilia (Strathmann et al. 1972). Strathmann and Leise (1979) observed that although longer preoral cilia were related to higher rates of water flow, they were less efficient in capturing particles in the smaller size ranges. The latter may be compensated for by an increase in total filtering area that increases the larval clearance rate. In addition to ciliary size, the length of the velar margin is important in larval feeding. Increase in ciliated area is accompanied by an increase in CR (Strathmann et al. 1972). The rate of clearance is not only influenced by the factors cited, but also by the ability of the organism to transport captured particles away from the primary sites of capture to the mouth. Thus, the rate at which the cilia in the food canal transport particles to the mouth directly affects the overall CR.

Crepidula fecunda, a sedentary filter-feeding gastropod, inhabits intertidal and shallow subtidal zones on the Chilean coast. It is a protandric hermaphrodite whose reproductive mechanism includes the deposition of egg capsules on rocky substrates, followed by parental brooding (Gallardo 1976, 1977, 1979). Most eggs in the capsules develop, and veliger larvae of approximately 500 μm in shell length are released from the capsules (Gallardo 1976, 1977, 1979) to complete their development in the inshore plankton prior to settlement.

Encapsulated larvae have no apparent source of food, and it is not known whether they are capable of suspension-feeding prior to eclosion. Development of the ciliated velum within the capsule (Gallardo 1977, 1979; Chaparro et al., in press) suggests that these larvae are capable of feeding and swimming before hatching, and that these properties would improve upon eclosion, since the pelagic larvae must actively feed and swim in preparation for settlement and metamorphosis (Gallardo 1977, 1979, 1989). We hypothesize that the ability of *C. fecunda* larvae to remove particles from suspension is initiated during the intracapsular phase and further developed during the pelagic phase, including increases in the size of the cilia, filtration area, and capacity for particle transport within the larval food groove.

MATERIALS AND METHODS

Stacks of adult specimens of *C. fecunda* were collected from the intertidal at Yaldad Bay, Chiloé (43°08′S; 73°44′W). In the laboratory, individuals were removed from the substrate in order to expose the egg capsules from which embryos and/or larvae were obtained for experimentation. Before each experiment, the capsules were observed under a stereomicroscope to identify the developmental stage of the embryos.

Developmental stages (egg, morula, blastula, trochophore, and veliger) were identified by their morphology. Fifteen to 20 individuals of each group of larvae were observed microscopically and videotaped as described below. The shell lengths of the larvae were determined from images captured on videotape (see later).

Clearance Rate

Clearance rate (CR) was quantified in several encapsulated stages and in pelagic veligers using the method of Coughlan (1969). Several thousands of embryos (egg, morula, trochophore) and veliger larvae from different spawning masses were manually removed from capsules and placed in individual 1-L glass aquaria containing 500 ml 0.45 μm filtered seawater (salinity 30 ‰, temperature 17°C). Embryos used in each aquarium came from the same egg mass. Laboratory cultured *Isochrysis galbana* was added to each experimental system (final concentration 3×10^4 cells/ml

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Aquaria were stirred to ensure adequate mixing. At intervals between one and two h, the concentrations of algae remaining in the aquaria were determined using an ELZONE 180 XY particle counter. Control aquaria were run during each experimental period under the same experimental conditions but without larvae.

At the end of each experiment, five samples of 1.5 ml each were obtained from each experimental system to determine the number of embryos or larvae in each aquarium. For measurement of shell length, larvae from each experiment were fixed in 5% formaldehyde and stored in Eppendorf tubes to await analysis. They were measured as previously described.

Larval cultures were maintained to determine the CR of pelagic larvae of *C. fecunda*. Naturally hatched larvae were collected on a 100 µl nylon screen as they emerged from the capsules and placed in 100-L aquaria containing filtered seawater as described above, with gentle aeration. Each day the larvae were fed *ad libitum* with *I. galbana*. The seawater was replaced every two days. For determination of clearance rate, a known number of larvae were placed in experimental aquaria as described above for the encapsulated larvae, and the same procedures used to determine CR. Groups of larvae from these experiments were also set aside for measurement of shell length.

Velar Morphology

Larvae obtained from capsules as well as pelagic larvae were videotaped by placing them in a plankton decantation chamber with seawater under an inverted microscope fitted with a video camera. Selected images were captured for subsequent processing on a computer equipped with an ATI Corp. "All in Wonder" video card and Scion Image 3.0 PC software. We obtained the velar area (extended velar lobe), length of the ciliated border, length of the preoral cilia, and shell length. The width of the food canal was also determined. The potential feeding area was calculated by multiplying the length of the ciliated border by the length (=height) of the cilia in the preoral band.

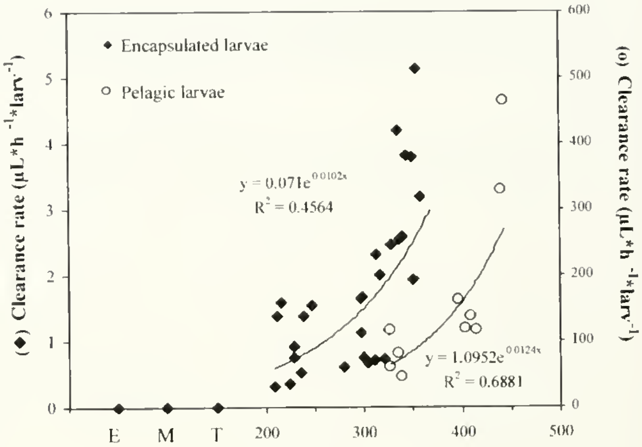

Figure 1. Clearance rate in different embryonic stages, encapsulated (n = 28) and pelagic (n = 10) larvae of *C. fecunda*. E = egg (n = 5), M = morula (n = 5) ,T = trochophore (n = 5).

Velocity of Particle Transport

A suspension of red plastic particles 2–10 µm in diameter was offered to larvae in a plankton settling chamber as previously described by Chaparro et al. (1993). Trajectories of these particles along the food groove were videotaped as described above for observing larvae, and the distances traversed by the particles were calculated using the Scion Image PC program. Simultaneously, the time taken by particles to traverse measured distances was determined (Ward 1996) for calculations of transport velocity. These determinations were carried out on larvae obtained from capsules and on pelagic (cultured) larvae.

RESULTS

Clearance Rate

No uptake of particles was observed in early developmental stages, including the egg, morula, blastula and trochophore (Fig. 1). The clearance rate (CR) of encapsulated veligers of *C. fecunda* increased with the size of the individuals. The initial CR was 0.31 µl h^{-1} larva^{-1} in larvae with shells 208 µm in length, increasing to 5.13 µl h^{-1} larva^{-1} in pre-hatched larvae 353 µm in shell length (CR = 0.071e$^{0.012*\text{larval shell length}}$, r^2 = 0.4564, n = 28) Figure 1.

Recently hatched veliger larvae showed much higher values for CR than those of the same size that remained encapsulated (Table 1). CR values ranged from 45.3µl h^{-1} larva^{-1} in larvae 337 µm in shell length at one day post-eclosion to 464 µl h^{-1} larva^{-1} in larvae 442 µm in shell length at 13 days post eclosion (CR = 1.0952 e$^{0.0124*\text{larval shell length}}$, r^2 = 0.6881, n = 10) Figure 1.

Velar Morphology

Velar Area

The velar area of encapsulated larvae showed linear growth during the developmental period, fluctuating between 0.012 mm^2

TABLE 1.

***Crepidula fecunda*. Comparison of clearance rate (CR), area of a velar lobe (VA), length of ciliated velar border (LCVB), length of preoral cilia (CL), potential filtering area (PFA), and width of food groove (WFG) in encapsulated and pelagic larvae. Values represent individuals of 340 µm in shell length and were obtained from appropriate regression equations. (%) = percent increase in variable when comparing pelagic stage (recently eclosed) with encapsulated larvae having the same shell length.**

Stage	Shell length (µm)	CR (µl · h^{-1} · larv^{-1})	VA (mm^2)	LCVB (mm^2)	CL (µm)	PFA (mm^2)	WFG (µm)
Encapsulate	340	2.28	0.037	688	66	0.034	19
Pelagic	340	74.21	0.049	739	83	0.061	21
%			32.4	7.4	25.8	79.4	10.5

in larvae 188 μm in shell length and 0.056 mm^2 in larvae 372 μm in shell length (velar area = 0.0002 × larval shell length − 0.0312, $r^2 = 0.8755$, $n = 52$) Figure 2.

In recently liberated pelagic larvae of *C. fecunda* the velar area was 32.4 % greater than in encapsulated larvae having the same shell length (Table 1). The velar area was 0.054 mm^2 in recently eclosed larvae 340 μm in shell length, reaching 0.198 mm^2 in larvae ready for metamorphosis at 16 days post-eclosion at 650 μm shell length (velar lobe area = $(6 \times 10^{-7}) \times (\text{larval shell length})^{1.9421}$, $r^2 = 0.9586$, $n = 5$) Figure 2.

Length of Ciliated Velar Border

The length of the ciliated border in encapsulated larvae showed gradual growth with development. The initial length, 287 μm in larvae 205 μm in shell length, increased to 755 μm in larvae 372 μm in shell length (length of ciliated velar border = 858.36 × ln (larval shell length) − 4315.5 , $r^2 = 0.9724$, $n = 19$) Figure 3.

The length of the ciliated velar border in recently liberated pelagic larvae of *C. fecunda* was 7.4% greater than that of encapsulated larvae having the same shell length (Table1). The length of the border varied from 772 μm in larvae of 340 μm shell length, to 1548 μm in larvae of 650 μm shell length (ciliated length of velar border = $1.7826 \times (\text{larval shell length})^{1.0341}$, $r^2 = 0.9307$, $n = 5$) Figure 3.

Length of Preoral Cilia

These cilia showed rapid growth during intracapsular development, reaching lengths of 80 μm in larvae with shell length 400 μm (ciliary length = $-0.0023\,(\text{larval shell length})^2 + (1.7307 \times \text{larval shell length}) - 256.29$, $r^2 = 0.8954$, $n = 29$) Figure 4.

The preoral cilia of recently hatched pelagic larvae were approximately 25.8% longer than the cilia of encapsulated larvae having the same shell length (Table 1). The length of these cilia in recently eclosed larvae was about 82 μm. This increased to 111 μm in those larvae at 15 days post eclosion that were ready to undergo metamorphosis (ciliary length = $61.316e^{0.0009 * \text{larval shell length}}$, $r^2 = 0.9041$, $n = 5$) Figure 4.

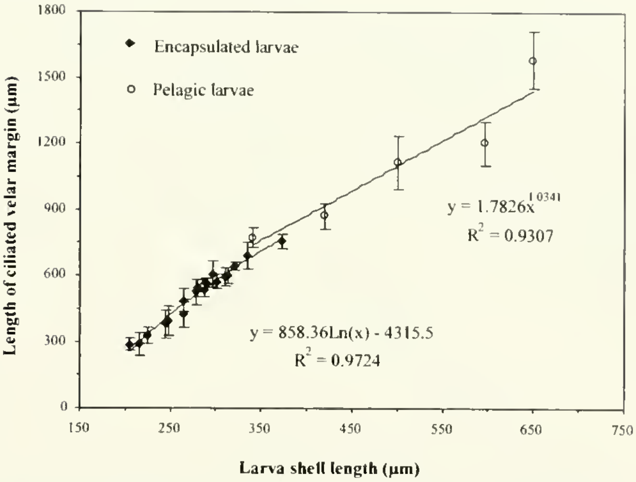

Figure 3. Length of ciliated velar margin (periphery of an extended velar lobe) in relation to larval size in encapsulated ($n = 19$) and pelagic ($n = 5$) larvae of *C. fecunda*. When the SD bar is not shown, it is smaller than the symbol size. Each data point represents the mean for at least 15 individuals for encapsulated larvae and 25 individuals for pelagic larvae.

Potential Filtering Area

The potential filtering area increased during larval development from 0.002 mm^2 in larvae of 205 μm shell length to 0.054 mm^2 in larvae of 372 μm shell length [potential filtering area = $(-5 \times 10^{-7}) \times (\text{larval shell length})^2 + (0.0006 \times \text{larval shell length}) - 0.1121$, $r^2 = 0.9417$, $n = 16$] Figure 5.

In recently eclosed pelagic larvae, the potential filtering area was about 79.4% greater than that of encapsulated larvae of comparable shell size (Table 1). This value was 0.06 mm^2 in one-day-old larvae, increasing to 0.18 mm^2 in larvae at 15 days post eclo-

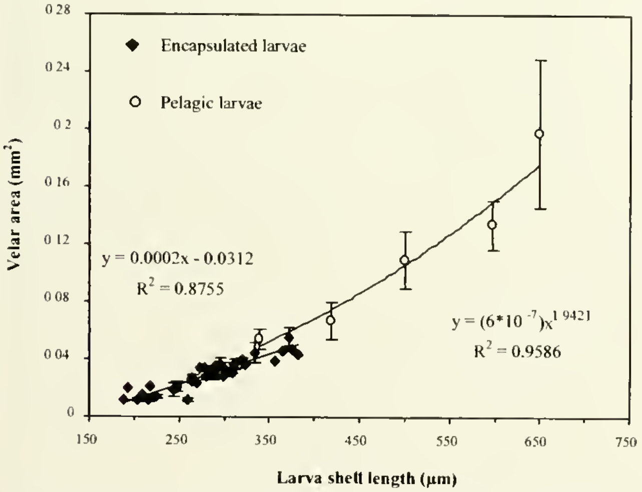

Figure 2. Area of an extended velar lobe in relation to size in encapsulated ($n = 52$) and pelagic ($n = 5$) larvae of *C. fecunda*. When the SD bar is not shown, it is smaller than the symbol size. Each data point represents the mean for at least 10 individuals for encapsulated larvae and 25 individuals for pelagic larvae.

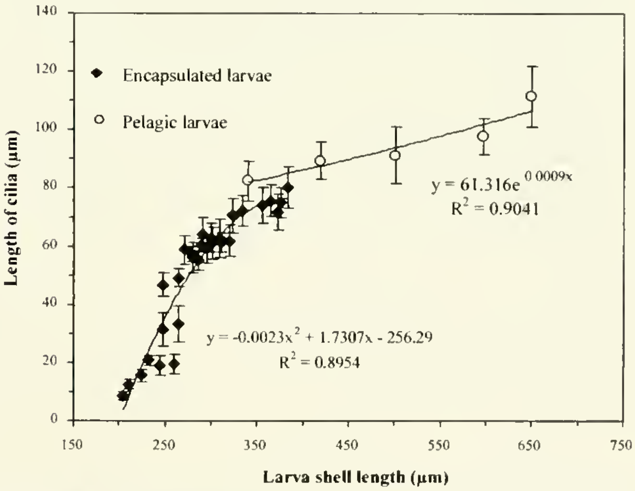

Figure 4. Length of preoral cilia in relation to larval size in encapsulated ($n = 29$) and pelagic ($n = 5$) larvae of *C. fecunda*. When the SD bar is not shown, it is smaller than the symbol size. Each data point represents the mean for at least 15 individuals for encapsulated larvae and 25 individuals for pelagic larvae.

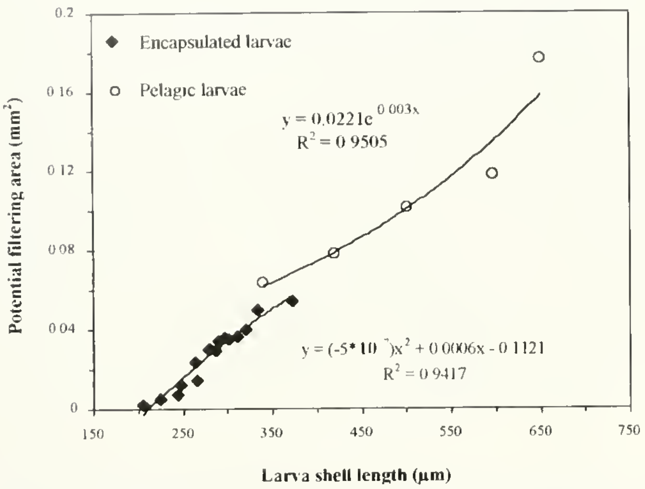

Figure 5. Potential filtering area in relation to larval size in encapsulated ($n = 16$) and pelagic ($n = 5$) larvae of *C. fecunda*. Each data point was calculated by multiplying the mean length of the ciliated velar edge by the mean length of the preoral cilia.

sion (potential filtering area = $0.0221e^{0.003*\text{larval shell length}}$, $r^2 = 0.9505$, $n = 5$) Figure 5.

Width of Food Groove

The width of the larval food groove increased with the size of the individual. The groove of encapsulated larvae 205 µm in shell length was 6.22 µm wide, increasing to 34.98 µm in pelagic larvae of 650 µm shell length (Fig. 6). In recently eclosed larvae, the width of the food groove was 10.5% greater than that of encapsulated larvae of the same shell length (Table 1).

Velocity of Particle Transport in the Food Groove

Particle transport velocity increased throughout the larval encapsulation period, with minimum values of 86 µm s^{-1} in larvae 225 µm in shell length, increasing to 270 µm s^{-1} in larvae 300 µm in shell length (approaching eclosion) [particle velocity = $0.02 \times (\text{shell length})^{1.622}$, $r^2 = 0.3562$, $n = 16$] Figure 7.

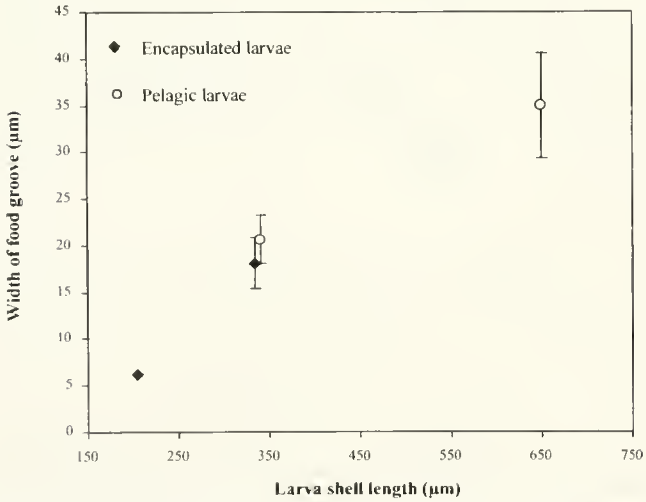

Figure 6. Width of larval feeding groove in relation to individual size in *C. fecunda*. Vertical bar = standard deviation.

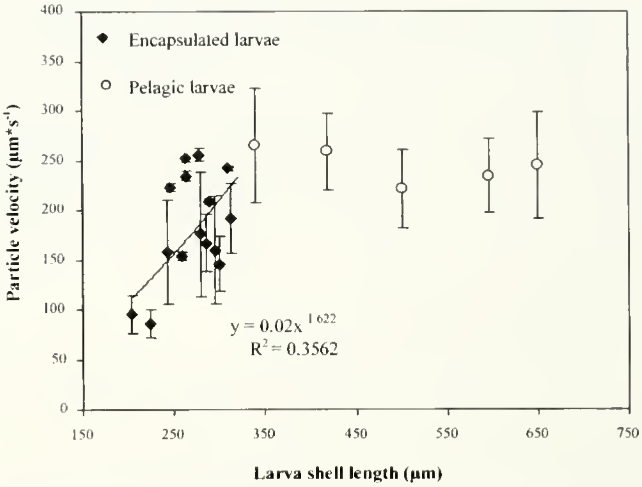

Figure 7. Transport velocity of particles in the larval food groove in relation to individual size in encapsulated ($n = 16$) and pelagic ($n = 5$) larvae of *C. fecunda*. When the SD bar is not shown, it is smaller than the symbol size. Each data point represents the mean for at least 10 individuals for encapsulated larvae and 20 individuals for pelagic larvae.

In pelagic larvae the velocity of the particles remained relatively constant with values near 250 µm s^{-1} (Fig. 7).

DISCUSSION

All encapsulated stages of *C. fecunda* veligers are able to ingest particulate material. This ability is related to the development of the ciliated velum (Gallardo, 1977, 1979), and particularly to the presence of opposed ciliary bands (Chaparro et al., in press). In the pelagic phase of the life cycle, development of the velum is of vital importance to swimming and feeding prior to settlement in this species, thus a large and well developed velum appears to be related to the mixed developmental strategy used by *C. fecunda* (Gallardo, 1979).

Encapsulated larvae of *C. fecunda* develop the ability to ingest particles before their transition to the planktotrophic habit. Chaparro et al. (in press) have observed particle capture and ingestion by pre-eclosed larvae which were fed immediately after artificial excapsulation, suggesting readiness for the planktotrophic phase.

The low CR observed in veligers removed from capsules is probably attributable to the early stage of development, as observed in larvae of other molluscan species (Hawkins et al. 1984; Welborn & Manahan 1990). Values for particle clearance by *C. fecunda* veligers obtained from capsules are lower in all cases than values reported in the literature for pelagic molluscan larvae (MacDonald 1988; Sprung 1984; Bayne 1965), although CR in *C. fecunda* pelagic larvae is well above the rates cited by these authors.

The rapid growth of the velum in encapsulated larvae of *C. fecunda* may be related to the mode of nutrition and the necessity for efficient swimming in the pelagic stage. Once the larva is free from the capsule, it is advantageous to have as large a velum as possible (Fretter & Graham 1962).

The increase in the length of the ciliated border of the velum in *C. fecunda* is a result of enlargement of the bilobed velum, not the formation of new lobes. The length of the preoral cilia in encapsulated and pelagic larvae of this species falls within the size range reported by Strathmann (1987), i.e., 30 to more than 100 µm, and also agrees with the 50 μm value given by Riedel (1992) for larvae of the gastropod *Cabestana spengleri* (200 μm shell length), which also exhibits mixed development.

The potential filtering area of recently eclosed pelagic larvae is nearly 80% larger than that of encapsulated larvae of the same size (370 μm shell length). This is due to the elongation of the ciliated border of the velum and growth of preoral cilia, and provides the larvae with a large surface area for particle capture, as suggested by the CR of pelagic larvae in this study. This is consistent with observations on the planktotrophic character of these larvae (Gallardo, 1977, 1979, 1989). The transport velocity of particles within the food groove of encapsulated larvae of *C. fecunda* increases with developmental stage to values near 250 μm s^{-1}. In pelagic larvae this velocity is more or less constant but the width of the food canal continues to increase during pelagic development, suggesting that pelagic larvae are able to capture and handle several particles at the same time, or increasingly larger particles (or particle aggregates) as they develop. Together with the increase in potential filtering area, this may explain the high values for CR in pelagic larvae of *C. fecunda*.

The greater capacity for particle clearance in pelagic larvae compared with encapsulated larvae in *C. fecunda* may be related to increases in the velar area, cilated length of the border of the velum, length of the preoral cilia, potential filtering area, and the width of the food groove, or to some combination of these variables. These changes are obviously adaptive as the larvae are released into the environment, where they must be able to swim and feed before settling. Some larvae remain in the capsule, yet have a shell size similar to that of newly released larvae, suggesting a differential rate of development between shell and velum, the latter requiring more time to develop before eclosion.

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DISTRIBUTION, DENSITY AND LENGTH-WEIGHT RELATIONSHIP OF *CHITON ARTICULATUS* SOWERBY, 1832 (MOLLUSCA-POLYPLACOPHORA) ON ISLA SOCORRO, REVILLAGIGEDO ARCHIPELAGO, MEXICO

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ABSTRACT *Chiton articulatus* is an abundant mollusk species on the Isla Socorro. In March 1992 *C. articulatus* populations were sampled at four sites on the southwestern and northern coast of the island. Average densities of *C. articulatus* ranged between 3.7 to 8.5 ind/m^2 among sites. The length-weight relationship for all sampled individuals (N = 385) is W(g) = 0.00022 L$^{2.73}$.

KEY WORDS: *Chiton articulatus*, mollusk, distribution, density, Isla Socorro

INTRODUCTION

Chitons are polyplacophoran mollusks that are common grazers of intertidal epilithic and endolithic algae. Since their first appearance in the late Cambrian, chitons have modified hard marine substrates using their strong radula (Glynn 1970; Rasmussen & Frankenberg 1990).

Chiton articulatus is a species that was recently very abundant along the Pacific tropical coast of Mexico, today *C. articulatus* is uncommon because of over exploitation by fishermen. The foot is commercially utilized and sought in subsistence fisheries. In the Revillagigedo Archipelago (Fig. 1) it is a potential resource that has integral usage possibilities, it is still an important member of the malacofauna since it has not been fished commercially (Villalobos 1960; as *C. laevigatus*, Keen 1971; Ferreira 1983; Holguin et al. 1992; Holguin 1994; Bautista-Romero et al. 1994; Mille-Pagaza et al. 1994; Emerson 1995).

Only one study has been conducted for the Mexican Pacific continental shore population of *C. articulatus*, in the Acapulco area (Rojas-Herrera 1988). Little is known about the Revillagigedo Archipelago chiton populations, because of its distance from the Mexican mainland. This article describes the distribution, density and length-weight relationship of *C. articulatus* sampled on Isla Socorro, Revillagigedo in March 1992.

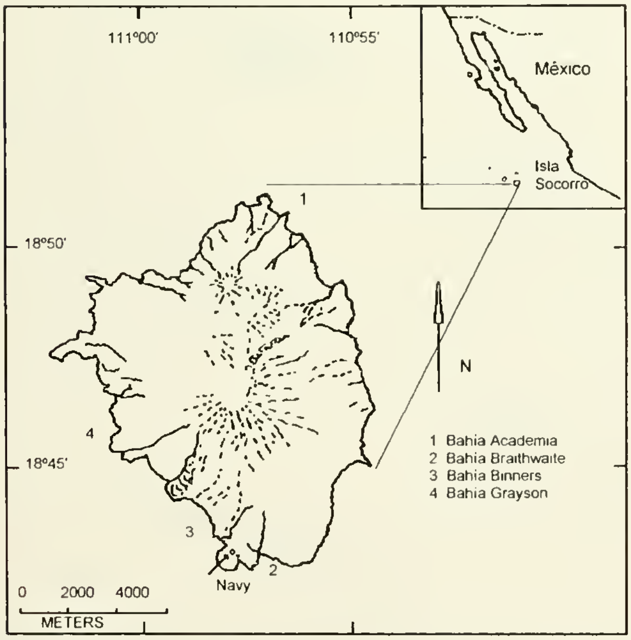

Figure 1. Map of the sampling sites on Isla Socorro, Revillagigedo, March 1992.

MATERIALS AND METHODS

The Revillagigedo Archipelago (Fig. 1) is located approximately 350–650 km, southeast of the Baja California Peninsula and 580 km west of the Colima coast (Llinas-Gutierrez et al. 1993). The Archipelago is comprised of four oceanic islands of volcanic origin, Socorro, Clarion, San Benedicto and Roca Partida.

The surface area of Isla Socorro, the largest island, is 167 km^2 with a maximum diameter of 16 km in a NW-SW direction. Geographically Isla Socorro is located at 18°41′57″N and 110°56′33″W (Troyo-Diéguez & Pedrín 1994). The island is composed primarily of braced igneous rock, with the presence of smooth stones and sandy or stone-sandy beaches (Holguin 1994).

During March 1992, *C articulatus* were sampled in the following rocky areas, from south to north (Fig. 1): Bahía Braithwaite (La Braulia), Bahía Binners, Bahía Grayson (Palmasola), and Bahía Academia (Playa Norte). Some of the areas are more accessible than other areas where there is a greater abundance of organisms. At each one of the four sampling locations, we took samples along a transect of 50 m^2 in the intertidal zone. We laid out transects parallel to the coast line, using 25 m cord which delineated a 2 m wide sampling swath. The entire sampling was accomplished during ebb tides. The density of *C. articulatus* in

TABLE 1.

Mean densities for *Chiton articulatus* at four sites on Isla Socorro, Revillagigedo, México, March 1992.

Locality	Area (m^2)	No. Total Organism	Relative Density (m^2)	Maximum Number Chitons Grouped
Bahía Binners	50	186	3.72	22
Bahía Braithwaite	50	424	8.48	35
Bahía Grayson	50	193	3.86	33
Bahía Academia	50	249	4.38	[illegible]

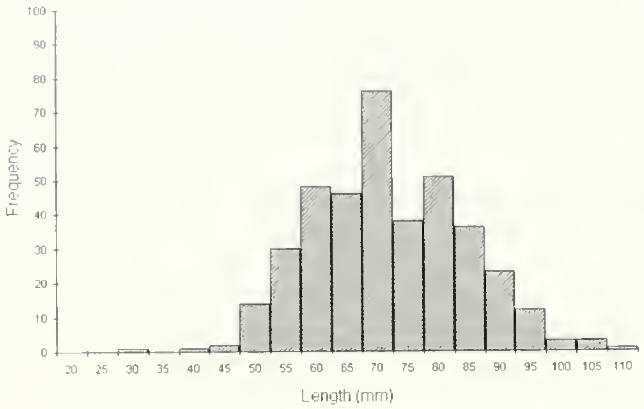

Figure 2. Length frequency distribution of *Chiton articulatus* sampled on Isla Socorro, Revillagigedo, March 1992.

each rocky shore area was determined from the count of all living organisms present in the transect. The maximum number of the chitons grouped was determined.

A sample of 30–40% of chitons (N = 385) was taken from the transects in order to be measured and weighed. The length and weight measurements from selected chitons were recorded. The length was measured using a vernier caliper, taking the major distance between the front and the posterior parts of the body. Weight, including the shell, was obtained with a three-arm scale having an accuracy of ±0.5 g. We liberated all the animals in the rocky intertidal zone after measuring them.

RESULTS AND DISCUSSION

On Isla Socorro all rocky coasts exposed to strong waves are populated by *Chiton articulatus*. The species is discontinuously distributed along the reef line; in some areas adult animals over 4 cm length were found crowded within a narrow belt along the coast, while in adjacent areas animals bigger than 3 cm were not found at all. According to Rojas-Herrera (1988) the size of the mollusk in its first sexual maturity is 40 mm. We observed juvenile *C. articulatus* (<4 cm) in Isla Socorro, mostly in the rock fissures and in the cracks of the intertidal belts occupied by the sea urchin *Echinometra vanbrunti*. We observed that for *C. articulatus* the

TABLE 2.

Mean lengths and weights for *Chiton articulatus* at four sites on Isla Socorro, Revillagigedo, México, March 1992.

		Length (mm)				Weight (g)			
Locality	*n*	min	max	mean	s.d.	min	max	mean	s.d.
Bahía Binners	55	42	108.0	67.9	15.9	4.5	86.5	25.5	19.1
Bahía Braithwaite	169	29	95.3	66.5	12.4	2.0	75.4	26.6	14.2
Bahía Grayson	75	49	102.0	75.6	10.6	9.0	83.5	33.5	14.9
Bahía Academia	86	49	90.0	71.9	8.9	8.3	49.0	27.4	9.3
TOTAL	385	29	108.0	69.6	12.5	2.0	86.5	27.9	14.5

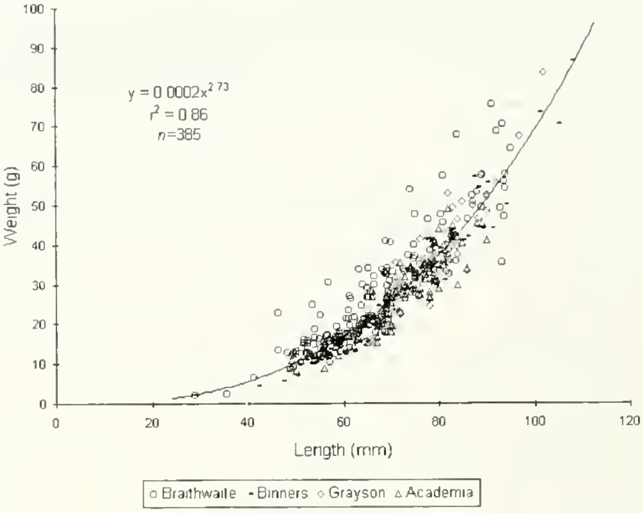

Figure 3. Length-weight relationship of *Chiton articulatus* sampled on Isla Socorro, Revillagigedo, March 1992.

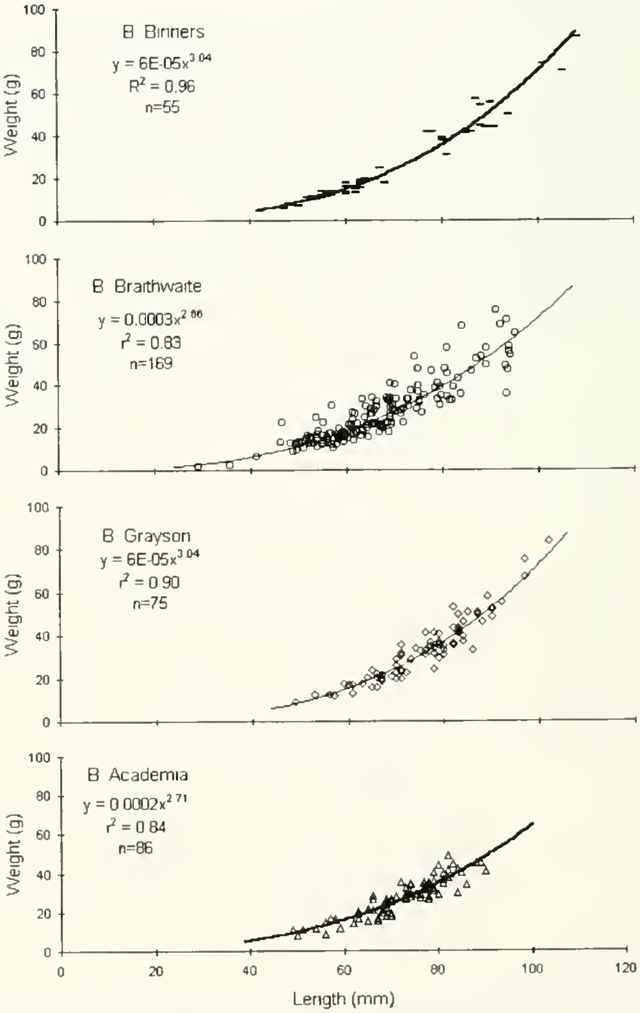

Figure 4. Length-weight relationship of *Chiton articulatus* for each sampling site on Isla Socorro, Revillagigedo, March 1992.

major feeding activity was nocturnal and food was generally composed of crusty filamentous algae and diatoms.

Several other species of invertebrates coexist with *C. articulatus*. Some are chiton predators such as the gastropods *Plicopurpura pansa* and *Thais* spp., as well as the cephalopod *Octopus* sp. Other coexisting species are *Littorina aspera*, *L. modesta* and *L. pullata*, *Nerita funiculata*, *Diodora inaequalis*, *Chama squamuligera*, *Colisella* spp. and *Fissurella spp*. Several crustacean species can also be found in those areas, such as the decapod crab *Grapsus grapsus*, the cirriped *Tetraclita* sp. and the isopod *Ligia exotica*.

We counted a total of 1,052 *C. articulatus* occurring in the sampling transects (Table 1). The highest relative density average (8.5 animals/m^2) was found in Bahía Braithwaite and the lowest in Bahía Binners (3.7 animals/m^2) with an average of 5.3 animals/m^2 for the four sampling sites combined. We observed the maximum densities of up to 41 chitons/m^2 in small sections of the transect (Bahía Academia, Table 1).

The abundance of chitons on Isla Socorro is greater than in Acapulco seaside (4.2 animals/m^2, Rojas-Herrera 1988). The high density of the mollusk, that we observed in Bahía Academia, is probably due to the fact that the bay is far away from any populated center. Bahía Binners which is most accessible to the navy village, shows lower densities because of fishery activity.

The length frequency distribution of the 385 chitons collected on Isla Socorro, ranged between 29 and 108 mm with a mode of 70 mm (Fig. 2). The smallest chiton sampled weighed 2.0 grams, while the longest weighed 86.5 grams (Table 2). The average values are of 69.6 mm and 27.9 g for length and weight respectively (Table 2). The length-weight relationship $W = 0.0022\ L^{2.73}$ where W is total weight (g) and L = total length (mm) was derived from all 385 *C. articulatus* measured in March 1992 on Isla Socorro (Fig. 3 and Fig. 4).

There are no significant differences between length-weight relationship in the sampling areas. The major length weight per unit was found in Bahía Braithwaite and the minor weight was found in Bahía Grayson.

It is important to point out that the predominance of Chiton bigger than 6 cm. in the rocky intertida zone of the Isla Socorro, is due mainly to its geographic location, this part of the island is almost inaccessible by land or sea. The population of this species in general, is unaltered and its partial exploitation under strict protection measures is possible.

ACKNOWLEDGMENTS

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CELLULAR IMMUNOLOGICAL PARAMETERS OF THE OCTOPUS, *OCTOPUS VULGARIS*

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ABSTRACT The white body is the main hematopoietic organ of cephalopods. In this study, we have investigated the capacity of the octopus (*Octopus vulgaris*) white body cells to perform common cellular defense parameters known to be done by hemocytes of other mollusks such as phagocytosis of zymosan particles, respiratory burst activity and nitric oxide (NO) production. White body cells were capable of respiratory burst and NO production, however, they exhibited a low phagocytic response. Similar capabilities were observed in hemocytes withdrawn from the hemolymph. We have studied the effects of in vitro incubation with bacterial lipopolysacharide (LPS) or zymosan for 24 hours on these two functions. Incubation of the white body cells with zymosan, but not with LPS, resulted in a significantly increased respiratory burst activity and NO production.

We have also investigated the capacity of circulating hemocytes and white body cells to increase their thymidine uptake (indicative of DNA synthesis) in response to LPS and phytohemaglutinin (PHA). In some animals, both mitogens induced a significant increase in thymidine uptake. If this thymidine uptake correlates with cell proliferation, this will be the first report of any proliferation of hemocytes in mollusks.

In the hemolymph, we observed two different morphologies under the electron microscope, however, we cannot conclude that they correspond to two distinct cell types. Among white body cells different morphologies that may correspond to intermediate stages were observed. All these findings represent a baseline for future studies to elucidate mechanisms of host defense in this mollusk.

KEY WORDS: *Octopus vulgaris*, hemocytes, white body, respiratory burst, thymidine uptake, nitric oxide (NO), phagocytosis

INTRODUCTION

Due to the decrease in Spanish cephalopod fisheries, the interest in the commercial culture of cepahalopod species has gained increasing attention. Diseases are one of the major obstacles in achieving this goal. Several pathogens have been identified in wild and aquarium-kept octopuses, including viruses, bacteria and fungi. Farley (1978) described the presence of viruses in the muscle of the octopus, *Octopus vulgaris*. Bacteria have been reported to cause several disease outbreaks in laboratory reared octopuses (Hanlon & Forsythe 1990; Hanlon et al. 1984). Parasites have been blamed for several pathogenic problems. Hochberg (1990) described a flagellated parasite in cultured octopuses. Fungi have been also described as causing problems in cephalopods maintained in captivity (Polglase 1980; Polglase et al. 1984). Despite the threat these pathogens may cause to octopus populations, elucidation of defense mechanisms in these species have received little attention.

Humoral defense factors have been identified and are reviewed by Ford (1992). Rogener et al. (1985) and Fisher and Dinuzzo (1991) described hemaglutinating activity and aglutinnins in cell free hemolymph of several molluskan species, including the octopus, *Octopus vulgaris*. In this species, an antiprotease of the α-macroglobulin family was also detected in the hemolymph (Thogersen et al. 1992). Malham et al. (1998) reported lysozyme and antiprotease activity in hemocytes and hemolymph of the lesser octopus *Eledone cirhhosa*. Like in other mollusks, cephalopod hemocytes are believed to play a role in host defense mechanisms, however, the function of the hemocytes has been poorly studied. Cowden and Curtis (1981) estimated that the phagocytic capacity of octopus hemocytes was low while high phagocytosis of carbon particles has been described in *Eledone cirrhosa* (Stuart 1968). Bayne (1983) reported a clearance of *Serratia marcescens* by hemocytes of the octopus, *Octopus dofleini*.

The generation of hemocytes of cephalopods is believed to take place in an organ situated around the optic nerve called the white body, gland of Hensen or gland of Faussek (Stuart 1968; Cowden 1983; Bolognari et al. 1980). Hence, the aim of this study was to determine whether white body cells from *Octopus vulgaris* are capable of performing certain functions believed to be of relevance to defense mechanisms like phagocytosis of zymosan, respiratory burst and nitric oxide (NO) production and to compare their functionality to that of circulating hemocytes. We have also examined the ability of two mitogens, bacterial lipopolysacharide (LPS) and phytohemaglutinin (PHA), to stimulate thymidine uptake, an indicative of DNA synthesis. In the case of the respiratory burst and NO production, we have also determined the effect of in vitro activation with bacterial lipopolysacharide (LPS) and zymosan.

MATERIALS AND METHODS

Sampling

Adult octopuses (*Octopus vulgaris*) of both sexes, weighing 2–3 kg were caught from a raft in the Ría de Vigo (Spain) and after a day of adaptation under laboratory conditions, were anesthetized with excess MS-222. When animals were fully anesthetized (identified by muscle relaxation and absence of movement), the visceral cavity was dissected. Once the heart and its associated vessels were reached, around 1 ml of hemolymph was drawn using a 27 gauge needle from the artery and subsequently from the heart, placed in an eppendorf and kept on ice until used. The white body was removed and kept on ice until used.

In order to obtain single cell suspensions, individual white bodies were passed through a 100 μm nylon mesh using Leibovitz medium (L-15, Gibco) supplemented with penicillin (100 IU/mL), streptomycin (100 μg/mL) and 2% fetal calf serum (FCS). The resulting cell suspension was centrifuged (500 × g for 15 min at 4°C) and the cells were resuspended in L-15 supplemented with penicillin, streptomycin and 2% (FCS). Cell viability was determined by Trypan blue exclusion. Cells were resuspended in L-15 supplemented with penicillin, streptomycin and 0.1% FCS a [illegible] density of 1×10^6 cells/mL.

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Hemocytes were obtained by centrifuging octopus hemolymph (500 × g for 15 min at 4°C) and hemocytes were resuspended in L-15 supplemented with penicillin, streptomycin and 2% FCS. The cell viability was determined by Trypan blue exclusion test. Cells were resuspended in L-15 supplemented with penicillin, streptomycin and 0.1% FCS at a concentration of 1 × 10^6 cells/mL.

Electron Microscopy

Pelleted cells (from white body and hemolymph) were fixed for one hour in 1% osmium tetroxide in cacodilate buffer 0.1 M (pH 7.3). In some cases, a previous step of fixation with 1% glutaraldehyde in cacodilate buffer 0.1 M (pH 7.3) for 15 minutes was performed. Following three washes in 0.1 M cacodilate, the cells were dehydrated with increasing percentages of ethanol and embedded in Araldite/Poly Bed (Polyscience). Ultrathin sections (50–70 nm) were stained with uranyl acetate and lead citrate and examined using a Phillips electron microscope CM100.

Phagocytosis Assay

To measure the phagocytic ability of octopus cells, 200 μL of cell suspensions (derived from white body or hemolymph of six animals) were incubated in chamber slides (Nunc) for 2 h at 18°C in moist incubation chambers to allow the cells to adhere. Medium was removed and the adherent cell layer was washed twice with L-15. Zymosan A (Sigma) resuspended in sterile phosphate buffered saline, PBS, at a concentration of 1 mg/mL, was heated at 100°C for 30 min, washed twice, and resuspended in the same PBS volume. Zymosan was added to the cells at a final concentration of 250 μg/mL, and the same volume of L-15 was added to the controls. Slides were incubated in a moist chamber for one hour at room temperature or 18°C to allow phagocytosis. Some slides were kept up to three hours. Slides were then washed twice in PBS, fixed in absolute ethanol, stained with Hemacolor, and mounted with DePex. Two replicas were made for each octopus and at least 150 cells were observed in each replica.

Respiratory Burst Activity

Respiratory burst activity of octopus cells was assayed by the reduction of ferricytochrome C (Cit C, Sigma) by released superoxide anion (O_2–), following stimulation of the cells with phorbol myristate acetate (PMA, Sigma) (Secombes 1990) in 4 octopuses. White body adherent cells and circulating hemocytes were obtained as described above, resuspended in L-15 supplemented with penicillin, streptomycin and 0.1% FCS and dispensed into 96-well tissue culture plates (Iwaki) at a concentration of 1 × 10^6 cells/mL (100 μL per well).

In a preliminary experiment, we determined the specificity of the respiratory burst by assaying the response of the cells to PMA. After 24 hours of incubation at 18°C, octopus cell monolayers were washed twice in phenol red-free Hank's balanced salt solution (HBSS, Gibco). One hundred μL of HBSS containing Cit C (2 mg/mL) and PMA (1 μg/mL) were added to each well. As a control for specificity, 300 IU/mL superoxide dismutase (SOD, Sigma) was added to some wells. The optical density (O.D.) was measured at 550 nm after 30 min in a multiscan spectrophotometer (Labsystems). Triplicate wells were used in all the experiments for each octopus and the mean ± SD was calculated.

Once the responsiveness of octopus cells to PMA had been determined, the effects of stimulation with zymosan or *Escherichia coli* serotype 0111: B4 lipopolysacharide (LPS) on the respiratory burst of octopus cells triggered by PMA was also studied. After three hours incubation of the cell monolayers at 18°C, LPS and zymosan were added to a final concentration of 50 and 250 μg/mL respectively. After an additional 24 hours of incubation at 18°C with these substances, the respiratory burst activity was measured by adding 100 μL of HBSS containing Cit C (2 mg/mL) and PMA (1 μg/mL) were added to each well. The O.D. at 550 nm was then determined as described above.

NO Production

The ability of octopus cells to produce NO in response to LPS and zymosan was also determined in 4 animals. Cells resuspended in L-15 with 0.1% FCS were disposed into 96-well plates at a concentration of 1 × 10^6 cells/mL. After 3 h of incubation at 18°C, LPS and zymosan were added at a final concentration 50 and 250 μg/mL respectively. After additional 24 h of incubation at 18°C, the NO concentration present in the cell supernatants was assayed through the Griess reaction (Green et al. 1982) that quantifies the nitrite content of the cell supernatants, since NO is an unstable molecule and degrades to nitrite and nitrate. Fifty μL of hemocyte supernatants were removed from individual wells and placed in a separate 96-well plate. One hundred microliters of 1% sulfanilamide (Sigma) in 2.5% phosphoric acid were added to each well, followed by the addition of 100 μL of 0.1% N-naphthylethylenediamine (Sigma) in 2.5% phosphoric acid. Optical density at 540 nm was determined using a multiscan spectrophotometer. The molar concentration of nitrite in the sample was determined from standard curves generated using known concentrations of sodium nitrite (100, 10, 5, 2.5, 1, 0.5, 0.25, and 0.1 μM).

Effect of Mitogens on (^{3}H)-Thymidine Uptake by Octopus Cells

The thymidine uptake by octopus cells was assayed following a modification of the method described by Marsden et al. (1994) in six octopuses. Briefly, hemocytes derived from the hemolymph or the white body were adjusted to a density of 5 × 10^6 cells/mL in RPMI 1640 medium (Gibco) supplemented with penicillin (100 IU/mL), streptomycin (100 μg/mL), 25 mM $NaHCO_3$ and 1 × 10^{-5} M 2-β-mercaptoethanol. Aliquots of 100 μL of cell suspensions were added to wells of 96-well plates containing 100 μL of LPS or PHA dilutions (Sigma) to make final concentrations of 50, 25 and 12.5 μg/mL or 5, 2.5, and 1.25 μg/mL, respectively. Controls without mitogens were also included. After 3 h of incubation at 18°C, FCS was added to the wells to give a final concentration of 10%. Following 48 h of incubation, the cells were pulsed with 0.5 μCi of (^{3}H)-thymidine (Amersham). After additional 24 h incubation at 18°C, DNA was harvested onto glass filter mats. One mL Xylofluor scintillant (Packard) was added to dried filter circles in vials and counts per minute (cpm) were recorded using a Packard liquid scintillation counter. Triplicate cultures were used in all cases.

Statistics

The data were compared using a Student's *t* test. Results are expressed as the mean ± standard deviation and differences were considered statistically significant at $P < 0.05$.

RESULTS

Cell Morphology

After two hours of incubation of the cell suspensions at 18°C, the white body adherent cells were adhered to the bottom of the wells, flatten and spread over the surface by extending pseudopodia. Circulating hemocytes presented the same aspect.

When visualized under the electron microscope (Fig. 1), what seems as two different morphologies, that were distinct under the inverted microscope, were observed among circulating hemocytes. No differences were found in the quality of fixation when the previous glutaraldehyde step was omitted and therefore the cells were always directly fixed in osmium tetroxide. We identified in the first cell type a kidney-shaped nucleus that occupied about 2/3 of the cell volume with a well-defined nucleolus and abundant heterochromatin in peripheral positions. Their cytoplasm was rich in vacuoles and electron-dense granules of various sizes. The second cell type had a nucleus with faint chromatin, a round nucleus that occupied about 1/3 of the cell volume. The cytoplasm was rich in vacuoles, but had only scarce granules. However, these results are not conclusive and we cannot assure that what looks as two different morphologies corresponds to two distinct cell types.

In the case of white body cells, we were able of identifying cells showing other morphologies that may correspond to intermediate stages between the two cell types found in the hemolymph.

Phagocytosis Activity of White Body Adherent Cells and Circulating Hemocytes

The phagocytic activity detected after incubation of octopus hemocytes and white body cells with zymosan was low. The percentage of phagocytosis observed in circulating hemocytes was 19.3% (SD = 14), while only 9.3% (SD = 8) of white body cells contained zymosan particles in their cytoplasm. In all cases, variations among individuals were high, since in some samples no phagocytosis was observed (0% of phagocytosis). The same results were obtained with the two incubation temperatures (18°C or room temperature) in both circulating and white body cells. No differences were observed when the hemocytes were incubated in their own hemolymph (data not shown).

Respiratory Burst Activity

Octopus white body cells showed a significant increase in the release of superoxide anion after stimulation with PMA compared with controls, as depicted in Figure 2A. The specificity of the reaction was demonstrated since SOD completely inhibited the respiratory burst response of octopus cells in all cases. This response was also observed with circulating hemocytes. The respiratory burst activity of hemocytes incubated directly in their own hemolymph was also assayed. In these conditions, some octopuses did not respond to PMA and did not elicit a respiratory burst response.

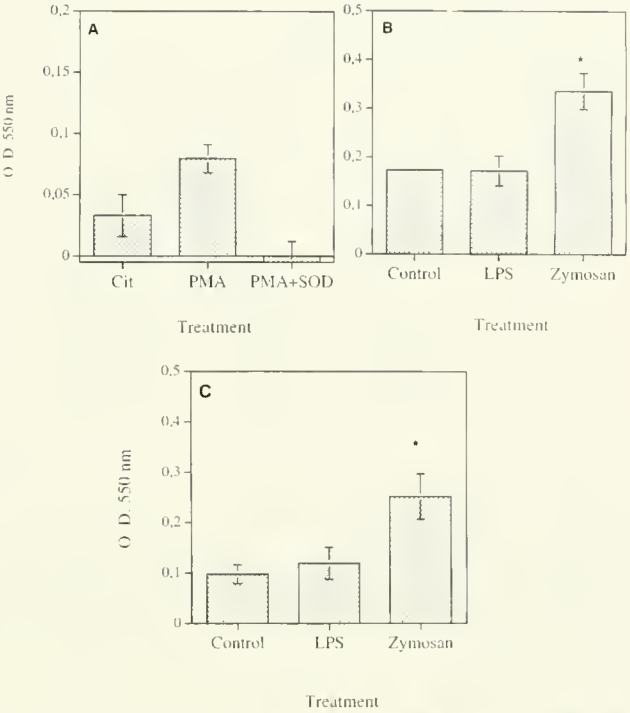

Figure 2. Respiratory burst activity of octopus white body cells. First, the capacity of PMA to stimulate the respiratory burst activity and the specificity of the reaction was assayed (A) (N = 2). Once, this was determined, the effects of LPS and zymosan on the respiratory burst activity of white body hemocytes (B) (N = 4) and circulating hemocytes (C) were studied through the reduction of Cit C, stimulating the cells with PMA. Data are shown as the mean O.D. at 550 nm. *Respiratory burst significantly higher than the respiratory burst obtained in controls. $P < 0.05$.

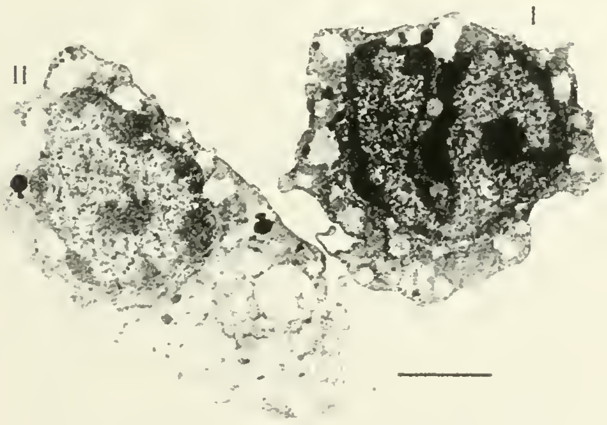

Figure 1. Under the electron microscope, two distinct morphologies were identified among circulating hemocytes. The first cell type (I) is characterized by its kidney-shaped nucleus and the high number of electron-dense granules. The second cell type (II) possessed a round nucleus with a few electron dense granules. Bar = 5 μm.

Figure 2B shows the effect of incubation for 24 hours with LPS or zymosan on the respiratory burst triggered by PMA of white body cells, compared to the respiratory burst observed in cells that had been incubated with L-15 only. The pre-incubation of the cells with zymosan, but not LPS, significantly increased the respiratory burst of white body cells in response to PMA. The same response was observed in cells obtained from hemolymph, showing a higher respiratory burst after zymosan treatment than controls (Fig. 2C).

NO Production

The NO production of white body cells in response to LPS and zymosan is shown in Figure 3. Zymosan significantly stimulated the cells for NO production in all individuals, however, as in the case of the respiratory burst activity, LPS failed to stimulate the cells.

In the case of hemolymph cells, a similar response was observed. The NO production in the cultures treated with zymosan (8.4; SD = 0.8) was higher than the response observed in controls (6.7; SD = 0.3).

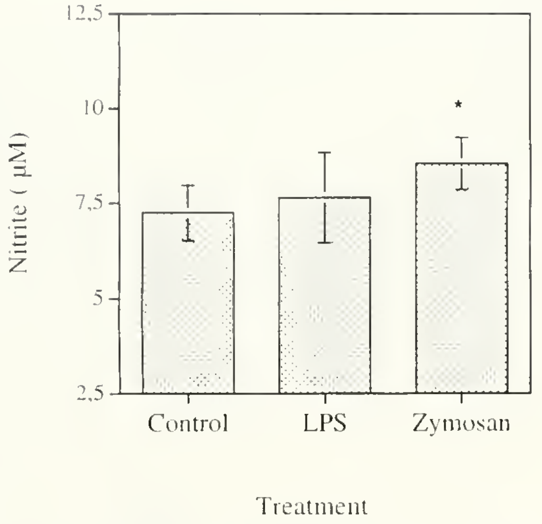

Figure 3. NO production of cells from the white body after incubation with LPS or zymosan. Data are presented as the mean nitrite concentration obtained with 4 octopus. *Nitrite concentration significantly higher than the one observed in controls only treated with L-15. $P < 0.05$.

(^{3}H)-Thymidine Uptake

Not all animals studied showed a significant response. Only two out of six individuals assayed responded with a significant increase of the thymidine uptake in response to mitogens. The individual responses observed in these two animals are shown in Table 1. When means were compared, concerning white body cells, a maximum thymidine uptake was observed with the higher LPS dose (687.3; SD = 6.7). In this case, the PHA dose that significantly stimulated the thymidine uptake was 2.5 µg/ml (495; SD = 20) in comparison to controls (378.5; SD = 2.5).

In these two responsive animals, hemocyte thymidine uptake was also significantly affected by the mitogens. All concentrations of LPS significantly increased (^{3}H)-thymidine uptake compared to non-stimulated controls, although the higher response was observed with the highest LPS dose. However, hemocyte thymidine uptake was only significantly increased with the higher dose of PHA.

TABLE 1.

Thymidine uptake by circulating hemocytes and white body cells in two responsive animals. Data are presented as the mean cpm obtained in the replicas (N = 3) ± SD.

	Octopus 1		Octopus 2	
Mitogens	Circulating Hemocytes	White Body Cells	Circulating Hemocytes	White Body Cells
LPS 50 µg/ml	630.6 ± 242	694 ± 351	515.3 ± 37	680.6 ± 104
LPS 25 µg/ml	340 ± 118	366 ± 144	457 ± 217	370 ± 70
LPS 12.5 µg/ml	458 ± 98	761 ± 418	427 ± 41	524 ± 142
PHA 5 µg/ml	340 ± 92	241 ± 171	615 ± 128	253 ± 58
PHA 2.5 µg/ml	238 ± 12	475 ± 147	141 ± 32	515 ± 154
PHA 1.125 µg/ml	178 ± 52	313 ± 79	222 ± 42	290 ± 55
Control	204 ± 64	381 ± 105	294 ± 94	376 ± 12

DISCUSSION

The findings in this study suggest that both octopus white body cells and circulating hemocytes are capable of performing functions associated with host defense mechanisms. This is particularly important to determine since scanty data is available on the immune response of this octopus species. This is the first work in which reagents and techniques usually used in vertebrate immunology have been successfully applied to study cellular responses of cephalopods.

Under the electron microscope, two distinct morphologies among circulating hemocytes were identified, although it had been described as only one cell type of the hemolymph of *Octopus vulgaris* (Bidder et al. 1989). Our results are not conclusive and more work should be done to determine whether these two morphologies correspond to different states of activation or they constitute two different cell types. Previous studies in bivalve mollusks have identified two main hemocyte types in the hemolymph (Fisher 1986; Lopez et al. 1997) that have been subdivided (Auffret 1988; Nakayama et al. 1997). In the white body, it is well known that there are cells, referred to as hemocytoblasts, with a large cytoplasmic volume and abundant rough endoplasmic reticulum and nucleoli (Ford 1992). Hemocytoblasts transform to leukoblasts by reducing its cytoplasmic volume and decreasing its nuclear size, whereas secondary leukoblasts (mature hemocytes) are cells with a larger size and a folded nucleus. Both under the light and electron microscope, we were able to identify different morphologies that may correspond to these intermediate stages among white body cells. As well, the two different morphologies that we describe in this work among circulating hemocytes seem to correspond to these two cell types (hemocytoblasts and leukoblasts). It may be possible that in these individuals maturation ends in the hemolymph or as will be discussed later, that these two cell types are functionally different.

The first cell type may correspond to what has been called granulocyes in bivalves (Auffret 1988). These cells possess a kidney-shaped nucleus that resembles those of vertebrate granulocytes. The second cell type had a round nucleus and a cytoplasm that was also rich in vacuoles, but had only scarce granules. This cell type may correspond to what in bivalves has been called hyalinocytes (agranular hemocytes) (Auffret 1988).

Results concerning in vitro phagocytosis by octopus hemocytes are in accordance with those previously reported (Crowden & Curtis 1981) where it was suggested that gill tissue or phagocytes from gill tissue could be responsible for clearing foreign substances and that circulating hemocytes had low activity (Crowden & Curtis 1981; Bayne 1983). However, studies in other cephalopod species, like *Eledone cirrhosa* reported bacterial phagocytic rates of even 40% (Malham et al. 1997). The low phagocytic activity that is observed in the octopus, *Octopus vulgaris*, circulating hemocytes also contrasts with the high phagocytic rate detected in bivalves (Mortensen & Glette 1996; Ordas et al. 1999), animals in which phagocytosis is a critical defense mechanism. Many factors can affect phagocytic rates in mollusks such as temperature (Carballal et al. 1997), time and pH (Abdul-Salam & Michelson 1980), size and nature of the particle presented for phagocytosis (Bayne 1983). In some cephalopod species, phagocytosis is not possible in the absence of hemolymph (Stuart 1968). It may be possible that on the contrary to what happens with the respiratory burst (that is inhibited by the presence of hemolymph), phagocytosis in this species needs humoral factors present in the hemolymph.

Octopus hemocytes, however, were able to respond to PMA and release significant amounts of superoxide anion. Respiratory burst activity is an interesting non-specific defense mechanism that vertebrate macrophages use to avoid bacterial and parasitic infections due to the potent microbiocidal effects of oxygen radicals. The reason why the respiratory burst activity was not detected in some of the octopuses while incubated in their own hemolymph is unknown. The immune system is regulated by multiple signals that can up or down modulate the animal responses. Maybe regulating factors, affecting this immune response, are present in the hemolymph of these animals, in the same way that other humoral factors such as lysozyme or antiproteases have been detected in cephalopod hemolymph (Malham et al. 1998).

We have also demonstrated that octopus cells derived from hemolymph and white body produced NO. In vertebrates, NO mediates many functions including neurotransmission, vasodilatation, as well as several immune functions. It is known that this molecule when secreted by macrophages is microbicidal against viruses, parasites and bacteria (Nathan & Hibbs 1991; Tafalla et al. 1999). In the octopus, NO production was significantly increased when the cells were incubated with zymosan for 24 hours. However, incubation with LPS did not have an effect on the NO secretion. This is the first report on NO production by cells of any cephalopod. NO production in other mollusks has been shown, in which NO production was demonstrated by indirect methods (Martinez 1995; Otaviani & Franchini 1995).

Octopus hemocytes were also able to increase their thymidine uptake after stimulation with LPS and PHA, although this response was not found in all individuals, maybe because this immune function is more related to a specific response not known to be present in cephalopods. Thymidine uptake implies DNA synthesis, so this increase may mean that the hemocytes are proliferating. If so, this will be the first report of any proliferation in mollusks, outside the hematopoietic organ. More work must be done to determine if this thymidine uptake detected correlates with cell proliferation. In higher vertebrates, these two mitogens correlate to B and T lymphocyte mitogens, respectively. Since we observed ultrastructurally two morphologies among hemocytes in responsive animals, it may be possible that LPS and PHA-induced proliferation are being supported by different hemocyte populations, and therefore imply a certain grade of heterogeneity, as in vertebrate immune cells.

In conclusion, we have effectively applied immunological techniques designed for vertebrates to evaluate cephalopod hemocyte functions. Among mollusks, cephalopods are highly evolved animals with a body design, nervous system and sense organs that often reach vertebrate standards (Budelmann et al. 1997). It seems possible that immune system may also be more developed than other mollusks in which modulation of immune responses by pathological agents or other factors has been reported (Ordas et al. 1999). We have set up different in vitro techniques, such us determination of respiratory burst activity, NO production and cell proliferation, which will let us further evaluate the octopus immune response against infections or the influence of environmental conditions. This latter aspect is especially relevant because of increasing interest that cephalopod aquaculture has had in the past years (Osako & Murata 1983; Guerra & Rocha 1994; Guerra et al. 1994). More work must be done to clarify the regulation of these immune functions in cephalopods and determine their role in the defense against pathogens.

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ESTIMATING GONADO-SOMATIC INDICES IN BIVALVES WITH FUSED GONADS

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ABSTRACT A new method was developed to estimate the gonado-somatic index in bivalves, taking into account that in most bivalves gonad and non reproductive tissues are fused. As examples, four tropical bivalve species (*Donax dentifer, Cardita affinis, Pinctada imbricata* and *Pteria colymbus*) from the Colombian Pacific and Caribbean were used. The fused gonad regions were replaced by geometric bodies, thus the gonad volumes were estimated with geometric equations (corresponding to geometric bodies) and linear measures obtained directly on frozen soft-body samples. The very good correlation between the gonado-somatic index and the gametogenetic cycle of the stage "ripe", leads to the conclusion that this method is a suitable tool to estimate gonad indices of bivalves with fused gonads.

KEY WORDS: method, gonado-somatic index, bivalves, *Donax dentifer, Cardita affinis, Pinctada imbricata, Pteria colymbus*

INTRODUCTION

A standard procedure in marine biological reproductive studies is to determine gametogenetic stages (Guillou et al. 1990) or to estimate indices (Moore 1934; Giese 1967; Pearse 1965). Among many existing indices the so called gonado-somatic indices, calculated from the relationship between a variable depending directly on reproductive processes and a variable independent from reproductive processes, are most frequent used. Examples for the variables used in gonado-somatic indices are the gonad volume and the total volume. Such a gonado-somatic index, followed periodically over a year, is a valuable tool because it is related directly to reproductive activities, such as spawning events. Among marine species, the estimation of gonado-somatic indices in bivalves often presents a problem because most species of this group have fused gonads, such that reproductive and non-reproductive tissues cannot—or are difficult to be separated.

Based on four tropical bivalve species, the objective of this article is to present and explain a new method, that permits the estimation of gonado-somatic indices in bivalves with fused gonads. In order to evaluate the new method, the gonado-somatic index is compared with gametogenetic stages obtained from smear samples.

MATERIALS & METHODS

Investigations were carried out in three areas: the *Tayrona National Nature Park*, close to the city of Santa Marta, Caribbean of Colombia (11°20′N, 74°10′W, Fig. 1a), the "*Cabo de la Vela*", a Peninsula located in the Guajira province, Caribbean of Colombia (12°10′N, 72°20′W, Fig. 1b) and the "*Bahía Málaga*", a bay close to the harbor town of Buenaventura, Pacific Coast of Colombia (3°56′N, 77°19′W, Fig. 1c). In *Tayrona Park* the pearl oyster *Pteria colymbus*, which lives attached to octocorals is a dominant species, while in *Cabo de la Vela* a characteristic *Thalassia testudinum* community gives a suitable substrate for a variety of bivalve species, especially a dense *Pinctada imbricata* popula-

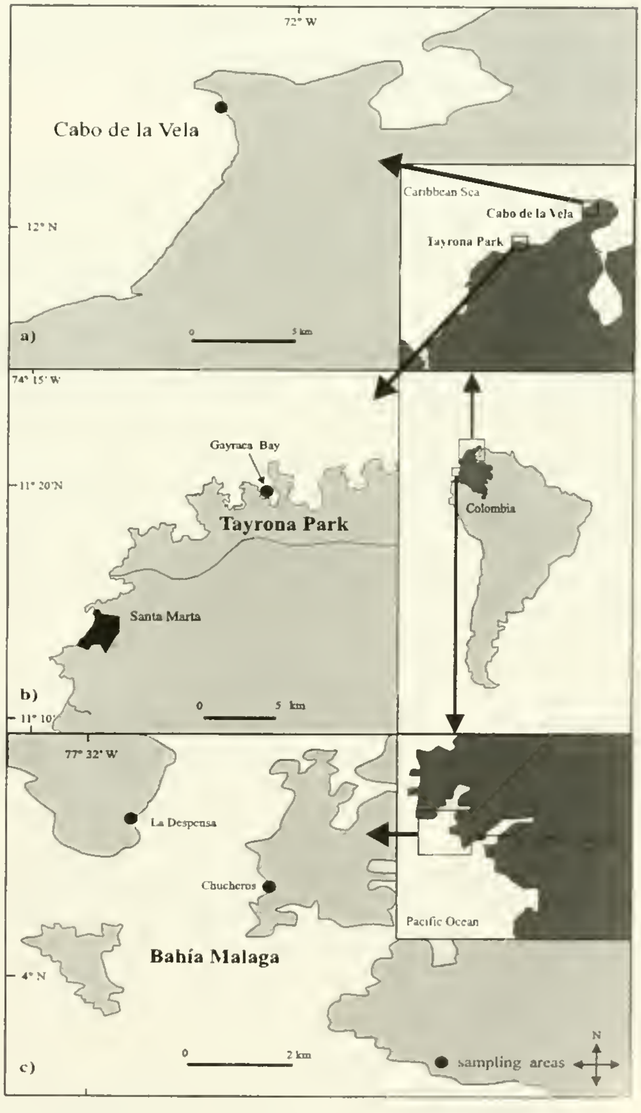

Figure 1. Study areas a) Cabo de la Vela, Colombian Caribbean; b) Tayrona Park; Colombian Caribbean; and c) Bahía Málaga, Colombian Pacific

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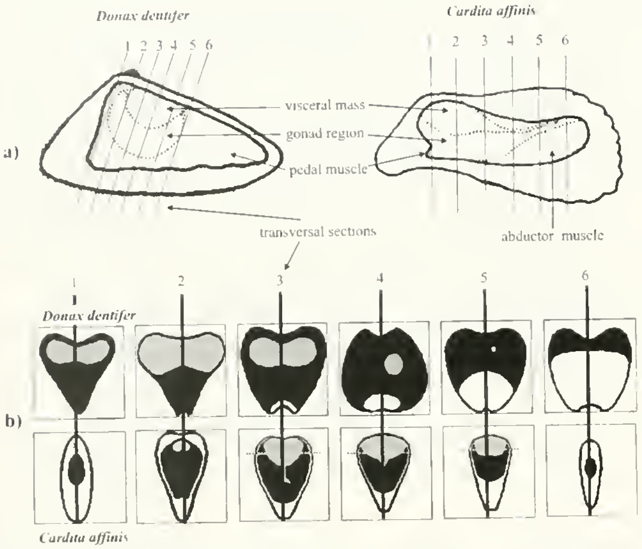
Donax dentifer
Cardita affinis
1 2 3 4 5 6
visceral mass
gonad region
pedal muscle
transversal sections
abductor muscle
a)
b)
1 2 3 4 5 6
Donax dentifer
Cardita affinis

Figure 2. a) Morphological description of body tissues (on left valve, mantle, siphons and gills removed) of *Donax dentifer* and *Cardita affinis*. Location where transversal sections were taken is indicated. b) Transversal section according to Figure 2a. Gonadal tissue is given in black, visceral tissue in gray and muscle tissue in white.

tion. Finally, at *Bahía Málaga* the infaunal *Donax dentifer* found in the intertidal of sandy beaches and the borer clam *Cardita affinis* from rocky shores were studied.

Sampling for *Donax dentifer* was carried out in 1997/98 as well as in 1999/00, while for the latter three species the study period lasted one year, but at different dates (*Pteria colymbus*: 1994/95, *Pinctada imbricata*: 1997/98, *Cardita affinis*: 2000/01). At monthly intervals, samples were taken and the shell length (anterior-posterior axis) of all specimens was recorded with vernier callipers. Two subsamples of 30 specimens per species each were taken. One subsample was used for length-weight relationships: soft parts were removed and dried at 70°C to constant weight to determine shell free dry weight (SFDW). Monthly length weight relationships were used to estimate a shell free dry weight (= condition) cycle of a standard individual (= 66% of maximum length) based on Equation (1):

$$SL = a \cdot SFDW^{b} \qquad (1)$$

where SL is the shell length [mm] and SFDW is the shell free dry weight [g]. The second subsample was deep frozen and used to obtain linear measures of gonad regions with a vernier callipers in order to estimate the gonado-somatic index (details are given under "Results" later). Finally, after taking the linear measures, gametogenetic stages of smear samples were determined according to a microscopic scale (Urban 2000): "developing 1 (= early developing)", "developing 2 (= late developing)", "ripe" and "spent".

RESULTS

Using frozen samples, six transversal sections (thick slices) were taken at different locations of the body; these were used to identify the gonad region geometrically. As an example only the morphological sections of two of the four species are presented. Figure 2a shows the soft parts (mantle, siphons and gills removed) on the left shell of *D. dentifer* and *C. affinis* demonstrating the fused character of somatic and gonad tissues. Figure 2b shows the six transversal sections. In *D. dentifer* the visceral mass is located in the middle-dorsal body part, partially enclosed by the gonad region. The pedal muscle occupies the major part of the total body volume extending in anterior-posterior direction and enclosing the gonad region. Contrary, in *C. affinis* the visceral mass is located in the posterior-dorsal part, decreasing towards the anterior end. The gonad region is located in the posterior-ventral zone, increasing its volume towards the middle part (section 3) and then extending

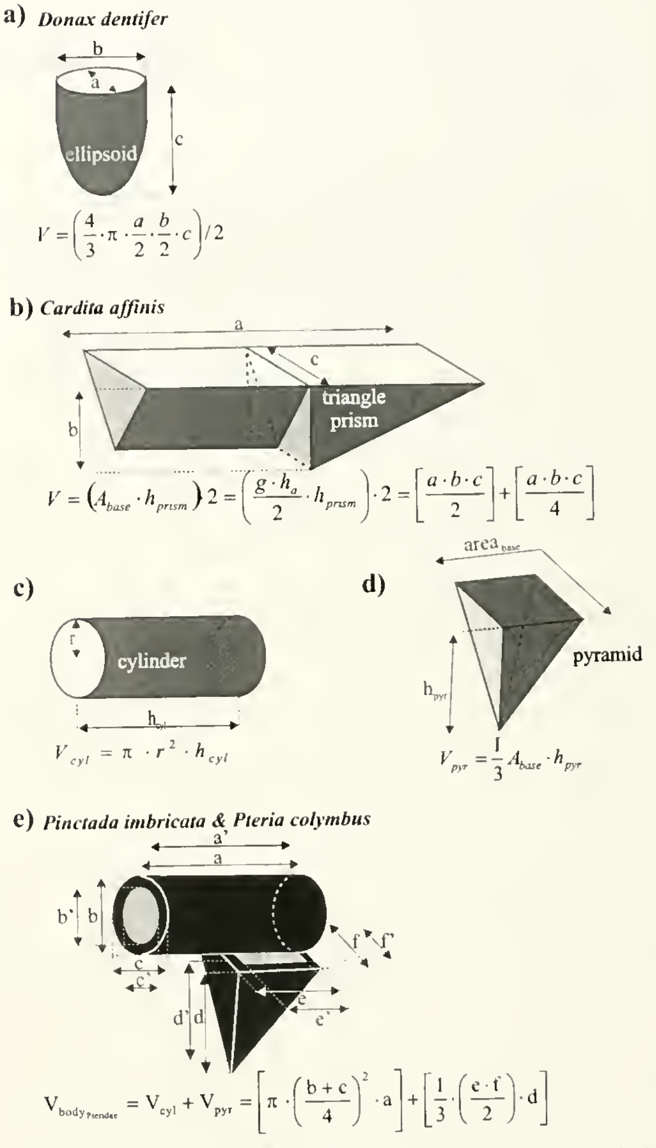
a) Donax dentifer
b
a
c
ellipsoid
$V = \left(\frac{4}{3} \cdot \pi \cdot \frac{a}{2} \cdot \frac{b}{2} \cdot c\right)/2$
b) Cardita affinis
a
c
b
triangle prism
$V = \left(A_{base} \cdot h_{prism}\right) 2 = \left(\frac{g \cdot h_a}{2} \cdot h_{prism}\right) \cdot 2 = \left[\frac{a \cdot b \cdot c}{2}\right] + \left[\frac{a \cdot b \cdot c}{4}\right]$
c)
r
cylinder
h_{cyl}
$V_{cyl} = \pi \cdot r^2 \cdot h_{cyl}$
d)
area base
pyramid
h_{pyr}
$V_{pyr} = \frac{1}{3} A_{base} \cdot h_{pyr}$
e) Pinctada imbricata & Pteria colymbus
a'
a
b'
b
c
c'
f
f'
d'
d
e
e'
$V_{body_{Pteridae}} = V_{cyl} + V_{pyr} = \left[\pi \cdot \left(\frac{b+c}{4}\right)^2 \cdot a\right] + \left[\frac{1}{3} \cdot \left(\frac{e \cdot f}{2}\right) \cdot d\right]$

Figure 3. Geometric bodies defined according to the morphological examinations of the transversal sections (Fig. 2b). The linear measures necessary to calculate the volume as well as the formulas are given (see text). a) Ellipsoid of *Donax dentifer*. b) Two fused triangle prisms of *Cardita affinis*. c, d, and e) Fused cylinder and pyramid of *Pinctada imbricata* and *Pteria colymbus*. Simple small letter correspond to linear measures of somatic and reproductive tissues, while gonadal tissues are given as small letters with "′" (e.g. a′).

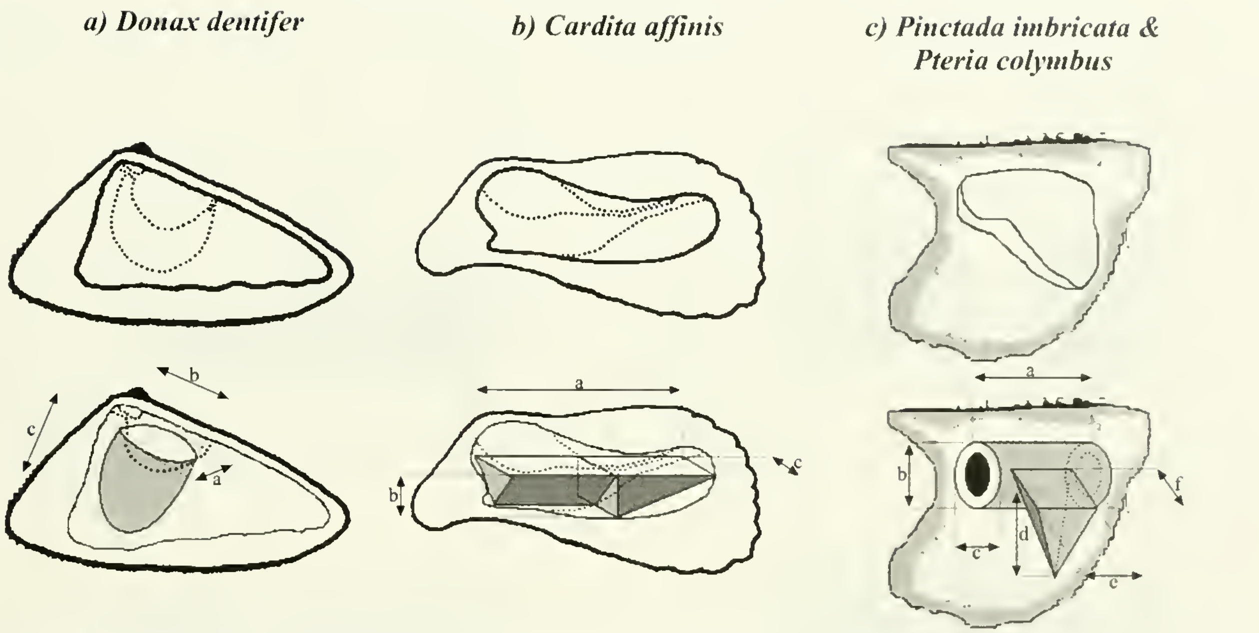

Figure 4. Body tissues (upper row) and geometric bodies replacing gonad region and or body tissues (lower row). a) ***Donax dentifer*****, b)** ***Cardita affinis*** **and c)** ***Pinctada imbricata*** **and** ***Pteria colymbus***

towards the anterior-dorsal part where its volume decreases gradually. The pedal muscle is reduced, corresponding to a small extension of the posterior-ventral end. The volume of the abductor muscle increases from the middle ventral zone, extending towards the anterior end.

Based on the morphological examination of the gonad region (Fig. 2) for all species three-dimensional bodies of the gonad region were defined. In other words, the gonad region was replaced with geometric bodies. The volumes of these bodies and thus the gonad volume, can be estimated using the following geometric equations and linear measures [mm] taken directly on the bivalves from frozen samples. Based on macroscopic criteria such as texture, color, etc., gonad tissue could be easily distinguished from somatic tissue. Thus, for each species during the study period the gonad volumes [ml] of approximately 30 specimens were obtained at monthly intervals.

Donax dentifer

The gonad morphology corresponds to an ellipsoid half (Figs. 3a & 4a), located between the foot and the visceral mass. The equation below Figure 3a corresponds to the volume of an ellipsoid half. It is clear, however, that a small visceral region is located in the dorsal part of the "gonadal ellipsoid" (Fig. 2a). On average, this visceral tissue occupied 10% (max: 19%, min: 6%) of the total ellipsoid volume. Thus, in order to yield the gonad volume, the formula was corrected multiplying with 0.9 (Eq. 2):

$$V_{gon.Donax} = \left[\left(\frac{4}{3}\cdot\pi\cdot\frac{a}{2}\cdot\frac{b}{2}\cdot c\right)/2\right]\cdot 0.9 \qquad (2)$$

Measures are indicated in Figures 3a & 4a; "a" is the gonad breadth (lateral to the body axis), "b" is the gonad length (anterior-posterior body axis) and "c" is the gonad height (dorsal-ventral body axis at transversal section number 3.

Cardita affinis

The geometry of the gonad region suggests the form of two fused triangle prisms (Fig. 3b & Fig. 4b). Thus, the formula of a single triangle prism has to be multiplied by 2 (Eq. 3):

$$V = [A_{base}\cdot h_{prism}]\cdot 2 = \left[\left(\frac{a\cdot h_a}{2}\right)\cdot h_{prism}\right]\cdot 2 \qquad (3)$$

Where A_{base} is the area of the base, which corresponds to a triangle with "a" being one side and "h_a" the height of the triangle corresponding to "a", finally "h_{prism}" is the height of the prism. The gonad volume was calculated as the sum of these two prisms (Eq. 4):

$$V_{gon.Cardita} = \left[\frac{a\cdot b\cdot c}{2}\right]+\left[\frac{a\cdot b\cdot c}{4}\right] \qquad (4)$$

Where "a" is the gonad length (anterior-posterior body axis), "b" is the gonad height (transversal to gonad length corresponding to transversal section number 3, Fig. 2) and "c" is the gonad breadth at the fusion point of both prisms. (Note: "a" corresponds to the length of both fused prisms, therefore for the two bodies "1/2 a" has to be used yielding Eq. 4.)

Pinctada imbricata *and* Pteria colymbus

These two species of the family Pteridae are different from the latter two because the gonad tissue covers the somatic tissues. In *D. dentifer* and *C. affinis* the total body volume was estimated with the water displacement method and gonad volume corresponds directly to the geometric abstractions, while in *P. imbricata* and *P. colymbus* first the total body volume (visceral mass and foot including gonads) and then in a second step the "covering" gonad volume was estimated. The total body volume can be defined by a cylinder (Fig. 3c & Fig. 4c, Eq. 5a) fused with a pyramid (Fig. 3d & Fig. 4c, Eq. 5b). The cylinder corresponds to the visceral mass

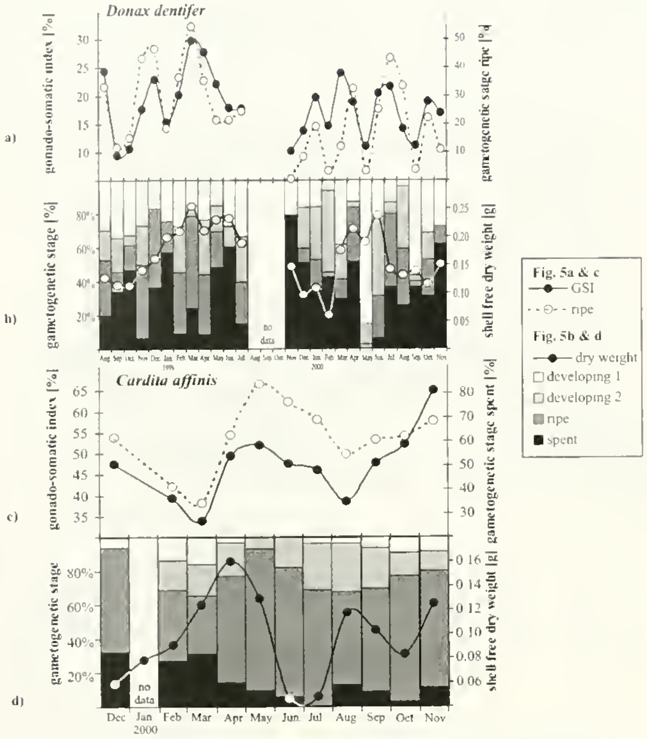

Figure 5. a) Overlay of gonado-somatic index and gametogenetic stage "ripe" cycle. b) Overlay of shell free dry weight cycle and distribution of gametogenetic stages of *Donax dentifer* from Bahía Málaga, Colombian Pacific. c & d) *Cardita affinis* from Bahía Málaga, Colombian Pacific.

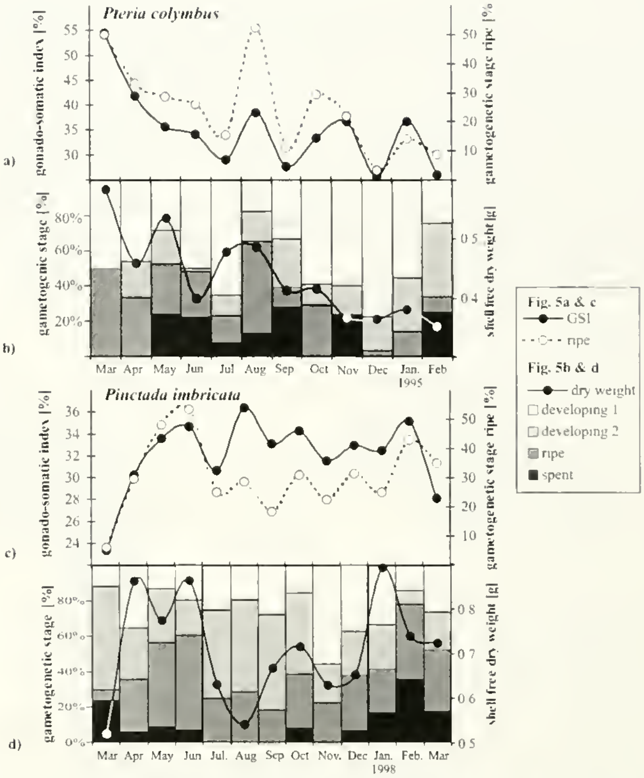

Figure 6. a) Overlay of gonado-somatic index and gametogenetic stage "ripe" cycle. b) Overlay of shell free dry weight cycle and distribution of gametogenetic stages of *Pteria colymbus* from Tayrona Park, Colombian Caribbean. c & d) *Pinctada imbricata* from Cabo de la Vela, Colombian Caribbean.

and the pyramid to the basal region of the foot. These two bodies have the general formula:

$$a)\ V_{cyl} = \pi \cdot r^2 \cdot h_{cyl},\ b)\ V_{pyr} = \frac{1}{3} A_{base} \cdot h_{pyr} \quad (5)$$

Where "r" is the radius, h_{cyl} is the height of the cylinder (Fig. 3c), "A_{base}" is the area of the base of the pyramid and "h_{pyr}" is the height of the pyramid (Fig. 3d). So the total body volume of these two species corresponds to the sum of the volume of a cylinder and a pyramid (Fig. 3e, Eq. 6 & 7):

$$\begin{aligned} V_{body\ tot_{Pteridae}} &= V_{cyl} + V_{pyr} \\ &= \left[\pi \cdot \left(\frac{b+c}{4}\right)^2 \cdot a\right] + \left[\frac{1}{3} \cdot \left(\frac{e \cdot f}{2}\right) \cdot d\right] \end{aligned} \quad (6)$$

Where "a" is the length, "b" is the height and "c" is the breadth of the visceral mass (including gonad region) and "d" is the length, "e" is the height and "f" is the breadth of the foot (including gonad region). In the second step the somatic volume was estimated using the same equation (Eq. 6), but with linear measures where gonad regions were excluded. Gonad volume was estimated subtracting the somatic volume from the total volume (Eq. 7):

$$\begin{aligned} V_{gon\ Pteridae} &= V_{body\text{-}tot} - V_{body\text{-}somat} \\ &= \left\{\left[\pi \cdot \left(\frac{b+c}{4}\right)^2 \cdot a\right] + \left[\frac{1}{3} \cdot \left(\frac{e \cdot f}{2}\right) \cdot d\right]\right\} \\ &\quad - \left\{\left[\pi \cdot \left(\frac{b'+c'}{4}\right)^2 \cdot a'\right] + \left[\frac{1}{3} \cdot \left(\frac{e' \cdot f'}{2}\right) \cdot d'\right]\right\} \end{aligned} \quad (7)$$

Where "$V_{body\ tot}$" is the total body volume and "$V_{body\text{-}somat}$" is the volume of the somatic tissues [ml], "a", "b", "c", "d", "e", and "f" are identical to the measures of Eq. (6), while a', b', c', d', e', and f' are linear measures parallel to "a", "b", "c", "d", "e", and "f" corresponding only to somatic tissues (Fig. 3e).

Figure 4 shows in the upper row for all four species the soft body parts on the right shell and below the geometric abstractions defined to estimate the gonad volume. Note that in *D. dentifer* and *C. affinis* only the gonad region corresponds to the (gray) geometric abstractions, while in *P. imbricata* and *P. colymbus* all soft parts were replaced with the geometric abstraction, and the gonad volume is covering non-reproductive tissues (in black).

TABLE 1.

Spearman correlation matrix of gonado-somatic index and gametogenetic stage "ripe" as well as shell free dry weight cycle

	P. colymbus	*P. imbricata*	*D. dentifer*	*C. affinis*
	Gonado-Somatic Index (GSI)			
Ripe	0.811	0.501	0.669	0.627
SFDW	0.594	0.170	0.488	0.264

Based on the total body and the gonad volume a gonado-somatic index (GSI, Eq. 8) was estimated.

$$GSI = \frac{V_{gon}}{V_{body}} \cdot 100 \quad (8)$$

Table 1 gives the Spearman correlation matrix of the gonado-somatic index (GSI) and the gametogenetic stage "ripe" as well as the condition (= shell free dry weight, SFDW) cycle. For all species a high correlation between GSI and the amount of ripe gonads is indicated ($p > 0.5$). For the correlation between GSI and SFDW only for *Pteria colymbus* and *Donax dentifer* high values can be observed.

This result is confirmed in Figure 5a & c and Figure 6a & c where GSI and ripe cycles are plotted in overlay graphs. In all species the pattern of GSI and ripe cycle is very similar. Figure 5b & d and Figure 6 b & d show the SFDW cycles together with the distribution of gametogenetic stages.

DISCUSSION

In order to evaluate a new method it is most convenient to compare the results with alternative information obtained completely independent. This can easily be accomplished for the GSI. It can be assumed that the GSI is positively correlated with the gametogenetic stage "ripe". GSI gives the mean gonad volume/total volume relationship per month. In other words if a large part of the population has voluminous gonads, the GSI is high. On the other hand the gametogenic stage "ripe" gives the percentage of specimens with ripe gonads (shortly before spawning). In this development stage, egg and sperm size is large, resulting in voluminous gonads. The results clearly confirm this assumption and thus prove the applicability of the GSI method. Graphically (Fig. 5a and c and Fig. 6a and c) as well as statistically (Table 1) a high correlation between the GSI and the gametogenic stage "ripe" exists. It should also be noted that GSI and stage "ripe" are completely independent measures: The GSI is a quantitative index based on linear measures, whereas the "ripe" cycle is a semi-quantitative measure obtained from microscopic observations of smear sample.

Together with the GSI and the "ripe" cycle, the shell free dry weight (SFDW = condition) cycle as well as the distribution of all gametogenetic stages is presented (Fig. 5b and d and Fig. 6b and d). Here, the typical pattern of tropical species with a very long or permanent spawning season and continuous reproduction strategy is demonstrated. All four species studied have ripe ovaries through out the year with no resting or inactive gonads found. This characteristic pattern is in contrast to cold temperate species. As an example, Urban and Campos (1994) studied the gonad cycles of the infaunal clam *Gari solida* from Chile at 37°S. In this species during 9 months of the year, developing 1 and/or spent stages dominate (85%). Only during the latter 3 months did ripe and/or developing 2 stages dominate indicating a short spawning period.

Contrasting to the "ripe" cycle only for *Pteria colymbus* and *Donax dentifer*, a high correlation between GSI and SFDW was observed. However, a high correlation between these two variables cannot be expected generally in a tropical environment with little annual variability of biotic and abiotic factors such as temperature, nutrients or phytoplankton. Thus, under rather continuous feeding conditions a condition cycle might be independent from the gonad activities, contrasting to cold temperate latitudes with typical spring/summer phytoplankton blooms.

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COMPARATIVE FECUNDITY OF THREE *PROCAMBARUS* SPECIES

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ABSTRACT Adult female *Procambarus acutus acutus*, *P. zonangulus*, and *P. clarkii* (n = 64) were held in simulated burrows for 160 days. Mean percentage hepatopancreas moisture for a subsample (n = 12) of *P. zonangulus* was significantly less than that for the other two species. *Procambarus clarkii* oviposited eggs 30 days earlier than the other two species; however, a significantly lower proportion of the *P. clarkii* (33.3%) oviposited eggs than did *P. a. acutus* (95.3%) and *P. zonangulus* (71.9%). The number of extruded eggs increased with crayfish total length (TL). Similar-sized *P. clarkii* oviposited significantly more eggs than *P. zonangulus* and the eggs/female for *P. a. acutus* were significantly less than for the other two species. The dry weight, protein, and lipid content per egg for *P. a. acutus* and *P. zonangulus* were similar and significantly greater than those for *P. clarkii* eggs. The smaller eggs of *P. clarkii* yielded shorter instars than *P. a. acutus* and *P. zonangulus* instar TL. A standard-sized *P. a. acutus* (104.6 mm TL) allocated 10.2%, 13.6%, and 22.2% of total-body dry weight, protein, and lipid to extruded eggs, respectively.

KEY WORDS: crayfish, fecundity, *Procambarus clarkii*, *Procambarus zonangulus*, *Procambarus acutus acutus*

INTRODUCTION

Red swamp crayfish, *Procambarus clarkii* (Girard), dominates crayfish aquaculture and wild harvests in the southeastern United States (Eversole & McClain 2000). Frequently, these harvests contain the white river crayfish, originally classified as *Procambarus acutus acutus* (Girard) until Hobbs and Hobbs (1990) revised the taxonomy. Upon revision, the white river crayfish, which overlapped *P. clarkii*'s range in Louisiana and in other states along the Gulf of Mexico, was named *Procambarus zonangulus* Hobbs and Hobbs. *P. a. acutus*, whose range extends from Maine to Georgia, occurs sympatrically with *P. clarkii* only in locations where the latter has been introduced.

Comparative studies of *P. clarkii* and *P. zonangulus* indicate significant differences in life history traits between the two species. For example, *P. zonangulus* produces fewer large eggs, whereas *P. clarkii* produces a greater number of small eggs (Noblitt et al. 1995; Noblitt & Payne 1995). Noblitt et al. (1995) suggested that *P. zonangulus* followed a prudent reproductive strategy that was characteristic of species adapted to an environment where nutrients are low or pulsed at an unpredictable rate. In contrast, *P. clarkii*'s reproductive strategy is consistent with existence in a nutrient-abundant, predictable environment. *P. zonangulus* also appears to be more conservative in mobilizing nutrients during starvation than is *P. clarkii* (Powell 2001). Other comparisons include length–weight relationships, production characteristics, and population studies in aquaculture systems (Romaire & Lutz 1989; Deng et al. 1995; Huner 1994).

P. clarkii was introduced into South Carolina for aquaculture in 1978 (Pomeroy & Kahl 1987). Although *P. clarkii* is the mainstay of the South Carolina aquaculture industry (Whetstone, pers. comm.), there are concerns about its and other crayfish introductions (Taylor et al. 1996). Comparative research on *P. clarkii* and *P. a. acutus* has been limited to investigations of the two species in experimental culture ponds. Denson and Eversole (1996) reported a dramatic shift in species composition from *P. clarkii*- to *P. a. acutus*-dominated culture ponds over a relatively short period. Follow-up 2 years of supplemental stockings (59–114 kg/ha) of *P. clarkii* failed to increase its long-term production in these *P. a. acutus*-dominated ponds (Eversole et al. 1999). Although reflooding of drained culture ponds in September increased *P. clarkii* percentage in the harvest in comparison to October and November reflooding dates, it did not improve production (Mazlum & Eversole 2000).

A series of investigations have been designed to gain a better understanding of *P. clarkii* and *P. a. acutus* interactions. The focus of this study was to provide comparative reproductive data for *P. a. acutus*, *P. zonangulus*, and *P. clarkii*. Specific objectives were to determine the number of extruded eggs, egg size and composition, egg development time, and instar size for the three species.

MATERIALS AND METHODS

Experimental Animals

Adult female *P. a. acutus* and *P. clarkii* were collected from harvests at two locations in South Carolina: Clemson University Aquaculture Research Facility at Clemson and a commercial farm in Fountain Inn on May 24 and June 1, 1999, respectively. Female *P. zonangulus* were collected from Ben Hur Farm, Louisiana State University, Baton Rouge, Louisiana, air shipped, and arrived at Clemson on May 19, 1999. Individuals (n = 76) of each species with a complete set of appendages were either placed in simulated burrows (n = 64) or sacrificed for hepatopancreas moisture determinations (n = 12).

Hepatopancreas Moisture

The total length (TL) of 12 individuals of each species was measured to the nearest millimeter. The hepatopancreas was excised, weighed to the nearest 0.01 g after blotting (H_{ww}) and dried in a convection oven (80°C) to a constant weight (H_{dw}). Moisture content of the hepatopancreas (HM) was calculated using the following formula:

$$\mathrm{HM} = \frac{\mathrm{H}_{\mathrm{ww}} - \mathrm{H}_{\mathrm{dw}}}{\mathrm{H}_{\mathrm{ww}}} \times 100$$

Reproductive Success

Individual crayfish (n = 64) were measured (TL) and placed in 1-L plastic containers (simulated burrows). Although these con-

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tainers were not intended to duplicate conditions in earthen burrows, they do represent a technique to compare fecundities across species under more uniform test conditions. The simulated burrows were suspended in restaurant glass racks held in four raceways receiving dechlorinated aerated water. Each raceway contained a rack of 16 individuals of each species. Holes drilled in the bottom and along the side of the container allowed water exchange and maintained 5-cm water depth. Crayfish were held at ambient water temperatures and not fed over the 160-day experimental period. Crayfish were checked 5 days/wk and every fifth day the water was exchanged in the raceway. Water temperature and dissolved oxygen were checked by meter (Model 55, YSI). Survival and the presence of extruded eggs were noted by date. A proportion of the ovigerous females were removed from burrows, usually within 24 h of oviposition, and the extruded eggs removed with forceps, counted, and fixed in 10% neutral formalin. Eggs on the other ovigerous females were allowed to develop and hatch. Development time was recorded and the TL of third instars measured.

Egg Characteristics

Egg subsamples ($n = 4$–5) from 12 individuals of each species dried at 80°C were weighed to the nearest 0.001 mg to estimate mean egg dry weight. Following drying, the nitrogen content of egg samples was determined by the Dumas method using a Carlo-Erba NA 1800 (Series 2) nitrogen analyzer. Nitrogen content (percent) in an egg sample was multiplied by 6.16 to estimate the percentage protein per egg on a dry weight basis (Noblitt & Payne 1995). Lipid content was determined on duplicate subsamples of 10 eggs each from six ovigerous females per species. Egg lipid content (percent) was determined as described by Mann and Gallager (1985). Egg lipid content (percent) was calculated on a dry weight basis as a mean of the duplicate subsamples.

Statistical Analysis

Data were analyzed with the analysis of variance (ANOVA), analysis of covariance (ANCOVA), and linear regression using Micro-SAS Statistical Software System Version 8 (SAS 1999). A split-block design with trays representing blocks was used to analyze the effects of raceways on the reproductive performance of the three species. The general linear model for ANOVA was used to compare species differences in initial TL, instar TL, hepatopancreas moisture content, time to ovipositing, hatching time, and egg size (dry weight, lipid, and protein content). The differences between ovipositing and nonovipositing female crayfish within species were compared by ANOVA. Linear regression was used to determine the relationship between crayfish TL and number of extruded eggs per ovipositing female. The ANCOVA procedure was used to compare slopes and intercepts of the regressions and eggs/female. Differences in means were detected with least-significant difference (LSD) test (Ott 1993). Level of significance was set at $P \leq 0.05$.

RESULTS

Experimental Animals

The TL of the species stocked in simulated burrows were significantly different; *P. a. acutus* was the longest, *P. zonangulus* the shortest, whereas *P. clarkii* was intermediate in TL (Table 1). *P. zonangulus* hepatopancreas contained the least amount of moisture and was assumed to be in better conditions than the other two species (Huner et al. 1985; Huner et al. 1990). Mortality in the simulated burrows was low; only two individuals each of *P. zonangulus* and *P. clarkii* died over the 160-day experimental period. Mean (±SD) water temperature and dissolved oxygen was 24.5 ± 2.21°C and 4.0 ± 1.38 mg/L, respectively, over the period.

TABLE 1.

Mean (±SD) and range of total length (TL) of the females used in the experiment, ovipositing and nonovipositing crayfish, hepatopancreas moisture, time from the start of experiment to ovipositing, and number of extruded eggs per female.

Variable*	*P. a. acutus*	*P. zonangulus*	*P. clarkii*
Initial female TL (mm)	109.1 ± 6.89^{a} (90–127) $n = 64$	95.7 ± 7.95^{b} (80–120) $n = 64$	100.5 ± 9.44^{c} (83–125) $n = 64$
Hepatopancreas moisture (%)	55.1 ± 8.31^{a} (41–69) $n = 12$	39.1 ± 6.17^{b} (29–50) $n = 12$	55.3 ± 5.93^{a} (48–64) $n = 12$
Ovipositing female TL (mm)	109.3 ± 6.99^{A} (90–127) $n = 61$	97.4 ± 7.22^{A} (85–120) $n = 46$	103.0 ± 7.50^{A} (88–115) $n = 24$
Nonovipositing TL (mm)	104.7 ± 1.15^{A} (104–106) $n = 3$	91.1 ± 8.38^{B} (80–112) $n = 16$	99.1 ± 10.51^{A} (83–125) $n = 38$
Time to ovipositing (days)	108.4 ± 6.87^{a} (89–122) $n = 61$	114.0 ± 8.00^{a} (92–146) $n = 46$	99.0 ± 19.72^{b} (58–131) $n = 24$
Eggs/female	304.9 ± 50.34^{a} (234–555) $n = 40$	367.2 ± 36.48^{b} (189–420) $n = 24$	586.5 ± 58.89^{c} (282–764) $n = 12$

* Those means sharing lower case superscripts within rows or uppercase superscripts within columns are not significantly different ($P > 0.05$).

Percentage of crayfish ovipositing was similar among the four raceways. Because of the lack of difference among the raceways, blocks (raceways) were pooled in subsequent statistical analysis of reproductive performance measures among species.

Reproductive Success

A significantly higher proportion of *P. a. acutus* (95.3%) oviposited eggs than *P. zonangulus* (74.2%), and the proportion of ovipositing *P. clarkii* (38.7%) was significantly less than the other two species. In contrast, *P. clarkii* was the first species to oviposit eggs, more than 30 days earlier than the other two species (Table 1). No crayfish were observed ovipositing eggs within the last 4 weeks of the experiment; however, glair gland development was not assessed in these crayfish.

Individuals of *P. a. acutus* entire size range (90–127 mm TL) oviposited eggs, whereas some smaller individuals of the other two species did not oviposit (Table 1). Although the ovipositing individuals of each species were larger than the nonovipositing individuals, the only significant difference in TL was observed with *P. zonangulus*.

Number of extruded eggs ranged from 189 on a 85-mm TL *P. zonangulus* to 764 on a 114-mm TL *P. clarkii* (Table 1). Extruded eggs per female varied significantly with increased TL in the three species (Fig. 1). These linear relationships explained 80%–90% of the variation among individuals of the species. Using TL as a covariant, ANCOVA indicated significant differences in extruded egg counts among the three species, with *P. clarkii* being most fecund (Table 1). The slopes and intercepts of the regressions were also significantly different among species.

Egg Characteristics

Eggs of *P. a. acutus* and *P. zonangulus* were similar in weight and significantly heavier than *P. clarkii* eggs (Table 2). The eggs of these two species also contained a significantly greater proportion of lipid and protein than did those of *P. clarkii*. The smaller eggs of *P. clarkii* hatched approximately 3 days earlier than those of the other two species; however, the TL of the third instars were significantly shorter than the instars of *P. a. acutus* and *P. zonangulus* (Table 2).

DISCUSSION

P. a. acutus and *P. zonangulus* share many morphological, ecological, and reproductive characteristics. Both species belong to the subgenus Ortmannicus, diagnosed by the terminal element of the first pleopod (Hobbs 1972; Hobbs & Hobbs 1990). Although this characteristic clearly distinguishes *P. zonangulus* as a species and distinct from *P. a. acutus*, these two species have similarly shaped chelipeds, carapace, and overall body proportions (Hobbs 1981; Hobbs & Hobbs 1990, pers. observations). Crayfish with similar body forms frequently occur in similar habitats (Hobbs 1975; Holdich & Reeve 1988). *P. a. acutus* and *P. zonangulus* occur in sluggish streams with aquatic vegetation and lentic habitats, including those with fluctuating water levels such as borrow pits, roadside ditches, sloughs, and farm ponds (Hobbs 1981; Hobbs & Hobbs 1990; Deng et al. 1993). Adults of both species retreat to burrows in drying habitats and also to oviposit eggs, which is characteristic of tertiary burrowers (Hobbs 1981).

P. a. acutus and *P. zonangulus* shared several of this study's measured reproductive characteristics with the exception of hepatopancreas moisture content and the number of extruded eggs per female (Table 1). Crayfish store large amounts of energy (lipid) in the hepatopancreas for oocyte development and survival while in the burrow (Huner 1989). For this reason, relative moisture content of the hepatopancreas has been suggested as an indicator of crayfish condition (Huner et al. 1985; Huner et al. 1990). It was expected, based on hepatopancreas moisture content, that *P. zonangulus* fecundity would be greater than a similar-sized *P. a. acutus*, which was the case. The "better conditioned" *P. zonangulus* oviposited more lipid as eggs (349.9 mg on a dry weight basis) than a comparable-sized *P. a. acutus* (271.6 mg lipid).

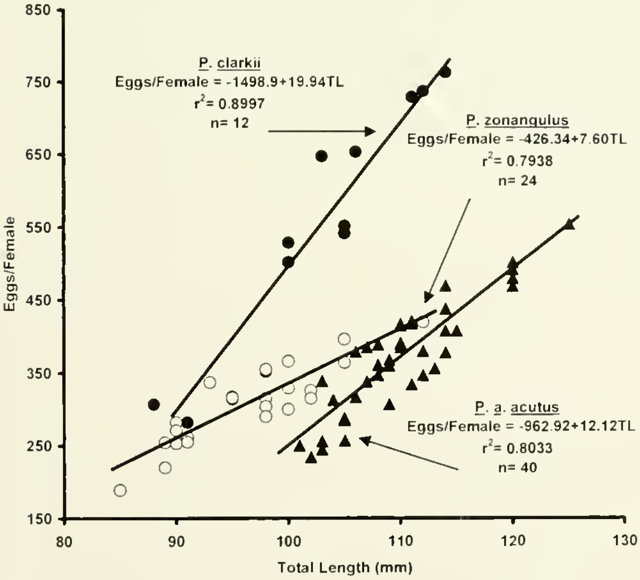

Figure 1. Relationships between total length (TL, mm) and number of extruded eggs per female for *Procambarus clarkii*, (closed circles), *P. a. acutus* (triangles), and *P. zonangulus* (open circles).

TABLE 2.

Mean (±SD) and range of egg dry weight, lipid, protein, hatching time from ovipositing date to the appearance of instars and the total length (TL) of instars.

Variable*	No.	*P. a. acutus*	*P. zonangulus*	*P. clarkii*
Dry weight (mg/egg)	12	3.49 ± 0.379^a (2.83–4.20)	3.44 ± 0.443^a (2.64–4.29)	1.94 ± 0.245^b (1.46–2.27)
Egg lipid (%)	6	26.0 ± 1.16^a (23.6–28.2)	26.6 ± 2.06^a (22.0–28.6)	17.6 ± 1.75^b (19.2–20.7)
Egg protein (%)	12	58.9 ± 3.67^a (47.4–60.1)	57.6 ± 4.37^a (43.4–58.8)	45.4 ± 3.21^b (40.5–50.9)
Hatching time (days)	9	21.1 ± 0.60^a (20–22)	21.6 ± 0.88^a (20–23)	18.6 ± 0.73^b (18–20)
Instar TL (mm)	200	9.5 ± 0.55^a (9–11)	9.6 ± 0.58^a (9–11)	7.6 ± 0.51^b (6–8)

* Those means within rows sharing lowercase superscripts are not significantly different ($P > 0.05$).

The reproductive characteristics of *P. a. acutus* and *P. zonangulus* contrasted that of *P. clarkii* (Tables 1 and 2). For example, *P. clarkii* fecundity was higher and the extruded egg size was smaller than that observed for *P. a. acutus* and *P. zonangulus*. Also, the hatching time and TL of hatchlings (instars) was shorter for *P. clarkii* than for the other two species. Our observations are consistent with the hypotheses of Noblitt et al. (1995) in that the reproductive characteristics of *P. zonangulus* and *P. a. acutus* follow a prudent strategy where more resources (energy) are invested in each egg, improving the survival or fitness of offspring in less predictable environments. Instar fitness is more dependent on size in this reproductive strategy scenario, and those species with bigger eggs yield larger instars. The larger instar *P. a. acutus* (10 mm TL) in allopatric and sympatric competition experiments survived at a higher rate than the smaller *P. clarkii* instars (8 mm TL) (Mazlum & Eversole, unpubl.).

The fraction of dry weight, protein, and lipid extruded in eggs were computed from linear regressions for whole-body female *P. a. acutus* dry weight, protein and lipid levels (calculated from data of Turker 1997) and for our fecundity estimates in terms of each of these parameters. A standard-sized *P. a. acutus* (104.6 mm TL) with an average fecundity of 249.4 eggs oviposited 1,065 mg dry weight, 573.7 mg protein, and 271.6 mg lipid. The fraction of the whole-body dry weight, protein, and lipid used for extruded eggs was 10.2%, 13.6%, and 22.2%, respectively. The average reproductive lipid output (extruded eggs) of 22.3% total-body lipid computed for 31 ovipositing *P. a. acutus* (Eversole et al. 2000) was almost identical to the estimate derived in this study. Similarities in the proportion of lipid devoted to reproduction are explicable in terms of fecundity, egg size, and the total-body content. Because of the differences in systematics and reproductive strategies among the species, it might be expected that the allocation of energy (lipid) to reproduction differs as well. Unfortunately, this comparison will have to wait until compatible data are available for the species or reproductive strategies.

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CONFIRMATION OF TWO COMMON MUD CRAB SPECIES (GENUS *SCYLLA*) IN THE MANGROVE ECOSYSTEM OF THE MEKONG DELTA, VIETNAM

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ABSTRACT Adult and juvenile mud crabs (genus *Scylla*) were collected from two sites in the Mekong Delta, Vietnam. The specimens were examined morphologically and analyzed by starch gel electrophoresis to confirm the species present based on a recently published revision of the genus *Scylla* that recognizes four species from adult morphological characters and electrophoresis. The Mekong Delta samples contained two species, S. *olivacea* ("red crab") and *S. paramamosain* ("green crab"). Ratios derived from measurements of the carapace width and the frontal spines, which are species diagnostic in adult mud crabs, did not separate juvenile *S. paramamosain* and *S. olivacea* reliably. However, in combination, differences in the shape of the frontal spines, the spination on the cheliped, and cheliped shape and color, make it possible to distinguish juveniles of these two species down to a size of about 1.5 mm carapace width. This is the modal body size of new recruits entering the mangroves of the Lower Mekong Delta. The diagnostic morphological features of adult and juvenile *S. olivacea* and *S. paramamosain* from the Mekong Delta are based on the architecture of the frontal lobe spines and the number of dorso anterior propodal spines and ventral carpal spines. These are described, together with features relating to their coloration and habitat preferences. The recognition of two named species of *Scylla* within the mangrove populations of mud crab in the delta is important to fisheries management and aquaculture development, as both adult and juvenile mud crabs are fished heavily for marketing and for stocking in aquaculture systems, respectively.

KEY WORDS: *Scylla*, mud crab, species identification, mangrove, Mekong Delta

INTRODUCTION

Mud crabs of the genus *Scylla* are the only swimming crabs (Family *Portunidae*) that are found habitually in tropical and subtropical mangrove forest ecosystems; their habitat extends to mangrove estuaries, embayments and surrounding coastal waters (Macnae 1968). As well as providing shelter from predation and desiccation, especially during moulting, mangroves also provide mud crabs with a plentiful supply of food in the form of other crustaceans and molluscs (Arriola 1940; Hill 1979).

Mud crabs have significant economic importance to artisanal fisheries throughout the Indo West Pacific region. They are also contributing increasingly to aquaculture production in several countries, most notably Vietnam and the Philippines (Johnston & Keenan 1999; Fortes 1999). Their rapid growth and high market value, coupled with easy post-harvest handling, make them an attractive alternative to farming shrimp within coastal areas (Overton & Macintosh 1997). The growing importance of mud crab farming is also supported by a rapidly expanding demand for crab products, both regionally and internationally.

The mangrove forests of Vietnam originally covered an area of up to 400,000 ha (Maraund 1943), of which about 250,000 ha flourished in the Mekong Delta. The greatest concentration of mangrove (150,000 ha) was in the Minh Hai Peninsula, which is now divided into Ca Mau and Bac Lieu provinces (Fig. 1). Despite heavy exploitation in recent decades, mangroves are still the dominant habitat in the extensive saltwater-influenced regions of the delta.

Within the coastal provinces of the Lower Mekong Delta (LMD), rural communities are heavily dependent on mangrove-based fisheries and aquaculture to support their livelihoods. These include crab fishing and crab culture as a secondary activity to shrimp farming within the mangrove forest ecosystem. Average mud crab production in the LMD ranges from 504 to 839 kg/ha/y (World Bank/Danida 2000). Nearly all the available brackishwater areas of the delta have been converted to extensive aquaculture ponds for shrimp and/or crab production integrated to varying degrees with the mangrove forest (these farming systems are described by Binh et al. 1997; and Johnson et al. 1999). To date, there are no commercial hatcheries in Vietnam to support mud crab culture and, consequently, the natural recruitment of juvenile mud crabs into the mangrove ecosystem provides the only source of seed available to crab farmers.

A previous study using morphometric analysis confirmed the existence of two common morphs of *Scylla* within the South China Sea region (Overton et al. 1997; Overton 2000), while Keenan et al. (1998) have proposed a total of four species of mud crab based on genetic data from a wider geographical range. They also describe morphological characters to distinguish the four species. Although Keenan et al. (1998) attribute two species to Vietnam, only one species is mentioned from the Mekong Delta, called *Scylla paramamosain* Estampador. Moreover, the study is based entirely on large adult specimens (carapace width greater than 95 mm), because the distinguishing characteristics of each species are more clearly defined in the adult stages. Until now, no species identifications have been reported for the juvenile stages. The people who catch or rear juvenile mud crabs in the Mekong Delta recognize two morphs of *Scylla* on the basis of color, using the local names "cua xanh" (green crab, due to its greenish carapace) and "cua lua" (red crab, due to dark red coloration on the lower chelae). These color differences are not distinct in the smaller juveniles, however, especially after capture.

In the present study, adult and juvenile mud crabs were collected from the Lower Mekong Delta. They were then examined morphologically and analyzed using electrophoresis to determine which species are recruiting into the mangrove crab fishery. The main objective was to produce scientific descriptions of both juveniles and adults of the species identified, which confirm [illegible]

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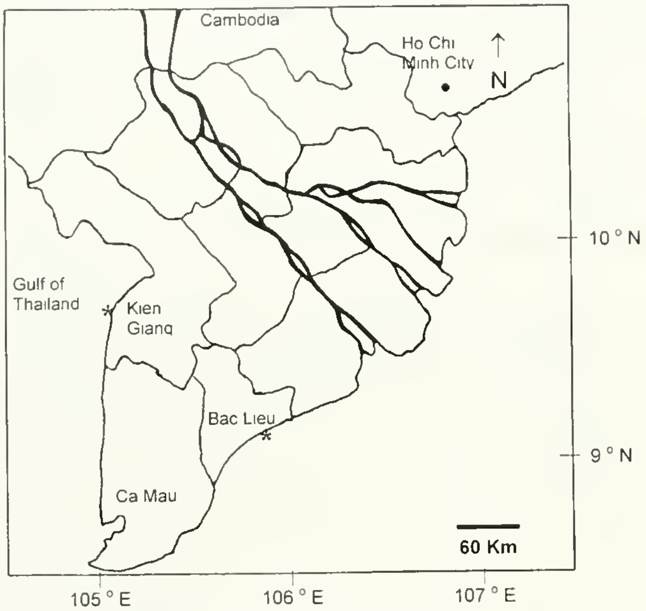

Figure 1. The Mekong Delta, Vietnam showing the location of the sampling sites in Bac Lieu and Kien Giang provinces.

local identities based on color and behavior. This knowledge is vital for mud-crab stock assessment and for the future development of mud crab culture using selected, known species of *Scylla*. Recruitment and growth studies on juvenile mud crabs are particularly needed in this regard.

MATERIALS AND METHODS

Crab Collection

Study sites were chosen within two of the most southerly coastal provinces of the Mekong Delta: Bac Lieu (9°00'N; 105°14'E) and Kien Giang (9°22'N; 104°26'E) (see Fig. 1). The crabs were collected from mud crab fishers or traders. They were interviewed to confirm that the crabs obtained were all fished from the selected locality.

In the first investigation, 23 adult crabs, 84 to 125 mm carapace width (CW), including both common morphs of *Scylla*, were sampled from the commercial mud crab fishery in Bac Lieu Province. Their species identities were confirmed by comparing their morphology and allozyme mobility with those published by Keenan et al. (1998). In the second investigation, juvenile mud crabs (CW 45–62 mm) from Bac Lieu and Kien Giang provinces (62 and 58 individuals, respectively) were studied. The majority of specimens from Kien Giang were the "red" morph, whereas all those collected from Bac Lieu in the second sample were typical of the "green" morph. In addition, a larger number of smaller juveniles (CW 10–45 mm) were available for examination from the commercial crab seed fishery in Ca Mau.

Crab Measurement

Three physical measurements were made on each sampled mud crab using digital calipers, namely Frontal Lobe Width (FLW), Internal Carapace Width (ICW) and Frontal Median Spine Height (FMSH) (Fig. 2). Measurements were recorded to the nearest 0.1 mm. From these measurements, two ratios described by Keenan et al. (1998) as discriminating between the four known species of *Scylla* were used to compare the red and green morphs of *Scylla* from the LMD. These ratios were FLW/ICW and FMSH/FLW.

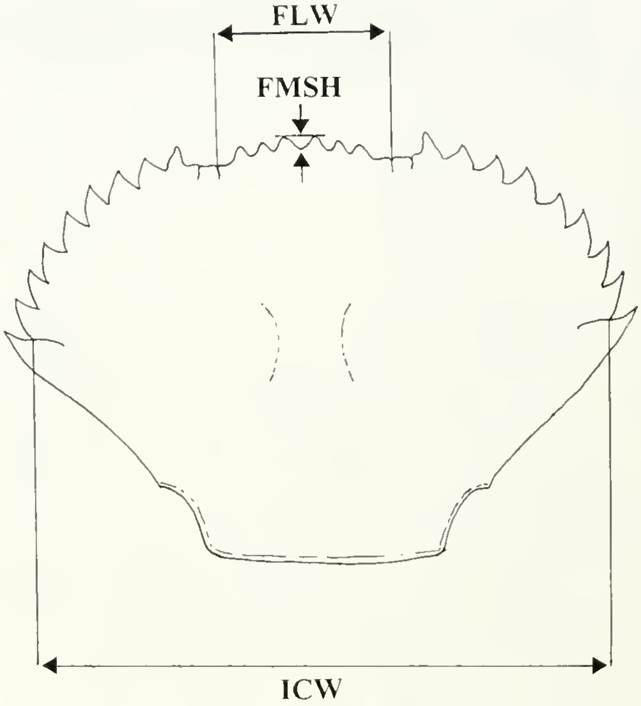

Figure 2. Illustration of the three measurements forming the ratios used to discriminate between the two *Scylla* species. FLW: Frontal Lobe Width; FMSH: Frontal Median Spine Height; ICW: Internal Carapace Width.

Allozyme Electrophoresis

Use of genetic markers, including allozyme electrophoresis, are the most reliable in identifying *Scylla* species (Keenan et al. 1998; Overton 2000). Other mud crab specimens representing the four *Scylla* species proposed by Keenan et al. (1998), which had been identified earlier (Overton 2000), were used as reference material to help interpret the results from electrophoresis using the mud crabs obtained from the Mekong Delta.

Muscle tissue was extracted from each crab specimen and stored at −80°C. The allozymes extracted from the muscle tissue were separated using starch gel electrophoresis. The electrophoretic techniques used followed those described by Harris and Hopkinson (1976), Shaklee and Keenan (1986) and Pasteur et al. (1988). Four allozymes, using two buffer systems that distinguish the four species of *Scylla* identified by Keenan et al. (1998), were used to identify the two common species collected from the LMD. These enzymes were alanine aminotransferase (ALAT), argenine kinase (ARGK), mannose phosphate isomerase (MPI) and phosphoglucomutase (PGM). They were visualized using standard staining procedures (Shaw & Prasad 1970; Harris & Hopkinson 1976; Shaklee & Keenan 1986; Pasteur et al. 1988).

RESULTS

The results of the allozyme electrophoresis confirmed that the two common species of *Scylla* in the Mekong Delta are *S. olivacea* Herbst (red morph) and *S. paramamosain* Estampador (green morph), as described by Keenan et al. (1998). Figure 3 and Table 1 illustrates the fixed mobility observed between the LMD specimens and the typed samples of the four species (also based on Keenan et al.).

The frontal spine morphology represented by the ratios FLW/ICW and FMSH/FLW did not prove to be conclusive in distinguishing the two species identified from the allozyme data. Both adults and juveniles of the green morph (*S. paramamosain*) have narrower frontal lobe widths (compared to their internal carapace width) and taller frontal lobe spines, than crabs of the red morph (*S. olivacea*). This conforms with the spinal architecture of sharp frontal spines on the green morph and smooth, obtuse frontal lobe spines on the red morph (Fig. 4). However, the ranges obtained for each of the ratios show that there is a large overlap between the two species and that it is not possible to identify an individual crab solely from either of these ratios.

Although both juveniles and adults of the two mud crab species from the Mekong Delta could be distinguished from each other using the above ratios, it is less clear which species they represent when they are compared to the equivalent ratios for the four *Scylla* species proposed by Keenan et al. (1998) (Table 2). Mean values of FLW/ICW for the juvenile red and green morphs of *Scylla* lie out with the ratios reported by these authors. The mean values for the adult specimens from Bac Lieu are within the published ranges for the four *Scylla* species recognized by Keenan et al. 1998, but they lie between the values of 0.037/0.038 for *S. serrata*/*S. paramamosain* and 0.412/0.415 for *S. tranquebarica* /*S. olivacea* (Table 2).

Mean values for the ratio FMSH/FLW for adults and juveniles of both Vietnamese morphs also fall within the ranges set by Keenan et al. 1998 for the four *Scylla* species they recognize (Table 2). FMSH/FLW ratios positively identified the adult green morph from Bac Lieu as *S. paramamosain* and the juvenile red morph as *S. olivacea*, but the other two sample groups cannot be classified reliably on the basis of this ratio. This is not surprising as there is also considerable overlap in the ranges of the FMSH/FLW values for each species as reported by Keenan et al. 1998.

DISCUSSION

The results from allozyme electrophoresis were conclusive in identifying the two common species of *Scylla* in the coastal mangrove ecosystem of the Mekong Delta. Based on the recent review of the genus *Scylla* by Keenan et al. (1998), the morphs recognized by fishers and crab farmers as "red crab" and "green crab" are *S. olivacea* and *S. paramamosain*, respectively. These confirmed identifications help to overcome the long-standing confusion about the identity of mud crab species when their fisheries biology, or use in aquaculture is described. As noted by Fortes (1999), several authors have assumed a single species, *Scylla serrata*, in aquaculture, despite evidence that this mud crab is actually rare in the Southeast Asian region compared to the other three species of *Scylla* now recognized (Keenan et al. 1998; Overton 2000).

It is not surprising that the morphometric ratios based on carapace measurements (FLW/ICW and FMSH/FLW) were inconclusive for the identification of juvenile mud crabs, or that the values obtained do not lie within the equivalent ranges for adult specimens. Developmental changes in exoskeletal dimensions between juvenile, sub-adult and adult crabs are well documented (Hartnoll 1982). Indeed the relative allometic changes in the dimensions of the body are often used to define the molt of maturity in crustacean species (e.g., Somerton 1980; Paul & Paul 1995). However, the same ratios obtained from the adult mud crabs sampled also did not conform to the equivalent values reported by Keenan et al. (1998). This apparent discrepancy may be explained by (a) operator differences when taking carapace measurements with calipers; or (b) natural variation in the morphology of these closely related species. It is notable that there is a high degree of overlap between the ratios recorded by Keenan et al. (1998), and clearly morphometrics alone cannot be used to identify individual mud crabs reliably.

The morphological characters used to identify the species in adult samples can also be extended to juvenile crabs down to a size of 1.5 cm carapace width, the modal size of new recruits into the Mekong Delta. In particular, the architecture of the frontal lobe spines and the number of spines on the carpus are reliable characters which can be used to distinguish juveniles of *S. olivacea* and *S. paramamosain*, even in the field.

The presence of two species in the Mekong Delta mud crab fisheries is important from a fisheries management and aquaculture development perspective, as natural recruitment of juvenile mud crabs into the mangrove ecosystem is the only source of seed available to crab farmers in the delta. It will now be possible to investigate and compare ecological differences between these two species, especially their potential in aquaculture.

The yield of mud crab in Ca Mau Province was 5000 tonnes in 1999, of which 1000 tonnes came from aquaculture production (Xuan 2001). In the ten-year fisheries development plan for Ca Mau Province (2000 to 2010), the production of cultured mud crab is expected to increase to 3,500 tonnes per annum. This increased emphasis on aquaculture has important implications regarding the sustainable level of exploitation of the mud crab seed recruiting into the coastal mangroves. From observations of the crab seed fishery in Ca Mau, it is clear that there are significant seasonal fluctuations in the supply and species composition of juvenile mud crabs caught by local fishers. It also appears that the recruitment dynamics of *S. paramamosain* and *S. olivacea* vary from province to province in the delta (Macintosh: personal observation). By being able to identify these two common species of *Scylla* down to the early juvenile stages entering the mangroves, a more accurate assessment of this important fisheries resource can now be attempted.

Scylla paramamosain and *S. olivacea* are farmed throughout the brackish water regions of the Mekong Delta, usually in earthen ponds constructed within the mangrove forests. The farmers generally stock juveniles of both species purchased from fishermen or crab dealers. Their general opinion is that *S. olivacea* is the more aggressive species. It also likes to burrow, which weakens the pond structure and making harvesting very difficult. In contrast, *Scylla paramamosain* is regarded as more suited to pond culture as it seldom burrows. However, female *S. olivacea* are very popular as "egg crab" (crabs with mature ovaries) because they develop large ovaries at a comparatively small body size. Differences in size at maturity and gonosomatic index have also been recorded in sympatric populations of *S. olivacea* and *S. paramamosain* in the Gulf of Thailand (Overton 2000). By confirming their species identity at the juvenile stage, it will now be possible to research the

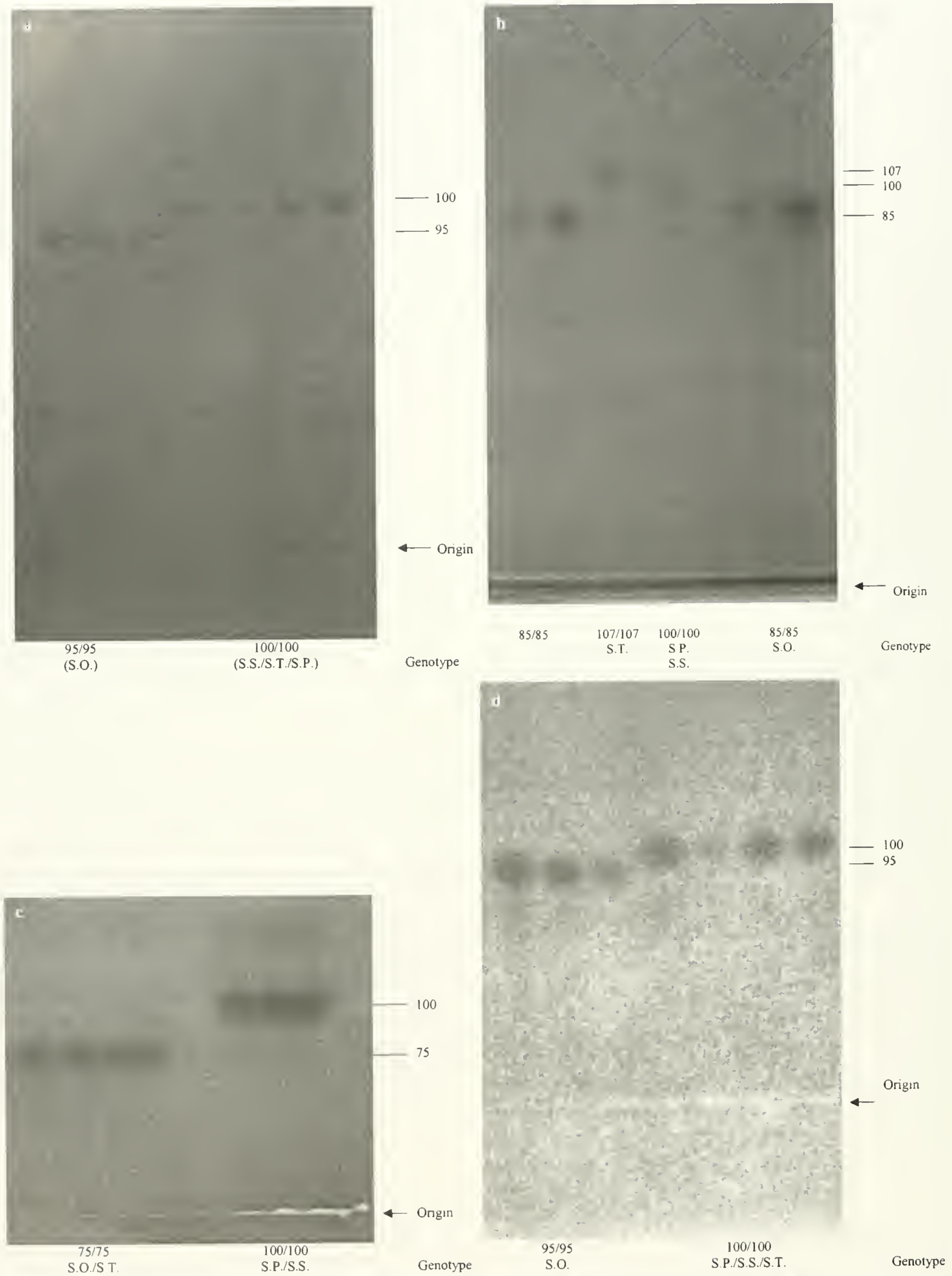
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Figure 3. Illustration of allozyme mobilites for *Scylla* species using a) mannose-6-phosphate isomerase (MPI); b) phosphoglucomutase (PGM); c) argenine kinase (ARGK); and d) alanine aminotransferase (ALAT, using ultra violet stain); from Overton (unpublished).
Note: S.O. = *Scylla olivacea*; S.S = *S. serrata; S.T. = S. tranquebarica* and S.P. = *S. paramamosain*.

TABLE 1.

Results of mobility for four allozymes used to compare two Scylla morphs from the Lower Mekong Delta with typed specimens of the four species of Scylla recognized by Keenan *et al.* (1998) used as markers (from Overton, 2000).

	Adults		Juveniles		Specimens from Overton (2000)			
Locus	Bac Lieu Green	Bac Lieu Red	Bac Lieu Green	Kien Giang Red	*Scylla serrata*	*Scylla tranquebarica*	*Scylla paramamosain*	*Scylla olivacea*
Numbers of individuals	11	12	8	8	6	5	5	6
ALAT	100	95	nd	nd	100	100	100	95
ARGK	75	75	nd	nd	100	75	75	75
MPI	100	95	100	95	100	100	100	95
PGM	100	85	100	85	100	107	100	85

biological basis for the farmers' observations about red crab (*S. olivacea*) and green crab (*S. paramamosain*) in aquaculture. Studies of their comparative tolerance to different combinations of salinity and temperature, their comparative growth rates and maturation under different pond conditions, and the possible affect of interspecific interaction between *S. olivacea* and *S. paramamosain* in mixed culture, could yield valuable information to improve mud crab farming in the Mekong Delta.

Diagnoses of the Two Common Species of Scylla in the Mekong Delta

Scylla olivacea Herbst

Dorsal carapace brown to dark green. Dorsal chelae and pereiopods range from dark brown to green, no reticulation present. Ventral propodus orange/red. Carapace rounded with blunt, obtuse frontal lobe teeth all equal in size, with shallow interspaces. Short,

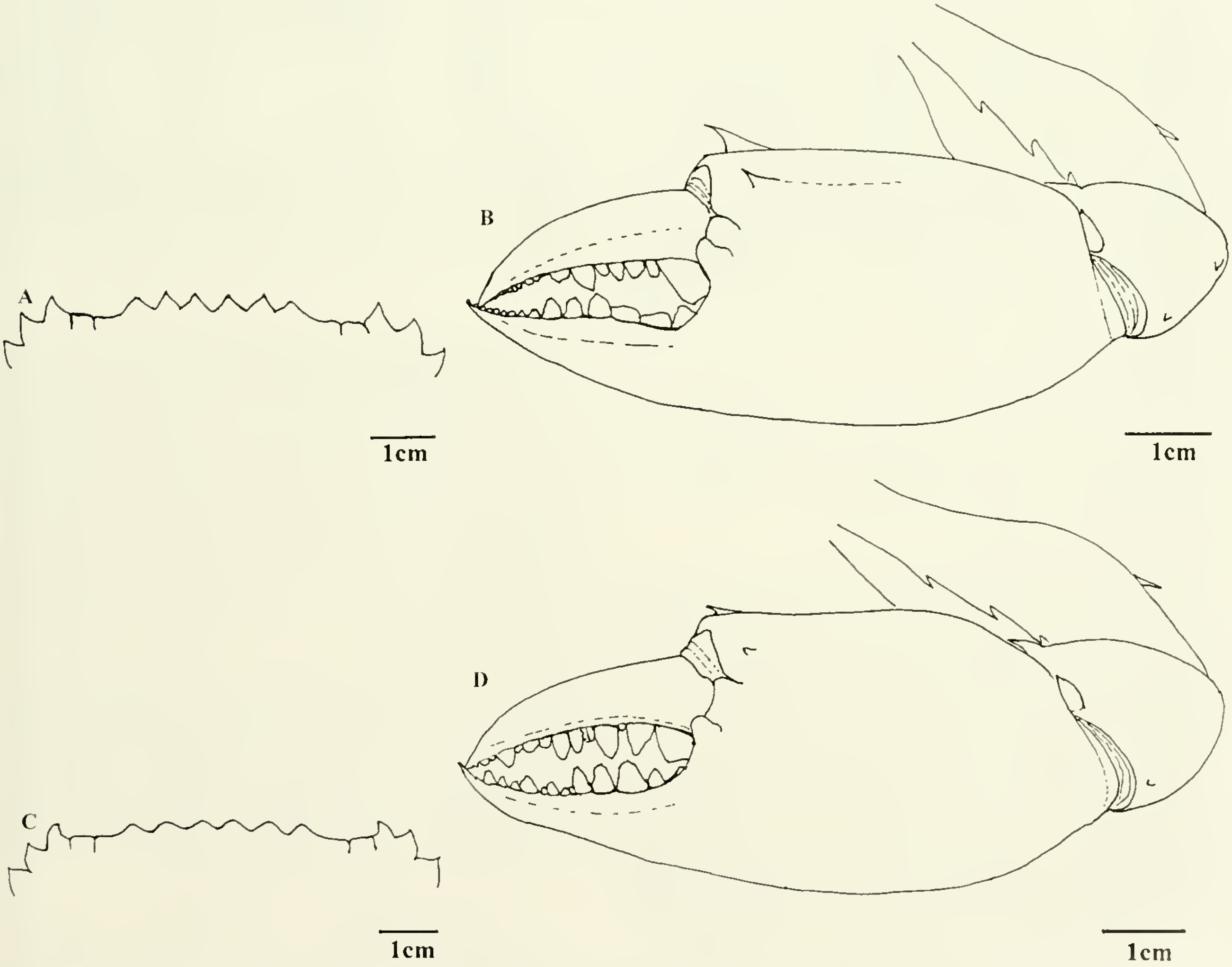

Figure 4. Drawings of the frontal carapace spination and left cheliped of adult *Scylla paramamosain* (a and b) and *Scylla olivacea* (c and d) based on specimens collected in the Mekong Delta, Vietnam.

TABLE 2.

Means, standard deviations (S.D.) and ranges for three morphometric characters (measured in mm) and two rations used to discriminate between four species of mud crab, Scylla described by keenan et al. (1998).

	Adults		**Juveniles**		**Keenan et al. (1998)**			
Character	**Bac Lieu Green**	**Bac Lieu Red**	**Bac Lieu Green**	**Kien Giang Red**	***Scylla serrata***	***Scylla paramamosain***	***Scylla tranquebarica***	***Scylla olivacea***
Numbers of individuals	11	12	62	58	68	9	25	66
ICW (mm)								
Mean	104.84	107.64	51.07	55.71	138.40	114.70	113.70	107.50
S.D.	10.24	13.67	3.81	3.87	23.20	9.00	11.40	10.10
Range	84.39–34.24	85.51–1333.54	45.00–61.30	47.11–61.65	95.50–191.70	104.80–134.10	97.10–137.80	95.00–133.90
FLW (mm)								
Mean	40.88	42.82	22.62	25.30	nd	nd	nd	nd
S.D.	3.57	3.99	1.94	1.65				
Range	34.24–45.68	38.84–48.41	19.80–31.70	21.70–27.85				
FMSH (mm)								
Mean	2.37	1.75	0.90	0.58	nd	nd	nd	nd
S.D.	0.44	0.28	0.24	0.17				
Range	1.72–2.92	1.16–2.05	0.40–1.40	0.40–1.20				
FLW/ICW								
Mean	0.39	0.40	0.44	0.45	0.37	0.38	0.41	0.42
S.D.	0.01	0.02	0.03	0.01	0.02	0.01	0.02	0.02
Range	0.38–0.41	0.36–0.42	0.41–0.63	0.42–0.48	0.34–0.41	0.36–0.39	0.38–0.44	0.37–0.45
FMSH/FLW								
Mean	0.06	0.04	0.04	0.02	0.06	0.06	0.04	0.03
S.D.	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.01
Range	0.04–0.07	0.03–0.05	0.01–0.05	0.02–0.05	0.04–0.10	0.04–0.08	0.03–0.05	0.02–0.04

obtuse anterolateral spines uniform in size and shape. None to two vestigial spines present on dorsal propodus of the chelae. Pronounced nodule present on inner lateral propodus. Spination on ventral carpus of chelae ranges from two vestigial, nodule-like spines to a total absence of spines. Abdominal flap of mature females dark brown to purple with a pale lateral stripe on each abdominal segment.

Coloration of juveniles similar to that of the adults. Chelae are elongate, with a distinctive dark red flash on the outer propodus and fixed dactyl. Chelae bear blunt, obtuse frontal lobe teeth that are equal in size, with shallow interspaces. Two sharp spines on dorsal propodus, and one pronounced nodule on inner lateral propodus. Single spine present on ventral carpus.

Scylla paramamosain Estampador

Adult carapace pale olive green. Dorsal chelae and pereiopods pale green with prominent dark green/brown reticulation. Reticulation breaks up and forms brown dots on outer upper half of propodus. Lower half of outer lateral propodus pale yellow to cream with orange to red flashes on tips of propodus and dactylus. A black patch is present on ventral surface of carpus.

Carapace flattened dorso-ventrally, with short, triangular frontal lobal teeth with angular interspaces. Central pair of frontal lobe teeth slightly protruded in some individuals. All anterolateral spines uniform in morphology, and compressed with small spaces between spines. Two sharp spines on dorsal propodus with ridges following posteriorly behind spines. One small spine present on inner lateral propodus. Two sharp spines on ventral carpus, on some individuals the anterior spine is vestigial, or missing.

Juveniles have similar body coloration to adult form. Carapace bears short frontal lobal teeth, sharp and triangular with angular interspaces. Two pronounced spines on the dorsal propodus. No nodule on inner lateral propodus. Ventral carpus usually bears two spines, but 6% of crabs examined have only one spine.

Habitat

Scylla olivacea is associated with mangrove forests, in particular estuarine and lower salinity areas associated with the river systems well within the mangrove zone or deltas where there is a high amount of freshwater runoff. *S. olivacea* prefers to burrow in the soft embankments during low tide. *S. olivacea* has an extensive distribution, populations of this species having been identified from the coastlines fringing the western Indian Ocean to northern and western Australia and islands of the Pacific Ocean (Keenan et al. 1998). Another species, *Scylla tranquebarica*, is often associated with *S. olivacea*. These two mud crabs occupy similar niches in the mangroves of East Malaysia and the Philippines (Macintosh, personal observation).

Scylla paramamosain inhabits a more subtidal environment than *Scylla olivacea*. This species is found in the mouth of estuarine areas and shallow subtidal mudflats and along the coastal fringes of mangrove areas. Keenan et al. 1998 also remarks that the habitat of this species extends to coral reefs areas, as in Singapore. *Scylla paramamosain* is widely distributed along the coastlines of the South China Sea particularly along the coast of Vietnam and the eastern seaboard of the Thai-Malay peninsula (Overton 2000).

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PHYSICO-CHEMICAL CHANGES IN ACID SULFATE SOIL DURING SEMI INTENSIVE CULTURE OF *PENAEUS MONODON* FABRICIUS, IN CLEARED MANGROVE AREAS OF THE CHAKARIA SUNDARBANS, BANGLADESH

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ABSTRACT The present study analyzed physico-chemical properties of pond soil during *Penaeus monodon* culture in a semi-intensive shrimp farm in Cox's Bazar district, Bangladesh during winter 1996 to 1997. Soils were acidic with high concentrations of SO_4-S, extractable Fe and Al. Soluble salt concentration were in order Na>Mg>Ca>K. The physico-chemical properties changed with flooding both under simple inundation and inundation with shrimp culture. The most noticeable change was soil P^H, SO_4-S and extractable Al. Extractable Al decreased a minimum value at 145 days after preparation of the culture ponds. Extractable Na and K were increased gradually while, Ca and Mg remained fairly constant throughout the entire period. The extent of change was different for the culture and the inundated ponds. Results of the present study indicated a positive effect on the improvement of acid sulphate soil in the cleared mangrove forest area because of shrimp culture.

KEY WORDS: *Penaeus monodon,* shrimp pond, culture

INTRODUCTION

Pond soil plays an important role in the balance of an aquacultural system and consequently on the growth and survival of aquatic organisms. Sediment/soil can function as a buffer. It provides water nutrients and serves as a biological filter through the adsorption of organic residues (Ray & Chien 1992). Soil thus playing a vital role in aquaculture practices is considered the "Chemical Laboratory" of the pond (Felix 1988). In ponds there is an intense interchange of organic and mineral compounds between the soil and the water (Wrobel 1983). Accumulation and decomposition of organic matter take place on the pond floor. As a result macro elements are eliminated from the pond water while the decomposition of organic matter releases inorganic substances and acids, and dissolutes minerals. These go into solution with water and influence aquatic biota.

A number of conditions are conducive to the formation of acid sulfate soils (Pons & Van Breemen 1982). Vast tracts of acid sulfate soils abound in Asia and Africa (Singh 1980; Brinkman & Singh 1982). In Bangladesh about 0.23 M ha of land constitute acid sulfate soils (FAO 1988). However, these are considered problem soils everywhere resulting in severe limitations for agricultural use (Nhung & Ponnamperuma 1982; Van Breemen & Pons 1978; Ponnamperuma & Solivas 1982; Hechanova 1983; Simpson et al. 1983).

Severe acidification of pond water in acid sulfate areas has been reported by many workers (Webber & Webber 1978; Lin 1986; Boyd 1989). The low P^H exerts its effects through the influence on most aspects of pond water chemistry. The main problems arising after construction or deeper excavation of fish ponds in acid sulfate soils comprise insufficient growth of algae, poor condition and consequent slow growth of phosphate fertilizers (Camacho 1977; Baylon 1981; Brinkman & Singh 1982; Poernomo & Singh 1982). Low P^H of the acid sulfate soils causes damage to gill tissues of fish (Ferguson 1988) and influences the impact of toxins (Alabaster & Lloyd 1980; Colt & Armstrong 1981) and heavy metals (Boyd 1989). In acid waters crustaceans and fish may experience impaired ionic regulation. P^H <4.8 is lethal for the Penaeids (Tsai 1990). Additionally, a high organic matter content in the bottom soil depletes O_2 of water (Wrobel 1983).

Characteristics of acid sulfate soils in some areas of Cox's Bazar have been reported by Rahman (1990), Rahman et al. (1993), and Mahmood and Saikat (1995). But no study has so far been done on the changes of pond bottom acid sulfate soil that take place by the amendments done for shrimp culture. Keeping these views in mind, this work was undertaken in a semi-intensive shrimp farm at Cox's Bazar. Ponds constructed on acid sulfate soils revealed physico-chemical changes in soils of shrimp (*Penaeus monodon*) culture ponds as well as inundated ponds.

MATERIALS AND METHODS

Location of the Farm

The semi-intensive shrimp farm namely "Chakaria Chingri Khamar" is situated on the western bank of the Matamuhuri River, about 5 km upstream from the estuary and about 65 km south of the Chittagong City.

Sampling

P. monodon was cultured in 14 ponds during winter of 1996 to 1997. Among those, three ponds (P-1, P-2 and P-3) were selected randomly on the basis of the previous years production data and soil P^H values to accommodate the maximum variability for the present study. From the rest of the fallow ponds (termed as inundated ponds), three others (P-4, P-5 and P-6) were selected where pond preparation was done similarly to the culture ponds but stocking was not.

Soil samples from 0–15 cm and 15–30 cm depths of pond bottom were taken from three spots diagonally of each pond (cultured and inundated), so that one spot was in the center and two were in the corners. Samples were drawn underwater with a specially designed sampler. Soil sampling was done 30 days before (DBP) and 7, 50, 95 days after completion of pond preparation (DAP). Soil samples were also taken at 145 DAP from the culture ponds.

Analysis of Soil Somples

Soil texture was determined according to Bouyouco's Hydrometer method (Piper 1950), P^H from 1:2 soil water suspensions and

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TABLE 1.

Textural classes of different soil samples collected from culture ponds and inundated ponds.

Pond Type	Pond No.	Depth (cm)	Sand (%)	Silt (%)	Clay (%)	Textural Class
Culture Ponds	P = 1	00–15	52	14	34	Sandy Clay
		15–30	65	13	22	Sandy Clay Loam
	P = 2	00–15	49	25	26	Clay Loam
		15–30	67	12	21	Sandy Loam
	P = 3	00–15	52	23	25	Sandy Clay Loam
		15–30	48	24	28	Clay Loam
Inundated Ponds	P = 4	00–15	66	13	21	Sandy Clay Loam
		15–30	70	14	16	Sandy Loam
	P = 5	00–15	45	28	27	Clay Loam
		15–30	65	13	22	Sandy Clay Loam
	P = 6	00–15	55	19	26	Sandy Clay Loam
		15–30	69	12	19	Sandy Loam

organic matter from loss of ignition. Total nitrogen was determined by micro-Kjeldhal digestion and distillation procedures. Available phosphorus was extracted with Bray and Kurtz No. 2 extractant (0.03 N NH_4F in 0.10 N HCl) followed by Spectrophotometric determination according to $SnCl_2$ reduced molybdophosphoric blue color method (Jackson 1958). Extractable Ca, Mg, Na, K and Mn were determined by 1 N NH_4OAC, P^H 7.0 ± 0.1 saturation and FE by 1 N NH_4OAC, P^H 4.6 saturation followed by Atomic Absorption Spectrophotometry (Allen et al. 1986).

RESULTS AND DISCUSSION

Physico-chemical parameters of the culture ponds and inundated ponds are tabulated in Table 2 and Table 3 respectively. The textural classes of the soil samples are shown in the Table 1.

Soil samples of the present study were sandy clay to clay loam in texture. The texture has been inherited from the deposition of the sediments. Such compositions of acid sulfate soils are also met with the saline soils of Chakaria Sundarbans (Rahman et al. 1990). The textural variation could have been created by the sedimentation processes during past mangrove swamp systems.

The soils were found rather poor in total nitrogen in comparison to the organic matter content resulting in high C/N ratio, that may inhibit the decomposition of organic matter. This feature shows the characteristics of peat layers. A high organic matter poor in nitrogen is also conducive to reduction processes. Similar total nitrogen values were reported earlier from other acid sulfate soils of the area in association with low organic matter (Rahman et al. 1993).

Values of soil P^H remained neutral. After submergence, the P^H of acid sulfate soils gradually increases due to reduction and inactivation of SO_4-S through microbial activities (Singh 1982). This reduction rate depends on the presence of sulfur reducing bacteria, P^H of the system, energy source (organic matter), etc. (Van Breemen 1976; Pons et al. 1982). In the present investigation, the P^H of soils was found to be reduced by air-drying with the average values ranging from 3.68 to 6.2. Further reduction could have been achieved by slow oxidation with prolonged drying. Additionally, there were significant differences in P^H values of the soils of the same pond at different spots. It might be due to the fact that the sulfidic horizon had not been uniformly parallel to the land surface and during excavation residual sulfidic materials were left. Evidence indicates that the pond soils had acid sulfate characters. In

TABLE 2.

Phsico-chemical parameters of different soil samples collected from culture ponds.

Pond No.	Parameters	Depth (cm)	pH Moist	pH Dry	EC (ds/m)	Salinity (ppt)	OM (percent)	N_t (percent)	P_a (ppm)	S_s (ppt)	S_o (ppt)	Na_e (ppt)	K_e (ppt)	Ca_e (ppt)	Mg_e (ppt)	Fe_e (ppm)	Mn_e (ppm)	Al_e (ppm)
P-1	BP	0–15	5.72	5.16	8.92	11.42	7.90	0.16	62.98	1.40	1.04	7.78	1.16	0.92	1.57	88.20	58.87	66.30
		15–30	6.64	5.08	16.93	21.67	10.42	0.13	53.50	1.26	3.80	8.30	0.81	0.84	2.01	115.87	45.00	128.13
	AP	0–15	6.06	5.59	11.34	14.57	9.70	0.16	63.20	0.57	5.23	6.88	1.05	0.89	1.50	128.44	48.70	43.37
		15–30	6.43	5.39	16.55	21.17	12.68	0.17	58.73	0.79	12.29	8.97	1.23	1.06	2.12	176.15	68.72	83.59
P-2	BP	0–15	6.78	4.79	12.88	16.48	10.51	0.19	69.30	0.92	3.97	6.33	0.48	0.89	1.94	124.33	73.33	99.40
		15–30	6.13	4.36	20.92	26.69	12.73	0.17	53.15	2.07	8.39	8.78	0.76	0.88	2.24	169.40	73.33	215.43
	AP	0–15	6.65	5.72	14.59	18.69	10.44	0.18	60.93	0.73	5.98	8.24	1.16	1.02	1.58	133.75	50.83	31.04
		15–30	6.38	5.14	18.65	23.86	12.26	0.15	55.04	0.89	11.41	9.77	1.04	0.94	1.99	172.13	73.08	58.47
P-3	BP	0–15	6.87	5.28	9.64	12.33	8.87	0.18	58.77	0.68	3.51	5.82	1.18	1.36	2.05	105.33	93.80	65.46
		15–30	6.54	4.57	13.69	17.52	10.84	0.17	48.95	0.58	6.58	6.95	0.79	0.70	2.04	122.46	52.00	139.63
	AP	0–15	6.50	5.53	14.82	18.98	10.53	0.17	62.31	0.78	6.42	8.27	1.37	0.97	1.78	154.42	50.48	30.33
		15–30	6.41	5.19	19.96	25.54	13.35	0.16	57.11	1.07	12.54	10.67	1.31	0.96	2.21	192.24	71.88	69.87

EC = Electrical Conductivity, OM = Organic Matter; N_t = Total Nitrogen; P_a = Available Phosphorus, S_s = Sulphate Sulfur; S_o = Oxidizable Sulfur, Na_e = Extractable Sodium, K_e = Extractable Potassium; Ca_e = Extractable Calcium; Mg_e = Extractable Magnesium; Fe_e = Extractable Iron; Mn_e = Extractable Manganese; Al_e = Extractable Aluminum; BP = 30 days Before Production & AP = Mean value of 5 days, 50 days, 95 and 145 days After production.

TABLE 3.

Phsico-chemical parameters nf different soil samples collected from inundated ponds.

Pond No.	Parameters	Depth (cm)	pH Moist	pH Dry	EC (ds/m)	Salinity (ppt)	OM (percent)	N_t (percent)	P_a (ppm)	S_s (ppt)	S_o (ppt)	Na_e (ppt)	K_e (ppt)	Ca_e (ppt)	Mg_e (ppt)	Fe_e (ppm)	Mn_e (ppm)	Al_e (ppm)
P-4	BP	0–15	5.69	4.33	19.50	24.96	15.75	0.22	36.66	2.50	5.52	11.10	0.52	0.66	2.18	177.53	71.13	219.50
		15–30	4.79	3.68	31.28	40.04	19.56	0.09	32.28	2.93	12.42	14.06	1.76	0.79	2.25	326.00	92.60	602.03
	AP	0–15	5.82	5.29	19.73	25.25	16.08	0.13	29.77	1.78	7.64	9.29	0.78	1.28	1.70	143.29	49.19	101.29
		15–30	5.67	4.86	29.30	37.22	16.62	0.12	26.31	1.83	13.87	12.71	1.14	1.61	2.56	212.58	87.39	216.69
P-5	BP	0–15	5.93	4.77	14.77	18.90	15.60	0.19	40.88	1.27	5.54	7.26	0.43	0.73	1.96	185.00	127.93	148.87
		15–30	6.75	4.27	37.51	48.00	12.99	0.15	41.05	1.53	11.57	7.778	0.57	0.62	2.23	178.54	107.33	262.33
	AP	0–15	5.78	5.77	13.56	14.59	10.02	0.17	57.48	1.07	3.75	7.88	1.15	1.17	1.68	110.33	68.33	37.25
		15–30	6.12	5.34	20.22	25.89	14.99	0.18	50.23	1.27	13.67	9.75	0.92	1.11	2.20	225.18	87.04	92.00
P-6	BP	0–15	5.81	4.67	12.05	15.43	11.00	0.16	39.30	1.07	4.51	6.09	0.54	0.60	1.66	190.73	71.40	141.67
		15–30	6.44	4.48	20.42	26.14	12.87	0.19	41.23	1.55	6.65	6.81	0.55	0.56	1.94	252.46	59.73	216.37
	AP	0–15	5.92	5.51	14.07	18.01	11.38	0.17	49.37	0.96	5.49	7.31	1.11	1.07	1.56	126.57	63.27	47.96
		15–30	5.98	4.87	18.76	24.00	13.19	0.16	49.41	1.43	11.59	8.27	0.66	0.87	1.98	210.04	93.18	126.48

EC = Electrical Conductivity; OM = Organic Matter; N_t = Total Nitrogen; P_a = Available Phosphorus; S_s = Sulpbate Sulfur; S_o = Oxidizable Sulfur: Na_e = Extractable Sodium; K_e = Extractable Potassium; Ca_e = Extractable Calcium; Mg_e = Extractable Magnesium: Fe_e = Extractable Iron: Mn_e = Extractable Manganese: Al_e = Extractable Aluminum; BP = 30 days Before Production & AP = Mean value of 5 days, 50 days, and 95 days After production.

TABLE 4.

Correlation and regression co-efficient between chemical parameters of soil in culture ponds.

St. No.	Independent Variable X	Dependent Variable Y	Correlation Co-efficient r	Regression a	Co-efficient b
1	P^H (air dry)	EC	-0.34109^{NS}	33.07123	3.34353
2	P^H	Salinity	0.38810*	44.82607	−4.68567
3	P^H	Organic Matter	-0.02294^{NS}	12.35626	−0.14819
4	P^H	Total-N	-0.02367^{NS}	0.17374	−0.00101
5	P^H	Available-P	0.35322^{NS}	29.36178	5.57392
6	P^H	Sulfate-S	-0.06742^{NS}	253.2928	−38.70149
7	P^H	Oxidizable-S	-0.04140^{NS}	10.10835	−0.37784
8	P^H	Extractable Na	-0.19885^{NS}	12.36769	−0.73409
9	P^H	Extractable K	-0.11505^{NS}	2.02837	−0.16984
10	P^H	Extractable Ca	0.16318^{NS}	0.66337	0.05491
11	P^H	Extractable Mg	0.48109**	3.41039	0.28362
12	P^H	Extractable Fe	-0.02689^{NS}	162.7490	−2.09753
13	P^H	Extractable Mn	-0.38776^{NS}	119.7637	−10.91958
14	P^H	Extractable Al	−0.55449**	389.0405	−60.73047
15	Organic Matter	Total-N	0.21277^{NS}	0.1595	0.001415
16	Organic Matter	Available-P	-0.25392^{NS}	66.77517	−0.62038
17	Organic Matter	Sulfate-S	-0.24085^{NS}	295.15233	−21.40631
18	Organic Matter	Oxidizable-S	0.75288***	−4.2054	1.06366
19	Organic Matter	Extractable Ca	0.76297***	−3.3018	1.06110
20	Organic Matter	Extractable Mg	0.01974^{NS}	0.94343	0.00102
21	Organic Matter	Extractable Fe	0.62131**	1.24631	0.05671
22	Organic Matter	Extractable Al	0.71889**	50.22998	8.76288
23	Organic Matter	Extractable Mg	0.11847^{NS}	42.89656	2.00984
24	Extractable Ca	Extractable K	0.13485^{NS}	1.67808	0.23581
25	Extractable Ca	Extractable Fe	0.29724^{NS}	0.87904	0.06789
26	Extractable Ca	Extractable Fe	0.08586^{NS}	132.4095	20.08978
27	Extractable Ca	Extractable Mn	0.41462*	23.6488	39.83474
28	Extractable Ca	Extractable Al	-0.20863^{NS}	131.01246	−67.90904
29	Extractable Ca	Total-N	0.25100^{NS}	0.62448	1.96549

NS: Not Significant

* Significant at 5% level

** Significant at 1% level

*** Highly Significant at 5% level

TABLE 5.

Correlation and regression co-efficient between chemical parameters of soil in inundated ponds.

St. No.	Independent Variable X	Dependent Variable Y	Correlation Co-efficient r	Regression a	Co-efficient b
1	P^H (air dry)	EC	–0.51448*	50.91311	–6.11398
2	P^H	Salinity	–0.57717**	71.19709	–9.09594
3	P^H	Organic Matter	–0.45927*	25.03221	–2.19786
4	P^H	Total-N	0.23651^{NS}	0.08123	0.01552
5	P^H	Available-P	0.47452*	–1.68086	8.69177
6	P^H	Sulfate-S	–0.76008**	5.12290	–0.72512
7	P^H	Oxidizable-S	-0.27316^{NS}	18.66437	–1.92903
8	P^H	Extractable Na	-0.25399^{NS}	14.18430	–1.0025
9	P^H	Extractable K	-0.19285^{NS}	0.17974	0.14421
10	P^H	Extractable Ca	0.23543^{NS}	0.13667	0.18206
11	P^H	Extractable Mg	0.44329*	3.26257	0.24920
12	P^H	Extractable Fe	–0.55978**	473.3843	–57.52583
13	P^H	Extractable Mn	-0.37716^{NS}	143.671	–12.96930
14	P^H	Extractable Al	0.81063***	1040.365	–177.6317
15	Organic Matter	Total-N	-0.39531^{NS}	0.23594	–0.00544
16	Organic Matter	Available-P	–0.71448**	80.31563	–2.7357
17	Organic Matter	Sulfate-S	0.58190**	–0.15350	0.12600
18	Organic Matter	Oxidizable-S	0.64261**	–4.29046	0.94829
19	Organic Matter	Extractable Ca	0.70217**	–4.57551	1.07604
20	Organic Matter	Extractable Mg	0.18387^{NS}	0.64117	0.02791
21	Organic Matter	Extractable Fe	0.78295**	0.72273	0.09197
22	Organic Matter	Extractable Al	0.60919**	0.71976	13.08166
23	Organic Matter	Extractable Mg	0.59527**	–236.0092	27.2572
24	Extractable Ca	Extractable K	0.29912^{NS}	1.82904	0.16691
25	Extractable Ca	Extractable Fe	0.41430*	0.66661	0.42843
26	Extractable Ca	Extractable Fe	-0.29640^{NS}	224.666	–39.3892
27	Extractable Ca	Extractable Mn	-0.19698^{NS}	87.4361	–8.7412
28	Extractable Ca	Extractable Al	-0.26591^{NS}	223.517	–75.3507
29	Extractable Ca	Total-N	-0.34532^{NS}	1.70459	–4.0679

NS: Not Significant

* Significant at 5% level

** Significant at 1% level

*** Highly Significant at 5% level

the Chakaria Sundarbans zone, dry P^H of surface soil was found as low as 3.15, which increased gradually with depth (Mahmood & Saikat 1995). The wet soil P^H in the surface soil of the present study was less than the sub-soil. Excavating a depth greater than 1 m for pond construction might have exposed the less acid sub-soil. Aeration of water might have caused some oxidation. The higher reduction in P^H of the sub-soil due to drying might, on the other hand, be responsible for downward leaching of sulfate. Air dry soil P^H was found to range from 2.6 to 4.3 in some acid sulfate soils of Cox's Bazar and Chakaria (Rahman et al. 1993). Liming during the past cropping might have also caused an increase in P^H value. Electrical Conductivity (EC) values of the presently studied pond soils are quite high. ECe values greater than 80 ds/m were reported from Senegambia (Vieillefon 1977). Some of the present values (ECe = EC 1:2 × 4 approximately) appear to be still higher and soil salinity may be responsible for the salinity of inundated water because of the similar trend of variation with ponds.

In the present findings, available P concentration was found frequently above 24 ppm. In acid sulfate soils, low available P is a result of solubility in acid reaction, insolubilization of fixation by Fe, Al and Mn, and low release from organic matter (Banerjea & Ghosh 1970; Andriesse et al. 1973; Subosa & Bautista 1991). Addition of phosphate fertilizer to acid sulfate soil is ineffective because of fixation of added P in the form of irreversible iron and aluminum phosphates. In alkaline conditions, colloidal materials in mud and organic matter may be inactive P (Tisdale & Nelson 1975; Singh 1982; Poernomo & Singh 1982). Available P in pond soil might have increased due to application of 25 kg TSP/ha.

The problems of extreme acidity of soils in agriculture and aquaculture arise from toxicities of H_2S, SO_4, Fe, Al, and Mn (IFP 1974; Singh 1982). In the present study SO_4-S values ranged from 0.26 to 2.93 ppt, oxidizable S from 1.04 to 15.41 ppt, extractable Fe from 88.2 to 326.0 ppm, extractable Al from 0 to 602.03 ppm and extractable Mn from 37.2 to 127.93 ppm. Extractable Ca was found to be low and the concentration followed the sequence Na>Mg>Ca>K. Another study of Rahman (1990) on acid sulfate soils of Cox's Bazar and Chakaria, the ranges of soluble Fe, Al and Mn were found to be from 52 to 75 ppm, from 3 to 260 ppm and from 2 to 22 ppm respectively (Rahman 1990). There was, however, a basic difference between the nature of the two studies and soil type. Concentrations of iron and aluminum in the present study were found lesser than the acid sulfate soils of many other countries (Baylon 1981; Hechanova 1983; Andriesse et al. 1973). The extractable Mn values in the present study were found corresponding to the active Mn concentrations reported from the Chakaria Sundarbans (Rahman 1990). In acid sulfate soils of Thailand, Vietnam, and Philippines active Mn ranges from 5 to 400 ppm (Ponnamperuma 1972). Actual acid sulfate soils have smaller amounts of active Mn (Tanaka & Yoshida 1970; Van Breemen 1976). Concentration of Mn in the present study were found higher than the flooded acid sulfate soil (Rahman 1990; Attanandana 1971).

From the present study it was observed that during shrimp culture or keeping the ponds inundated without stocking, soil P^H increased gradually while SO_4-S and extractable Al decreased rapidly. Such phenomena are characteristic of acid sulfate soils under flooding and may be exploited in their reclamation and utilization. Extractable Al was found negatively correlated (Table 4 and 5) with the soil P^H, which was also observed by some other workers (Van Breemen 1973, 1976; Baylon 1981; Rahman 1990). On the other hand, oxidizable S and extractable Fe were positively correlated with the organic matter. It appears that transformation of aluminum was P^H dependent and transformation of S and Fe in submerged systems were organic matter dependent. Both S and Fe transformations are microbial processes, the agents of which may utilize organic matter as energy source. So, submergence of many acid sulfate soils may eliminate two most important problems, low P^H and high Al, but the removal of S seems to be inadequate if the soil/sediment contains high organic matter. Therefore, flooding these soils is a temporary relief. Together with liming, this might keep the soils more tolerable. To remove pyrite, drying of pond bottom with subsequent repeated flushing has been advised (Felix 1988) but prolonged drying, or intense pyrite oxidation may render the soils more acidic.

CONCLUSION

The extents of increase in P^H and decrease in extractable Al and SO_4-S were higher in the culture ponds than those under simple inundation. This suggests that shrimp culture had a positive effect on the improvement of acid sulfate soils under submergence. This could be due to fish fed, mechanical aeration of water, frequent water exchange, shrimp excretion, mixing of surface soil by burrowing, leaving Ca rich in shells during molting, etc. In the light of the above discussion, it seems that clearance of mangrove forests would lead to destruction of environmental balance. In cleared areas, however, cultivation of shrimp under careful management should be preferred to other forms of land use because of the better scope of soil improvement. Research on integrated soil-water and crop management is necessary to address these problems.

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PHYSICAL, CHEMICAL AND BIOLOGICAL VARIATION AMONG FRESHWATER CRAYFISH (*CHERAX ALBIDUS* CLARK, 1936) RESEARCH PONDS

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ABSTRACT Previous workers performing classical agricultural field experiments and aquaculture pond trials have emphasized that in order for results from experiments to be analyzed and evaluated, it is important to either demonstrate that the experimental units are homogeneous or quantify any variation between plots, ponds or blocks of these experimental units. Moreover to ensure that results of aquaculture experiments are applicable to industry, research ponds must have similar characteristics to those of industry. Physical, chemical and biological characteristics of 24 research ponds were recorded at the Avondale Research Station, Western Australia. This demonstrated that: (a) The ponds behaved in a manner similar to farm dams typical of the Western Australian wheat belt; (b) The water chemistry and turbidity profiles of the ponds were within the range recorded for wheat belt farm dams; (c) The average % organic matter of the sediments was 3.49%, which was within the range recorded for farm dams in Western Australia (d). The coefficient of variation for growth of yabbies (*Cherax albidus*) among the ponds at the Avondale Research Station was 9.26%. The comparatively low level of variation among the ponds was attributed to a number of factors including the age and design of the facility, and the homogeneous water supply for all ponds. Power analysis has been applied to determine the number of replicates required for experiments in these research ponds.

KEY WORDS: variation, ponds, crayfish, *Cherax albidus*, power analyses

INTRODUCTION

Agricultural plot experiments on field research stations provide a bridge between glass house experiments and industry paddocks (Fisher 1958; Fisher 1960). Similarly, aquaculture research pond experiments provide both a bridge between aquarium studies and commercial ponds, and a more realistic estimate of production than laboratory aquarium or tank studies that tend to underestimate yields (Shell 1983).

For the results of aquaculture experiments to be applicable to industry it is essential to be able to measure production characteristics both accurately and in a system that mimics the natural environment in ponds as closely as possible. Aquarium studies provide an opportunity to measure single characters accurately in a tightly controlled environment. However, they do not simulate production in ponds well because they do not provide the same natural biota, or algal and clay turbidity. Consequently, long-term growth performance and nutritional health of freshwater crayfish, such as yabbies (*Cherax albidus* Clark 1936), is usually so poor in clean aquaria that experimental comparisons are compromised (Morrissy 1984a). Generally, aquaria studies tend to underestimate yields (Shell 1983). Furthermore, laboratory tank experimentation is impractical at industry densities of 1–5 yabbies/m^2. Since density and growth of freshwater crayfish are related inversely (Morrissy 1992; Mills & McCloud 1983; Brown et al. 1995; McClain 1995a; Morrissy et al. 1995), tank experiments in small containers at high densities are unrealistic and give poor growth and survival (Ackefors et al. 1989; Verhoef & Austin 1999a; Verhoef & Austin 1999b).

Another alternative is to estimate production characteristics of yabbies (*C. albidus*) directly from commercial ponds. In contrast to the smaller Eastern Australian yabby (*C. destructor*) industry that relies on wild caught and pond reared animals, the much larger farmed Western Australian yabby (*C. albidus*) industry is based on large "commercial ponds" that are argillotrophic, clay-based, paddock catchment dams filled by rainfall runoff to provide drinking water for sheep (Lawrence 1998; Lawrence & Jones 2001). However, it is difficult to obtain useful information from the large ponds and farm dams used for rearing yabbies (*C. albidus*) because of the variability in physical and biological characteristics (Morrissy 1974; Lawrence, et al. 1998). In addition there are other limitations of farm dams for efficient research. Management practices differ between dams, they are difficult to drain and hence measure the total population, and they generally contain so many yabbies (*C. albidus*) that the labor required to measure production traits like growth and population size is excessive.

Research station experiments include most of the advantages of true ecological studies, where the population of animals is influenced by many uncontrolled natural factors, as is experienced in commercial ponds, and classical experimentation, usually single factor, under highly controlled conditions in the laboratory.

To carry out yabby (*C. albidus*) experiments a research facility, consisting of 25 ponds supplied by a homogeneous water supply from a header dam, was built near Beverley Western Australia (32°7′S, 116°55′E). This was capable of supporting replicated, randomized and reproducible experiments, with controls, in an environment that simulated farm dams.

Large variability among ponds in aquaculture experiments often leads to imprecise estimates of treatment effects. Previous workers in classical agricultural field experiments such as at Rothamsted (Fisher 1958; Fisher 1960) and aquaculture pond trials at Auburn (Shell 1983) emphasized that in order for results from experiments to be analyzed and evaluated, it is important first to: (a) demonstrate that the experimental units are relatively homogeneous; or (b) to quantify any variation between plots, ponds or blocks of these experimental units.

Cross-over (Change-over) designs, which make comparisons

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directly within the same pond instead of between ponds, have been proposed for use in aquaculture experiments to eliminate the variation between ponds (Smart et al. 1997). The construction of an appropriate cross-over design is challenging (Cheng 1996; Cheng & Street 1997) as: (a) due to environmental factors most species in aquaculture respond significantly differently according to seasonal variations over a year; and (b) the interaction between seasonal and other treatment effects are unknown. It is likely that seasonal variation and the effect of the interaction between seasons and other treatments in each pond within a year may be greater than the variation among ponds within the same time frame. Consequently cross-over designs require a longer experimental period and the analysis may involve a more complex statistical model. To increase the degree of precision for estimation of treatment effects, the most efficient way to account for between pond variation is to minimize the variability between ponds and quantify that level of variation.

By measuring the level of variation between ponds prior to commencing a field trial program it was possible to (a) take variation between experimental units into account when planning the randomization and replication of treatments in future experiments, and (b) determine whether results recorded from future experiments will be due to the application of treatments or merely a result of naturally occurring variability between ponds.

This study aimed at testing the homogeneity of experimental units, quantifying the variation between these units, determining the number of replicates required when planning experiments and confirming that the ponds had similar characteristics to wheat belt farm dams.

MATERIALS AND METHODS

The site for ponds was selected according to clay profiles from the region and to ensure that all ponds were placed as close as practicable to each other. All 25 ponds were constructed within the same soil type with the same dimensions (10 m × 10 m water surface area, 1.5 m deep and 3:1 side, or batter slopes). The 25 ponds all received water from the same supply dam.

The twenty-five 0.01 ha ponds were filled from the water storage dam four weeks prior to stocking. Two weeks prior to stocking each pond with yabbies (*C. albidus*) for the first experiment, the ponds received the addition of 50 L of sheep manure to condition pond sediment and increase organic matter to the level commonly found in farm dams.

Twenty-four of the 25 ponds were divided into 6 blocks according to possible sources of variation (clay type, location—north, south, east, west, upper row, lower row); a randomized block design was used, each block contained 4 ponds which received the same four treatments (yabbies 4.5/m^2 unfed, yabbies 4.5/m^2 fed lupins at the rate of 2.5 g/m^2/week, yabbies 1/m^2 unfed, yabbies 1/m^2 fed lupins at the rate of 2.5 g/m^2/week). The remaining pond received yabbies 4.5/m^2 fed lupins at the rate of 2.5 g/m^2/week. Prior to stocking each yabby (*C. albidus*) was weighed, sexed and 10% of animals were tagged by tail punching (Morrissy 1980; Getchell 1987). The ponds were stocked with 7,050 yabbies (*C. albidus*) (mean weight 19.41 g ± 0.22 SE) at a sex ratio of 1 male:1 female on the 29th November 1994 and the trial was harvested between the 8th to the 15th of March 1995.

At the commencement and conclusion of the experiment samples were collected for water chemistry analyses. Samples were submitted to the Chemistry Centre of WA for analyses of Ca, N-NO$_2$, N-NO$_3$, Cl, Cu, Fe (dissolved), Fe-total (unfiltered sample), HCO$_3$, K, Mn (dissolved), Mn-total (unfiltered sample), Na, P-SR (Phosphorous, soluble reactive), SO$_4$-S (Sulphate, expressed as sulphur), Zn-total (unfiltered sample), CO$_3$, pH, Electrical conductivity (25°C) (Econd.), Alkalinity and Hardness. Max-Min thermometers were used to record water temperature in the ponds.

At fortnightly intervals subsurface water samples (10-cm depth) and benthos core samples were collected from each pond. The benthos corer removed a 2-cm diameter core of sediment and clay to a depth of 15 cm. The percentage organic matter of water samples and sediment was determined by drying samples in pre-dried and weighed crucibles and then ashing out. As the loss in weight is due to combustion of organics, percentage organic matter was calculated according to the formula:

$$\text{Organic Matter} = [\text{weight of organic matter/dry weight}] \times 100\%$$

At fortnightly intervals Secchi disk depth was measured in each pond as an index of turbidity.

All data in the randomized block design were analyzed using analysis of variance (ANOVA) to determine significant differences among treatment means. Data were considered significantly different at the 0.05 level of significance. The coefficient of variation (C.V.) is a measure of variation and was calculated according to the formula:

$$\text{C.V.} = \text{standard deviation/mean (Shell 1983).}$$

Power analyses was applied using the methods described by Searcy-Bernal (1994) to determine the number of replicates required for a given number of treatments to measure a difference of 5, 10, and 20% in the growth of yabbies (*C. albidus*) from the research ponds.

RESULTS

Water Chemistry

Water chemistry parameters at the commencement and conclusion of the experiment are presented below (Table 1). Using the nonparametric Wilcoxon-Mann-Whitney test (Wilcoxon 1945; Mann & Whitney 1947) to examine paired observations, there was no significant difference between the initial chemical parameters and final chemical parameters ($P = 0.73$). During the experiment water temperature ranged from 13 – 35°C (mean 22°C ± 0.6 SE). There was no significant difference in minimum ($P = 0.51$) (C.V. = 9.63%) or maximum ($P = 0.21$) (C.V. = 12.38%) water temperature among blocks of ponds.

Turbidity

There was no significant difference in turbidity among blocks of ponds at either the commencement ($P = 0.67$) (C.V. = 4.74%) or conclusion of the experiment ($P = 0.73$) (C.V. = 11.75%). However, there was a significant decrease in turbidity over the course of the experiment across all ponds ($P < 0.0001$) by two tailed *t*-test with paired observations (Fig. 1). As there was no variation in salinity (measured as electrical conductivity Table 1), it is probable that the decrease in turbidity was due to a reduction in suspended organic matter (see Fig. 3).

TABLE I.

Water chemistry parameters of Avondale research ponds at commencement and conclusion of experiment ($n = 25$).

		Commencement				Conclusion			
Parameter	**Units**	**Mean**	**SE**	**Min**	**Max**	**Mean**	**SE**	**Min**	**Max**
Alkalinity	mg/L	186	9	130	200	172	15	130	200
CO_3	mg/L	11	2	<2	22	13	4	<2	18
Ca	mg/L	33	2	26	38	30	3	26	38
Cl	mg/L	918	49	662	1150	936	103	662	1150
Cu total	mg/L	0.05	0.02	<0.02	0.21	0.09	0.04	0.04	0.21
Econd	mS/m	343	14	259	404	346	31	259	404
Fe	mg/L	0.07	0.01	<0.05	0.13	0.08	0.02	<0.05	0.13
Fe total	mg/L	0.8	0.2	0.1	1.4	0.6	0.3	0.1	1.4
HCO_3	mg/L	206	10	160	230	185	12	160	210
Hardness	mg/L	348	18	270	430	365	35	270	430
K	mg/L	7.8	0.5	6.0	10.0	8.8	0.6	7.0	10.0
Mn	mg/L	0.02	0.00	<0.02	0.02	0.02	0.00	<0.02	0.02
Mn total	mg/L	0.02	0.00	<0.02	0.02	0.02	0.00	<0.02	0.02
$N\text{-}NO_3$	mg/L	0.46	0.24	0.02	2.00	0.52	0.49	0.02	2.00
Na	mg/L	593	28	412	678	578	58	412	678
P-SR	mg/L	0.05	0.01	0.03	0.08	0.06	0.01	0.04	0.08
$SO_4\text{-}S$	mg/L	139	6	121	173	146	11	121	173
Zn total	mg/L	0.41	0.24	0.03	2.00	0.10	0.05	0.03	0.23
pH		8.4	0.1	7.5	8.8	8.4	0.3	7.5	8.8

Organic Matter

The organic matter of the pond sediment (Fig. 2) was not significantly different among blocks of ponds at the commencement ($P = 0.16$) (C.V. = 19.88%), conclusion ($P = 0.07$) (C.V. = 31.03%) or during the experiment ($P = 0.09$) by two tailed *t*-test. All ponds ranged between 1.6% and 5.3% sediment organic matter content.

There was no significant difference in the suspended organic matter in water among blocks of ponds at either the commencement ($P = 0.10$) (C.V. = 24.87%) or conclusion ($P = 0.13$) (C.V. = 21.13%) of the experiment (Fig. 3). However, there was a significant decrease in suspended organic matter over the course of the experiment across all ponds ($P < 0.0001$) by two tailed *t*-test with paired observations (Fig. 3). The low levels of variation in organic matter during this trial may be largely attributed to the low industry based feeding rates (0–2.5 g/m^2/week) in this experiment.

Yabby (C. albidus) *Survival, Growth and Biomass*

There was no significant block effect on yabby (*C. albidus*) production determined as either survival (65% ± 2 SE) ($P = 0.24$), change in biomass of adult yabbies (*C. albidus*) stocked ($P = 0.26$), total change in biomass of all yabbies (*C. albidus*) harvested (including juveniles) ($P = 0.50$) or final mean weight of yabbies (*C. albidus*) ($P = 0.47$) (Fig. 4). The coefficient of variation for yabby (*C. albidus*) growth (expressed as final mean weight-initial mean weight) between the blocks of research ponds was 9.26% ($n = 6$).

These results indicate that there was no trend across the ponds

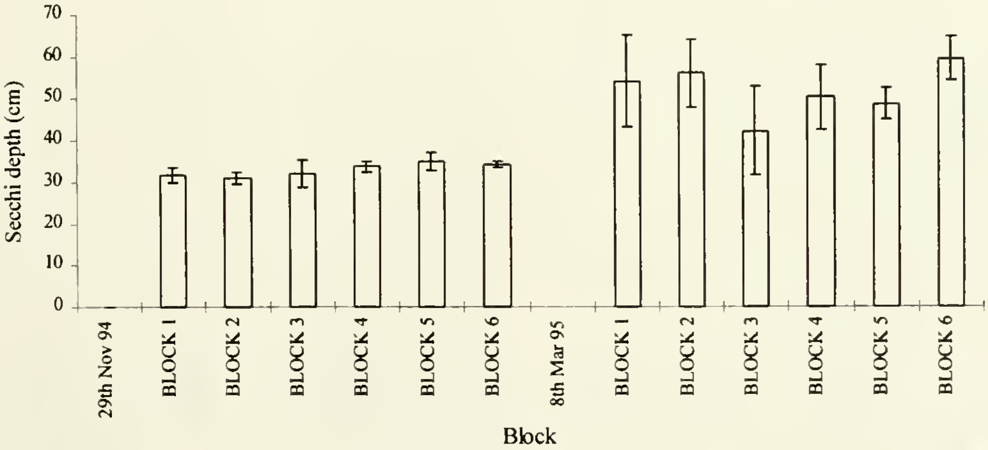

Figure 1. Turbidity of research ponds (cm ± SE) at the commencement and conclusion of experiment - the higher the Secchi disk depth, the lower the turbidity of the water.

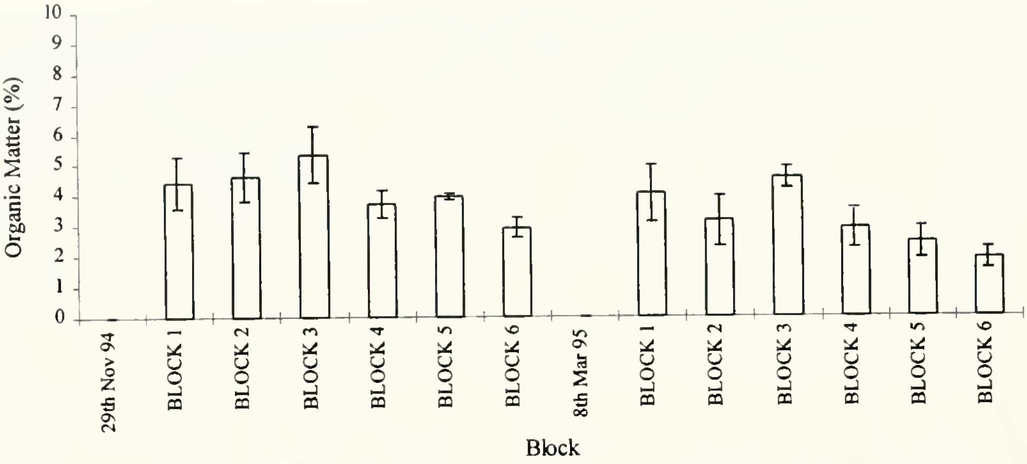

Figure 2. Organic matter (% ± SE) of pond sediment at the commencement and conclusion of experiment.

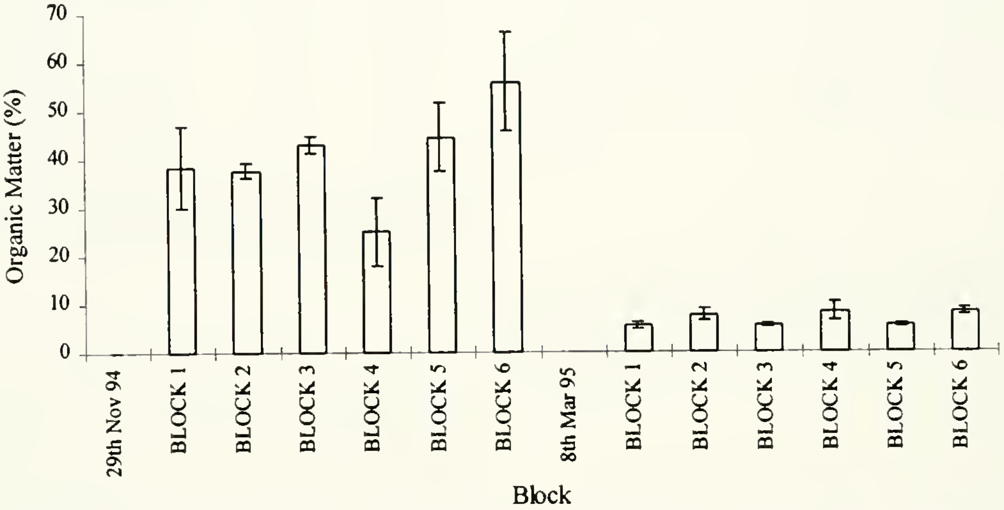

Figure 3. Suspended organic matter (% ± SE) of pond water at the commencement and conclusion of experiment.

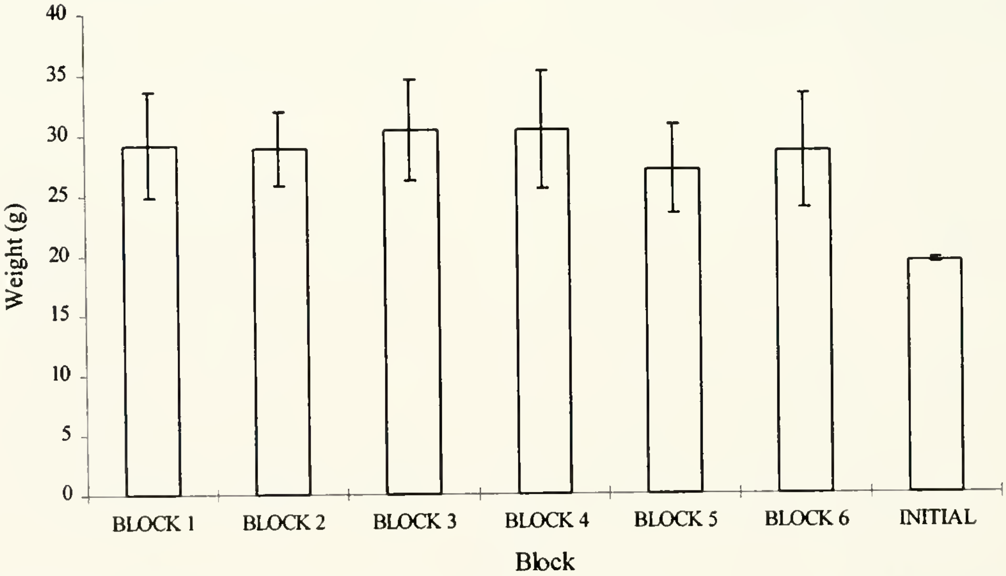

Figure 4. Mean yabby (*C. albidus*) weight (g ± SE) at commencement of experiment (Initial) and variation in final yabby (*C. albidus*) weights (g ± SE) among pond blocks at conclusion of experiment (Block 1–6).

for change in yabby (*C. albidus*) growth due to inherent pond factors at the site. The low variation in yabby (*C. albidus*) growth (C.V. = 9.26%) between individual ponds treated identically showed a highly acceptable homogeneity in the pond site for future experiments.

DISCUSSION

To ensure that results of aquaculture experiments are applicable to industry, research ponds must have similar characteristics to those of commercial ponds. In addition the variation (C.V.) in yabby (*C. albidus*) growth among ponds due to inherent site factors needs to be as small as possible to minimize the replication needed for future experiments to be effective in showing effects due to different treatments. If differences between treatments are smaller than the coefficient of variation then the observed differences are likely to be due to chance variation rather than a treatment effect.

The coefficient of variation for yabbie growth among the ponds at the Avondale Research Station was 9.26%.

Experiments within earthen ponds at the Auburn University aquaculture research station in Alabama have produced coefficients of variation for fish production ranging from 4.2–34.6%, with an average of 20% (Shell 1983). Previous research using adjacent ponds for marron (*Cherax tenuimanus*) experiments attributed 11% of variation in growth to differences between ponds. (Morrissy 1992; Morrissy et al. 1995).

The comparatively low level of variation between the ponds at the Avondale Research Station may be attributed to a number of factors including: (1) the age of the facility; (2) the planning and design of the facility to ensure a homogeneous environment; and (3) the homogeneous water supply for all ponds.

Comparative trials between ponds, cages and lined tanks have shown that the primary causes of variation are differences in environmental conditions (including water quality, productivity, soil types, and water source) (Shell 1983). This experiment has shown that the pond site was homogeneous and although environmental parameters (i.e., turbidity and % suspended organic matter which showed an inverse relationship due to the initial algal bloom from pond fertilization declining) change over time, all ponds followed similar patterns of change. The ponds were therefore suitable for testing treatments (such as density, diet, monosex culture etc.) because any observed difference in yabbie growth greater than 9.26% (C.V.) was likely to be due to the effect of the experimental treatment.

For a given number of treatments, power analysis can be used to determine the number of replicates required. Power analyses are therefore considered to be a basic tool in experimental and sampling design (Searcy-Bernal 1994). In general, aquaculture experiments can only detect relatively large effect sizes with a reasonable power. This is particularly true with pond experiments that are often characterized by high within-treatment variability and low numbers of replicates (Searcy-Bernal 1994; Shell 1983). A power value of 0.80 has been proposed as the minimum desirable to avoid committing a Type II error (i.e., accepting a false null hypothesis) (Searcy-Bernal 1994). Applying the methods described by Searcy-Bernal (1994) a power table (Table 2) using the data from this experiment shows the minimum number of replicates (n) required for treatments (k) to measure a difference of 5, 10, and 20% in the growth of yabbies (*C. albidus*) from the research ponds, using the $\alpha = 0.05$ level of significance, with a power of 0.8. As would be expected from the C.V. of 9.26% an unrealistically high number of replicates (>20) would be required to measure a difference in yabby (*C. albidus*) growth of 5% between 2 or more treatments (Table 2). In contrast, to record a difference in growth of 10% requires considerably less replicates (i.e., 3–5 replicates depending on number of treatments under investigation).

TABLE 2.

Number of replicates (n) required to identify a difference in growth of 5, 10, and 20% with mean 60g and standard deviation 4.5g for $\alpha = 0.05$ level of significance with a power of 0.8 for (k) treatments.

Difference in Growth	No. Treatments (k)	No. Replicates (n)
5%	2	20
	3	30
	4	60
	5	70
10%	2	3
	3	3
	4	4
	5	5
20%	2	2
	3	2
	4	2
	5	2

The condition of the ponds was similar in manner to typical farm dams of the Western Australian wheat belt. The water chemistry of the ponds was within the range recorded for farm dams in the Western Australian wheat belt (Morrissy 1980; Lawrence et al. 1998; Cheng et al. 2001), which is dominated by sea salt ions, (Na and Cl), rather than by salts from catchment erosion, (Ca, Mg, SO_4), as found in other world freshwaters (Francesconi et al. 1995b). The high salinity recorded 346 mS/m (1885 mg/l), is typical of cleared catchments in the Western Australian wheat belt (Lawrence et al. 1998; Cheng et al. 2001). The values of a number of parameters (i.e., Cl, Na, SO_4-S and hardness) were higher than those recorded from most wheat belt farm dams but within the range suitable for yabbies (Morrissy 1980; Lawrence et al. 1998; Cheng et al. 2001) (Table 1). This may be attributed to initial disturbance of the catchment due to dam construction and subsequent flushing of the catchment.

The turbidity of the ponds was within the range recorded for farm dams in the Western Australian wheatbelt (Lawrence et al. 1998). Similarly, the percentage of organic matter in the sediments of the experimental ponds, mean 3.49%, was also within the range recorded for farm dams in the Western Australian wheat belt, which range from 0.27–12.13% organic matter (Lawrence et al. 1998).

The research ponds were therefore suitable for conducting experiments on yabbie farming and the results were likely to be directly applicable to the farm dam environment.

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POPULATION DYNAMICS OF THE SPINY LOBSTER *PANULIRUS GUTTATUS* (LATREILLE) IN A CORAL REEF ON THE MEXICAN CARIBBEAN

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ABSTRACT The dynamics of a population of adult spiny lobsters, *Panulirus guttatus*, was studied in a group of coral reef patches in Puerto Morelos, Quintana Roo (Caribbean coast of Mexico), from October 1986 to November 1987. Lobsters were extracted by divers from traps deployed in the perimeter of reef patches for 10–13 days every month. In total, 778 *P. guttatus* were caught. Males (size range: 42.4–87.5 mm carapace length (CL), were significantly larger than females (45.5–73.5 mm CL). The highly biased sex ratio (2.6 males: 1 female) was partly due to a differential catchability in traps of males and females. We tagged 227 males and 90 females with spaghetti-type tags and recaptured 62 males and 12 females, some on multiple occasions, yielding 119 recaptures of males and 26 of females. The monthly population size in the patch complex, as separately estimated for each sex with the Fisher-Ford multiple-recapture model, was higher from June to November 1987, when both the percentage of ovigerous females and the mean size of individuals was smaller, indicating a possible recruitment of young adults into the trappable population during the summer and early autumn. The average density of lobsters was 126 lobsters ha^{-1}. Growth data were scarce, but suggest that growth rates decrease as size increases, and that males can molt at least 2–3 times per year. The egg-incubation period of recaptured females was 2–3 weeks. Females >50 mm CL may produce 3–4 broods per year. In addition to *P. guttatus*, 120 individuals of *P. argus*, mostly sub-adults (<80 mm CL) were also caught. Size of male and female *P. argus* was similar, but the size of *P. argus* was significantly larger than that of *P. guttatus*. Time at large of recaptured individuals suggests that the reef patches are a temporary habitat for *P. argus*, but a more permanent residence site for *P. guttatus*.

KEY WORDS: *Panulirus guttatus*, *Panulirus argus*, population dynamics, spiny lobster, abundance, growth, reproduction, Mexico

INTRODUCTION

Spiny lobsters are among the most valuable fishing resources in the world. Population studies provide information on the abundance, movements, growth rates, reproductive dynamics, and survival of these species, and are therefore useful to improve their fishery management (Morgan 1980). However, such studies are also important to increase the knowledge on the ecology of the species, whether they support major fisheries or not. In the Caribbean Sea and adjacent Western Atlantic coasts, two sympatric species of spiny lobsters occur, *Panulirus argus* (Latreille) and *P. guttatus* (Latreille). *Panulirus argus* is a migratory, large-sized species that undergoes several ontogenetic changes in habitat. The postlarvae (pueruli) of *P. argus* settle in shallow, vegetated areas, where they remain throughout the so-called "algal juvenile" phase (6–15 mm carapace length (CL)). The "postalgal juveniles" (15–45 mm CL) move to crevice-type shelters, also in shallow areas, whereas the "subadults" (45–80 mm CL) move to coral reef habitats. Further on, the adults (>80 mm CL) migrate to deeper, more diverse habitats (Butler & Herrnkind 1997). In contrast, *P. guttatus* is a small, rather sedentary species. The pueruli of *P. guttatus* are believed to settle directly on the coral reef habitat, and remain in this habitat throughout their entire benthic life (Briones-Fourzán & McWilliam 1997; Sharp et al. 1997).

Panulirus argus supports major fisheries throughout its geographic range, but *P. guttatus* is mostly a by-catch or secondary catch in most areas, with specific fisheries only in Bermuda and the French West Indies (Evans & Lockwood 1994). Consequently, numerous and extensive population studies of *P. argus* have been conducted in locations such as Florida (e.g., Lyons et al. 1981; Forcucci et al. 1994), Cuba (review in Baisre 2000), Jamaica (Munro 1974), and Mexico (review in Briones-Fourzán & Lozano-Álvarez 2000). In contrast, most population studies on *P. guttatus* have been conducted in Martinique (Farrugio 1975, 1976; Farrugio & Saint-Felix 1975; Marfin 1978), and Bermuda (Sutcliffe 1953; Evans & Lockwood 1994; Evans & Evans 1995, 1996; Evans et al. 1995, 1996), but also in Florida (Caillouet et al. 1971; Chitty 1973; Sharp et al. 1997) and in Mexico.

In the Caribbean coast of Mexico (coast of the state of Quintana Roo), *P. guttatus* amounts to ~6% of the lobster catch (Padilla-Ramos & Briones-Fourzán 1997), and has been the subject of specific studies on size distribution (Briones-Fourzán 1991), movement patterns (Carrasco-Zanini 1985; Lozano-Álvarez et al. unpubl. data), reproductive dynamics (Briones-Fourzán & Contreras-Ortiz 1999), and the description of its puerulus (Briones-Fourzán & McWilliam 1997). Also, comparative studies have been conducted on biological, ecological and fisheries aspects of *P. guttatus* and *P. argus* (Colinas-Sánchez & Briones-Fourzán 1990; Briones-Fourzán 1995; Padilla-Ramos & Briones-Fourzán 1997; Briones et al. 1997), and on the den choice and occupation patterns of shelters by these two sympatric species (Lozano-Álvarez & Briones-Fourzán 2001).

The present paper provides information on a field investigation into the population dynamics of adult *P. guttatus* in a coral reef of northern Quintana Roo. Monthly changes in the population size of adult *P. guttatus* were explored, by means of capture-recapture techniques, in a group of coral reef patches separated, but not entirely isolated, from adjacent coral patches. Because *P. guttatus* is a sedentary species, we hypothesized that population additions would be due mostly to recruitment of young adults, and population losses to predation-induced mortality. We also aimed to determine the growth rates of *P. guttatus* and to compare them to those reported for *P. argus*, as well as to obtain direct evidence for repetitive breeding of females throughout the year. Although our study was focused on *P. guttatus*, our samplings also yielded individuals of *P. argus*, providing an opportunity to compare the size ranges and time at large of individuals of both lobster species in this reef patch habitat.

MATERIALS AND METHODS

Study Area

The study was conducted in the coral reef at Puerto Morelos, in northern Quintana Roo (Fig. 1). Puerto Morelos is located in the northern portion of a barrier-fringing reef tract that extends from Belize to the Yucatan Strait. Rather than a continuous barrier, the coral reef in Puerto Morelos consists of a series of reef patches, separated from the coast by a reef lagoon 300–1000 m in width. The reef lagoon (<5 m in depth) is covered by seagrass meadows, a habitat where juveniles of *P. argus* dwell, but where *P. guttatus* does not occur (Briones-Fourzán 1995). Along the reef tract, the sloping fore-reef has relatively few high-relief features, but hard coral cover is dense at the reef crest and in the back-reef zone (Ruiz-Rentería et al. 1998), providing an intricate habitat with numerous crevices and caves where both *P. guttatus* and *P. argus* occur (Briones-Fourzán 1995; Lozano-Álvarez & Briones-Fourzán 2001).

Lobster Sampling and Tagging

The main study site (site 1) consisted of a group of several reef patches, close to each other (maximum distance between adjacent patches: 50 m), but relatively separated (~200 m) from the rest of the reef tract. In addition to their relative isolation, we chose these reef patches because they are not fished for lobsters, owing to their proximity to the navigational channel to the port. The area of the patch complex was 2.5 ha, excluding the areas between patches, which consisted mostly of sand and sparse sea grasses. Average depth around the reef patches was 4 to 5 m. From October 1986 to November 1987, 20 lobster traps (mesh size: 5 × 2.5 cm) were deployed by divers in the underside of ledges or coral formations around these patches. The distance between adjacent traps ranged from ~20 to 100 m. The traps remained fixed for 10–13 days every month, during the dark portion of the lunar cycle, after which the traps were recovered and relocated the following month. The divers carefully extracted the lobsters from within the traps every morning during each sampling period, unless impeded by bad weather. Lobsters were tagged with modified Australian spaghetti-type tags (Lozano-Álvarez et al. 1991; Lozano-Álvarez 1992) that were manually assembled in the laboratory as described by Chittleborough (1974). These tags consist of an individually numbered vinyl "spaghetti" and a small plastic toggle, joined by a thin nylon thread. The toggle is inserted in the dorsolateral muscle of the lobster, between the cephalothorax and abdomen, with a stainless steel applicator. The original toggles measured 11 × 3 × 0.5 mm, but we shortened them to 9 mm in length with a grinding machine before assembling the tags, in order to reduce their possible deleterious effects on the relatively small-sized *P. guttatus*.

All lobsters were measured (carapace length, CL, in mm, from between the rostral horns to the posterior margin of the cephalothorax) with digital calipers (±0.1 mm), and injuries (i.e. number and type of missing appendages) were recorded. The reproductive stage of females was determined according to the following scale (Briones-Fourzán & Contreras-Ortiz 1999): (1) clean carapace, no extruded eggs or traces of spermatophore; (2) new and intact spermatophore on sternum; (3) newly extruded eggs (bright orange); (4) dark orange eggs, with eyespots visible; (5) brown eggs, embryo and eyes clearly visible; (6) remnants of empty egg capsules and/or eroded spermatophore. After tagging, the lobsters were returned immediately to natural crevices in the coral patches. To reduce tag loss, individuals that were about to molt or recently molted ("soft-shelled") were not tagged. All lobsters were carefully examined to ascertain whether they had lost a tag, as evidenced by a distinctive scar in the site of tag application.

Additional information on size composition and sex-ratios was obtained from a large, elongated patch (site 2) located 4 km north of site 1, where 13 traps were used in a similar fashion as in site 1. Lobsters from site 2 were not tagged because they were sacrificed to study their diet (Colinas-Sánchez & Briones-Fourzán 1990) and the fecundity of females (Briones-Fourzán & Contreras-Ortiz 1999).

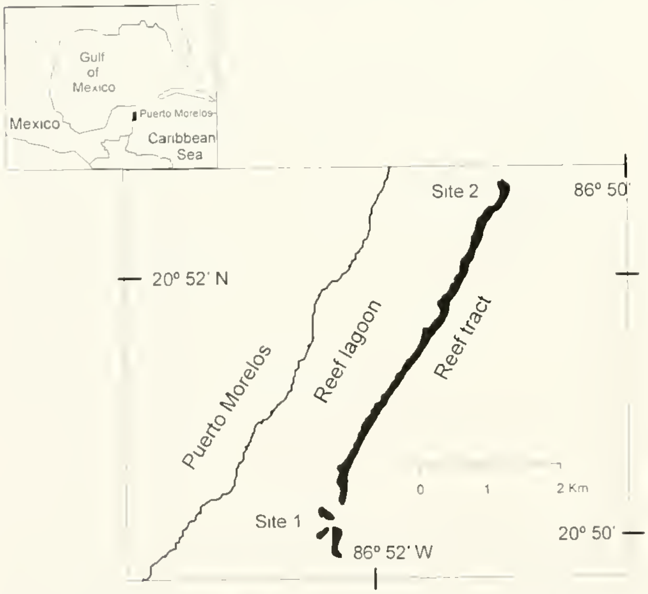


Figure 1. Location of study sites on the coral reef tract at Puerto Morelos, México.

Size Distribution

We compared the overall size distribution of *P. argus* and *P. guttatus*; of *P. guttatus* between sites 1 and 2, and of males and females of each species, with Student's *t*-tests for unequal sample sizes (Zar 1984). When necessary, data were log-transformed to homogenize variances. To explore temporal changes in the size distribution of *P. guttatus* in site 1, monthly data were grouped in 2-mm size classes and analyzed with a one-way repeated measures ANOVA, with time as the repeated factor, followed by a Tukey's test for unequal sample sizes (Winer 1971).

Population Size and Survival

The population size of *P. guttatus* was monthly estimated by means of the Fisher-Ford model (Fisher & Ford 1947), which relies on several tagging occasions and several recaptures. We chose this model because the capture-recapture data were relatively scarce and the survival rate was fairly constant (see Results). In these circumstances, the Fisher-Ford model tends to yield more reliable results than other models based on multiple-recapture data (Bishop & Sheppard 1973; Begon 1979; Lozano et al. 1982), while still providing estimates of population losses (death + emigration), and population additions (recruitment + immigration). The Fisher-Ford model assumes a constant survival rate (ϕ), but this assumption can be analyzed by means of two goodness-of-fit tests (Begon

1979): a test of the period-to-period differences in ϕ (observed vs. expected periods survived) (test 1), and a test of the independence of ϕ from the age of tags (observed vs. expected recaptures of various ages) (test 2). To avoid bias due to a possible "trap addiction" of lobsters, individuals that were recaptured more than once within a sampling period were only considered to have been captured once (Bishop & Hartley 1976). Owing to differences in catchability between sexes (see Results), we applied the Fisher-Ford model separately to males and females, and then summed the monthly estimates of both sexes to obtain the monthly and average population sizes and sex ratios. Estimates of population size were further standardized as lobster density (number of adult lobsters ha^{-1}).

Growth

Because of possible measurement errors, only lobsters whose CL increased over 1 mm between recaptures were considered as having grown (Forcucci et al. 1994). Growth rate (mm CL $week^{-1}$) was calculated by dividing the increase in CL of recaptured individuals by the number of weeks between recaptures. To calculate molt increments (increment in CL per molt), we followed the technique proposed by Forcucci et al. (1994), in which each observation in change in CL is plotted against time at large (in weeks). In these plots, the data points cluster in groups representing single and multiple molting events. Since growth may vary as a function of sex, size, and injury condition (Hunt & Lyons 1986), we produced different plots for uninjured and injured males and females, and analyzed the data for each sex by size class (<60 mm CL, 60–70 mm, and >70 mm CL). Only single-molt observations were used to estimate molt increments. The intermolt period (in weeks) was then calculated by dividing the average molt increment (mm CL) by the average growth rate. The results were then summarized in a table.

Reproductive Aspects

The monthly percentage of ovigerous females was obtained from the total sample to determine the main reproductive season. Data from females of *P. guttatus* that were recaptured in different reproductive stages allowed for a preliminary estimate of the egg-incubation period, and of the duration of a complete breeding cycle.

RESULTS

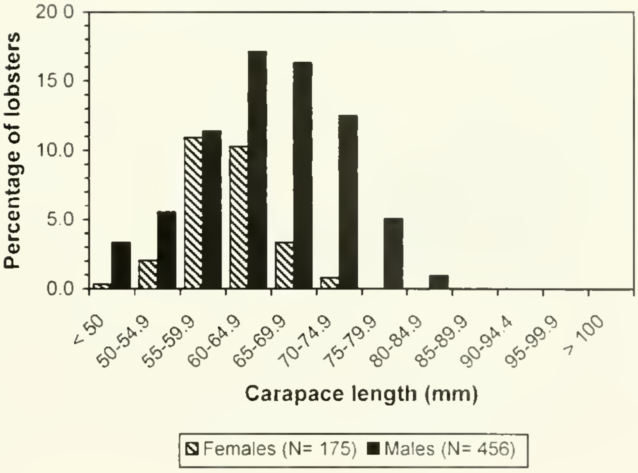
In total, 920 lobsters (including recaptures) were obtained, 778 (85%) *P. guttatus* and 142 (15%) *P. argus*. Results on the issues explored are given for each species separately.

Panulirus guttatus

Size Distribution

In all, 551 *P. guttatus* were caught in site 1 (410 males and 141 females), and 227 in site 2 (160 males and 67 females). Mean size of *P. guttatus* was similar between sites (t = 0.782, df = 777, P = 0.495). Overall, male *P. guttatus* ranged in size from 42.4 to 87.5 mm CL (mean ± SD: 64.5 ± 7.6 mm CL), significantly larger than females (range: 44.5 to 73.5 mm CL; mean ± SD: 59.3 ± 5.2 mm CL) (log-transformed data, t = 8.748, df = 774, $P < 0.0001$) (Fig. 2A).

(a) *Panulirus guttatus*



(b) *Panulirus argus*

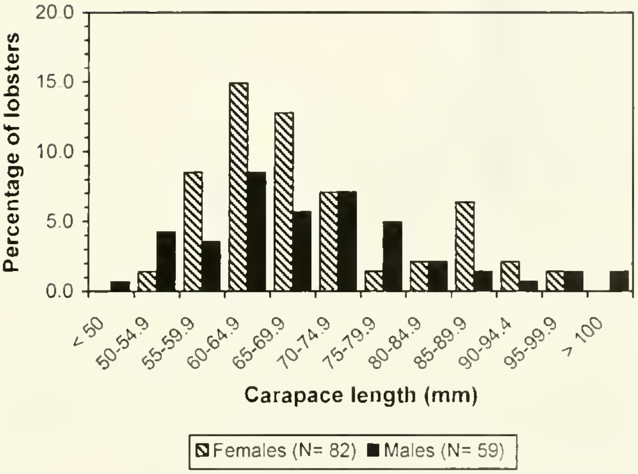
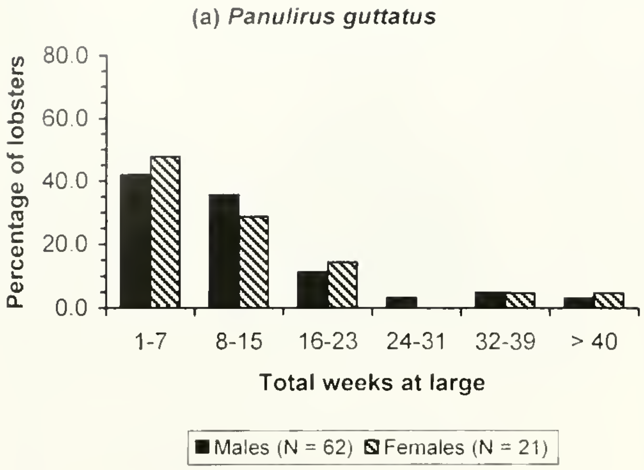


Figure 2. Size distribution of the total sample (including recaptures) of (a) *Panulirus guttatus* and (b) *Panulirus argus* caught in traps in coral reef patches at Puerto Morelos, México.

Tagged and Recaptured Individuals

Individuals of *P. guttatus* (excluding recaptures) in site 1 were 331 males and 127 females (sex ratio 2.6:1). Of these, we tagged 227 males and 90 females, and recaptured 62 males (27.3%) and 12 females (13.3%). Individual males remained at large for 1–50 weeks, and females for 1–43 weeks (Fig. 3a). No significant differences were found in time at large of males and females (χ^2 = 1.22, df = 5, $P > 0.95$). Some individuals were recaptured on multiple occasions (from two to seven), yielding a total of 119 recaptures of males and 26 recaptures of females. Fifteen lobsters (10.3% of recaptures) lost their tags, but were taken into account when estimating the population size by calculating the "average age" of their tags, based on the average age of the tags of the individuals concurrently recaptured (Lozano et al. 1982). Of the recaptured individuals, 53% were recaptured within a distance of 20 m from their site of release, 45% at distances from 20 to 50 m, and 2% at distances from 50 to 100 m. Predation-induced mortality within the traps was high (28%), accounting for 94 males (69 untagged and 25 tagged) and 33 females (28 untagged and five



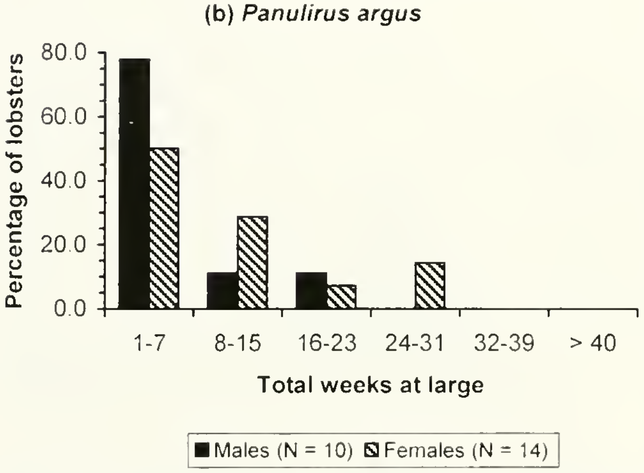


Figure 3. Percentage of individual lobsters recaptured in different periods at large (in weeks). (a) *Panulirus guttatus*, (b) *Panulirus argus*.

tagged). Predators found in traps included groupers (*Epinephelus* spp.), triggerfish (*Balistes vetula*), snappers (*Lutjanus* spp.), and moray eels (*Gymnothorax* spp.).

Population Size and Survival

A lower catchability of female *P. guttatus* in traps was reported by Evans and Lockwood (1994) in Bermuda. A differential catchability of males and females in our traps would contradict one of the principal assumptions of the Fisher-Ford model, namely that all individuals are equally catchable. Therefore, before applying the Fisher-Ford model, we explored whether the biased sex ratio of *P. guttatus* was (a) the actual sex ratio over the size range of trappable lobsters, caused by a lower number of females in the largest size classes due to a differential growth rate of males and females (Wenner 1972), or (b) an artifact due to a differential catchability of males and females in traps. To test (a), we plotted the percentage of males in each 5-mm size class of the total catch. In species where males reach significantly greater sizes than females, such as in palinurids (Morgan 1980), male percentages tend to fluctuate around 50 in the small size classes, then decrease as adult females accumulate in one or a few size classes, and further rise as size increases until approaching 100% in the largest size classes (Wenner 1972; Herrnkind & Lipcius 1989). However, our curve showed higher percentages of males in most size classes, indicating that females were undersampled, and suggesting a differential catchability of males and females. This was further confirmed by a test of difference in catchability between population sub-groups (i.e., males and females) (Begon 1979), which was highly significant ($\chi^2 = 28.756$, df = 12, $P < 0.001$).

The most likely cause for this differential catchability is a different behavior of males and females (Lozano et al. 1982). Apparently, the protracted reproductive season of *P. guttatus* (Farrugio 1976; Sharp et al. 1997; Briones-Fourzán & Contreras-Ortiz 1999) results in females being overall less active than males, as evidenced by the higher occurrence of male *P. guttatus* in fixed gear, such as nets and traps compared to samples obtained by divers, who search actively for lobsters (Table 1). Sex ratios, then, may depend on the sampling method, the location, and the size

TABLE 1.

***Panulirus guttatus*. Size-ranges and sex-ratios of individuals sampled with different methods in several locations of its geographic range.**

Location	Sampling Method	Sample Size	Sex-Ratio (M:F)	Size Range (CL, mm) Males	Size Range (CL, mm) Females	Source
Florida	Diving	894	1.2:1	32–85	36–71	Caillouet et al. (1971)
Florida	Diving	1477	0.7:1	32–84	26–71	Chitty (1973)
Florida	Diving	342	0.5:1	18–75	21–63	Sharp et al. (1997)
Mexico	Diving	212	1.7:1	40–89	44–78	Padilla-Ramos & Briones-Fourzán (1997)
Martinique	Nets	234	2.8:1	33–70	40–60	Farrugio (1975)
Martinique	Nets	772	1.7:1	36–66	30–66	Farrugio (1976)
Martinique	Nets	1469	1.9:1	37–74	34–68	Marfin (1978)
Bermuda	Traps	152	10.0:1	64–88	58–74	Sutcliffe (1953)
Bermuda	Traps	919	13.0:1	51–81	49–69	Evans & Lockwood (1994)
Bermuda	Traps	1656	16.0:1	51–83	51–69	Evans et al. (1996)
Jamaica	Traps	114	2.1:1	43–61	43–70	Munro (1974)
Mexico	Traps	136	1.9:1	55–82	54–65	Carrasco-Zanini (1985)
Mexico	Traps	631	2.6:1	42–88	45–74	Present paper

CL is carapace length; M is males; F is females range of individuals in the samples. Allowing for a higher proportion of males in the largest size classes, the 1.7:1 sex ratio of the *P. guttatus* sampled by Padilla-Ramos and Briones-Fourzán (1997) from fisher divers in Puerto Morelos over a fishing season (Table 1), appears more realistic than ours. Consequently, we applied the Fisher-Ford model separately to the male and female data, and obtained separate estimates of population sizes and survival for each sex (Begon 1979).

The estimated survival rates (ϕ) were 0.727 for males and 0.867 for females. The two tests for the constancy of ϕ showed that this assumption was not violated in any one period (month) for either sex, so the monthly χ^2s were added and further tested for their significance. For males, the overall result of test 1 was $\chi^2 = 18.504$ (df = 12, $P > 0.10$) and of test 2 $\chi^2 = 6.901$ (df = 12, $P > 0.75$). For females, the overall result of test 1 was $\chi^2 = 4.770$ (df = 12, $P > 0.95$) and of test 2 $\chi^2 = 13.840$ (df = 12, $P > 0.25$). Hence, the assumption of a constant survival rate for both males and females was reasonably supported.

Male population size ranged from 97 to 373 individuals, with higher values in November 1986, and from April to September 1987 (Table 2a). The size of the female population varied between 69 and 435, with higher values in April, and from June to November 1987 (Table 2b). When adding the male and female population estimates, higher abundances of adult *P. guttatus* occurred in April, and from June to November 1987 (Table 3). In contrast to the more heavily biased sex ratios in our samples, monthly sex ratios in the population varied from 0.3 to 2.8 males:1 female, with an overall sex ratio of 1.3:1 (Table 3).

The monthly population size was converted to density (number of lobsters ha^{-1}) and confronted with the monthly mean size of lobsters (Table 4). The mean size of *P. guttatus* was significantly different in time (F = 2.637, df = 13, 208; $P = 0.002$), with

TABLE 2.

Panulirus guttatus. **Statistics of the Fisher-Ford model for (a) males; and (b) females in the reef patches. Losses include mortality and emigration; additions include recruitment and immigration.**

(a) Males

	Number of Males			Male Population		
Sampling Period	**Caught**	**Released**	**New Tags in Period**	**Size (N)**	**Losses**	**Additions**
Oct. 1986	31	28	28			
Nov.	42	41	38	219	60	−62.2
Dec.	23	20	13	97	27	43
Jan. 1987	28	19	14	114	31	68
Feb.	46	28	17	150	41	−4.3
Mar.	18	14	9	105	29	218
Apr.	34	23	19	294	80	−47
May	24	14	11	167	46	156
Jun.	33	21	20	278	76	171
Jul.	21	14	12	373	102	−89.5
Aug.	26	15	11	182	50	83
Sep.	18	18	15	215	59	14
Oct.	25	23	19	171	47	−8.8
Nov.	22	0	0	115		
Total	**391**	**278**	**226**			

(b) Females

	Number of Females			Female Population		
Sampling Period	**Caught**	**Released**	**New Tags in Period**	**Size (N)**	**Losses**	**Additions**
Oct. 1986	8	6	6			
Nov.	14	14	14	78	10	49
Dec.	6	6	6	117	16	−32
Jan. 1987	6	5	5	69	9	20
Feb.	14	11	8	80	11	8
Mar.	10	9	7	77	10	94
Apr.	14	7	7	161	21	−38
May	5	2	2	102	14	45
Jun.	16	10	8	133	18	28
Jul.	7	6	5	143	19	93
Aug.	5	3	3	217	29	−52
Sep.	11	10	8	136	18	60
Oct.	13	12	11	178	24	281
Nov.	9	0	0	435		
Total	**138**	**101**	**90**			

TABLE 3.

Panulirus guttatus. **Total population size in the reef patches estimated by the Fisher-Ford model, and comparison of sex ratios of individuals sampled in the reef patches with those obtained from the model population estimates.**

	Number of Lobsters Sampled (n)				Population Size Estimates (N)			
Period	**Males (n_M)**	**Females (n_F)**	**Total ($n_M + n_F$)**	**Sex Ratio (M:F)**	**Males (N_M)**	**Females (N_F)**	**Total ($N_M + N_F$)**	**Sex Ratio (M:F)**
Oct. 1986	31	9	40	3.4:1				
Nov.	46	14	60	3.3:1	219	78	297	2.8:1
Dec.	27	6	33	4.5:1	97	117	214	0.8:1
Jan. 1987	29	6	35	4.8:1	114	69	183	1.7:1
Feb.	51	14	65	3.6:1	150	80	230	1.9:1
Mar.	20	10	30	2.0:1	105	77	182	1.4:1
Apr.	35	14	49	2.5:1	294	161	455	1.8:1
May	25	5	30	5.0:1	167	102	269	1.6:1
Jun.	33	16	49	2.1:1	278	133	411	2.1:1
Jul.	21	7	28	3.0:1	373	143	516	2.6:1
Aug.	26	6	32	4.3:1	182	217	399	0.8:1
Sep.	19	11	30	1.7:1	215	136	351	1.6:1
Oct.	25	13	38	1.9:1	171	178	349	1.0:1
Nov.	22	9	31	2.4:1	115	435	550	0.3:1
Total					**410**	**140**	**550**	**2.9:1**
Average	***191***	***148***	***339***	***1.3:1***				

smaller values from June to October 1987 than throughout the remaining months (Table 4). In this period, some of the highest population density values also occurred. The overall average population density of *P. guttatus* was 126 individuals ha^{-1}.

Reproductive Aspects

We recorded the reproductive stage of 202 female *P. guttatus* in the total sample. Of these, 85 (42.1%) were ovigerous. Ovigerous females occurred every month, except in August 1987. In general, the occurrence of ovigerous females was higher (39.3–65.4%) from December 1986 to July 1987, and lower (0–35%) in August to November 1987 (Table 4).

TABLE 4.

Panulirus guttatus. **Population characteristics over the study period. Mean carapace lengths were grouped with a Tukey's test for unequal sample sizes after a repeated-measures ANOVA. Population density was derived by dividing the total population size (males + females) estimated with the Fisher-Ford model by the surface area of the reef patches (2.5 ha).**

Sampling Period	**Sample Size (n)**	**Carapace Length (mm) (Mean ± SD)**	**Groups of Means**	**Population Density (Lobsters ha^{-1})**	**Percentage of Ovigerous Females**
Oct. 1986	40	68.5 ± 6.5	c		33.3
Nov.	60	65.1 ± 5.8	abc	110	35.3
Dec.	33	67.1 ± 6.4	bc	79	42.9
Jan. 1987	35	65.5 ± 6.3	abc	68	54.5
Feb.	66	63.6 ± 8.4	ab	85	52.2
Mar.	30	62.0 ± 5.2	ab	67	53.8
Apr.	49	63.1 ± 6.8	ab	169	65.4
May	30	61.6 ± 7.9	ab	100	42.9
Jun.	49	60.4 ± 6.8	a	152	39.3
Jul.	28	59.9 ± 6.3	a	191	50.0
Aug.	32	60.6 ± 6.5	a	148	0.0
Sep.	30	61.1 ± 4.9	a	130	8.3
Oct.	38	61.2 ± 5.7	a	129	33.3
Nov.	31	62.3 ± 5.9	ab	204	27.3
Average		**63.1 ± 7.4**		**126**	**42.1**

Of the 21 recaptured females, three changed from reproductive stage 3 to 6 in 13, 13, and 21 days, indicating an egg incubation period of 2–3 weeks. This agrees with Chitty (1973), who estimated the egg-incubation period of *P. guttatus* in less than 30 days. Evidence for repetitive breeding occurred in six females, which had different broods when captured and when recaptured after 25, 31, 46, 97, 101, and 240 days. Repetitive breeding of female *P. guttatus* has been reported before (e.g., Chitty 1973; Farrugio 1976; Sharp et al. 1997; Briones-Fourzán & Contreras-Ortiz 1999) and, in Puerto Morelos, large females (>50 mm CL) breed more times in a year than small females (<50 mm CL) (Briones-Fourzán & Contreras-Ortiz 1999). Based on the short incubation period, the large size (>50 mm CL) of all our recaptured females, and the repetitive breeding evidence from recaptured females, we conservatively propose an average duration of 90–120 days for a full breeding cycle in large females, i.e., up to 3–4 broods per year.

Growth

In total, 119 recaptures of males (57 uninjured and 62 injured) and 26 recaptures of females (19 uninjured and 7 injured) were considered in the growth analyses. Size range at capture of males was 47.8–81.1 mm CL, and of females 53.7–69.0 mm CL. Time between subsequent captures was 1–24 weeks in males, and 2–34 weeks in females. Only 19 males (10 uninjured, 9 injured) and three females (1 uninjured, 2 injured) grew between recaptures (Table 5). In general, injured individuals had smaller molt increments than uninjured individuals. Small uninjured males showed higher growth rates and molt increments than larger males, but their intermolt periods were similar. The uninjured female had a growth rate of 0.32 mm CL week^{-1}. Thirteen injured males and two injured females molted and regenerated between 1 and 5 appendages in periods of 4 to 17 weeks, but showed no increment in CL.

TABLE 5.

***Panulirus guttatus*. Summary of growth data for males (size range: 47.5–81.0 mm carapace length, CL) and females (size range: 53.7–65.3 mm CL) by size class and injury condition at time of release (uninjured: complete individuals; injured; individuals missing one or more appendages). For each sex and size class, average growth rate (mm CL week^{-1}) was estimated by dividing the increase in CL of recaptured individuals by the number of weeks at large. Average molt increments in CL were calculated as in Forcucci et al. (1994), by plotting each observation of change in CL against weeks at large and considering only those individuals that underwent one single molt. Intermolt interval was then calculated by dividing the average molt increment by the average growth rate.**

		(a) Uninjured Individuals					
		That Did Not Grow		That Grew			
Size Class (CL, mm)	Number Recaptured	N	Time at Large (weeks)	N	Average Intermolt Period (weeks)	Average Molt Increment (CL, mm)	Average Growth Rate (mm CL week^{-1})
Males							
<60	11	8	5–18	3	10	4.0	0.40
60–65	21	16	1–17	5	8	2.8	0.37
65–70	14	12	1–14	2	12	2.2	0.19
>70	11	11	1–24	0			
Total	57	47		10			
Females							
<60	10	9	2–18	1	7	2.1	0.32
>60	9	9	2–34	0			
Total	19	18		1			

		(b) Injured Individuals					
		That Did Not Grow		That Grew			
Size Class (CL, mm)	Number Recaptured	N	Time at Large (weeks)	N	Average Intermolt Period (weeks)	Average Molt Increment (CL, mm)	Average Growth Rate (mm CL week^{-1})
Males							
<60	9	5	1–16	4	10	2.4	0.16
60–65	20	19	1–24	1	4	1.2	0.30
65–70	18	16	1–21	2	8	1.1	0.14
>70	15	13	1–12	2	10	1.2	0.12
Total	62	53		9			
Females							
<60	4	3	3–8	1	14	1.6	0.11
>60	3	2	2–7	1	9	1.4	0.17
Total	7	5		2			

Panulirus argus

The mean size of *P. argus* (range: 49.0–123.6 mm CL; mean ± SD: 69.8 ± 12.7) (see Fig. 2b) was significantly larger than that of *P. guttatus* (range: 42.4–87.5 mm CL; mean ± SD: 63.1 ± 7.4 mm CL) (log-transformed data; $t = 8.055$, $df = 914$, $P < 0.0001$).

We caught 120 *P. argus* in site 1 (including recaptures) and only 22 in site 2. Overall, there were 48 males (size range: 49.0–123.6 mm CL) and 72 females (size range: 50.1–98.4 mm CL). Mean size of males and females was similar (mean ± SD of males: 70.3 ± 14.2 mm CL; of females: 69.5 ± 11.6 mm CL; $t = 0.362$, $df = 138$, $P = 0.716$).

In site 1, new individuals of *P. argus* (excluding recaptures) accounted for 35 males and 41 females (sex ratio 0.85:1). We tagged 33 males and 39 females, and recaptured 9 males (27%) and 14 females (36%). Most recaptures of *P. argus* were obtained within 15 weeks; only four individuals remained at large for longer periods (up to 31 weeks) (see Fig. 3b). No significant differences in time at large were observed between males and females ($\chi^2 = 2.92$, $df = 3$, $P > 0.25$). Distance of recapture varied from 20 to 150 m. Some individuals were recaptured more than once, yielding a total of 12 recaptures of males and 30 of females. Of these, four males (60.8–75.8 mm CL) and seven females (50.1–78.0 mm CL) grew. The average molt increment, intermolt period, and growth rate of males were, respectively, 5.2 mm CL, 8.4 weeks, and 0.67 mm CL week^{-1}; whereas those for females were 4.3 mm CL, 6.5 weeks, and 0.68 mm CL week^{-1}. These data were too scanty to attempt any further analyses on population size or growth, but indicate that individuals of *P. argus* were less abundant, had higher growth rates, and remained in the reef patches for shorter periods than individuals of *P. guttatus*. Also, in contrast to *P. guttatus*, there was no evidence of reproductive activity of *P. argus* in the coral patches: all female *P. argus* were in reproductive stage 1.

DISCUSSION

In the reef patches surveyed in this study, we caught over five times as many *P. guttatus* as *P. argus*. The underlying causes for these disparate results could be interspecific competition, a differential catchability in traps, or a real difference in abundance of both species. Competition is not likely to occur, because *P. argus* and *P. guttatus* do not seem to compete for food or shelter resources (Sharp et al. 1997; Lozano-Álvarez & Briones-Fourzán 2001). Although differences in catchability of both species cannot be discarded, we believe that our results reflect a true scarcity of subadult *P. argus* recruiting to the reef habitat. In Puerto Morelos, densities of algal juveniles of *P. argus* in the shallow vegetated reef lagoon are high (146–263 individuals ha^{-1}, Briones-Fourzán & Lozano-Álvarez 2001a), but the density of the subsequent postalgal juveniles is drastically reduced (0–31 individuals ha^{-1}), owing to a lack of crevice-type shelters throughout the reef lagoon (Briones-Fourzán & Lozano-Álvarez 2001b). This would further result in low numbers of subadult *P. argus* moving to the reef habitat. Over 80% of the individuals of *P. argus* in our sample were subadults (<80 mm CL) as opposed to the individuals of *P. guttatus*, which were all adults. In addition, each species appears to make a differential use of the reef habitat: subadult *P. argus* shelter in the reefs during the day, but forage at night in rubble areas or seagrass meadows adjacent to the reefs (Cox et al. 1997), whereas individuals of *P. guttatus* forage on the reef itself (Sharp et al. 1997) and never abandon this habitat completely.

Our estimates on population sizes and survival rates of *P. guttatus* were similar to those estimated in the Florida Keys (Sharp et al. 1997), but, although we did acknowledge the tag loss, our estimates may be biased owing to an unquantified tag-induced mortality. When analyzing the effects of the Australian tag on juvenile *P. cygnus* (40–75 mm CL), Chittleborough (1974) estimated an overall "tag loss" of 23% over 39 weeks, but he could not separate tag-induced mortality from tag loss. In addition, although the size range of Chittleborough's *P. cygnus* and our *P. guttatus* was similar, Chittleborough's tags had considerably larger toggles (14 mm long) than ours (9 mm). Mortality of *Homarus americanus* (43.5–97.5 mm CL) induced by sphyrion tags (similar to the Australian tags but with a steel anchor instead of a plastic toggle) was significantly lower (≤ 5%) in lobsters tagged during intermolt or premolt than in those tagged during postmolt (soft-shelled) (Moriyasu et al. 1995), a precaution we observed in our study. Lozano-Álvarez (1992), using exactly the same type of tag as ours on a population of juvenile and young adults of *P. argus* (25–90 mm CL), estimated a tag-induced mortality of 5%. If a similar tag-induced mortality occurred shortly after tagging in our individual *P. guttatus*, it might have not affected the estimates of survival, but it may have contributed to an underestimation of the population size (Begon 1979, Moriyasu et al. 1995).

Therefore, our population size values are probably underestimates. However, the estimates of survival rates for males (0.73) and females (0.87), and the average density of adult *P. guttatus* in our coral patches (126 lobsters ha^{-1}), were comparable to the mean densities of 118 and 115 adult *P. guttatus* ha^{-1} estimated by Sharp et al. (1997) in two patch reef areas in a lobster sanctuary in Florida, based on nighttime samplings by divers, with overall survival rates of 0.87 and 0.67 in each area. In contrast, in reef-crest areas of Bermuda, where a specific fishery for *P. guttatus* exists, Evans and Lockwood (1994) obtained a mean density of 29 ± 7.6 trappable *P. guttatus* ha^{-1}, but with an overall male:female ratio of 7:1.

The largest population sizes of adult *P. guttatus* in our study site were obtained in June to November. Within this period, the lowest percentages of ovigerous females and the smallest mean size of lobsters also occurred, suggesting a recruitment of small adults to the trappable population during the summer and early autumn. The juveniles of *P. guttatus* presumably live in the same habitat as adults (Sharp et al. 1997), but we have never observed individuals <34 mm CL during daytime divings in these reefs. However, the coral reef is a very intricate habitat, and the small phases of *P. guttatus* may hide deep within small recesses and crevices during the day, warranting future extensive surveys by nighttime divings to reveal their occurrence and their contribution to the whole population size.

Because *P. guttatus* is a rather sedentary species, we hypothesized that the population additions would be mostly ascribed to the recruitment of small adults. However, we cannot rule out entirely the contribution to the population additions and losses of movements of males and females throughout the reef habitat, and from and to our limited study area. Most of our individual *P. guttatus* were recaptured from distances <50 m, and our reef patches were relatively separated from adjacent reef habitats, but low-relief hard grounds between these and other patches may have been traversed by some individuals. In displacement experiments with tagged adult *P. guttatus*, Carrasco-Zanini (1985) and Lozano-Álvarez et al. (unpubl. data) estimated the home range of adult *P. guttatus* to lie within a 100 m radius along the reef tract, but some of their individuals were able to return to their initial shelters from up to 200 m. Evans and Lockwood (1994) documented an autumnal offshore migration, associated with reproductive activities, of females and young males of *P. guttatus* in an extensive reef habitat in Bermuda. However, the fore-reef habitat in our study area is neither as extensive nor as developed as in other coral reef areas (Ruiz-Rentería et al. 1998), so offshore migrations of *P. guttatus* are not likely to occur.

Sharp et al. (1997) proposed that the availability of suitable shelters in the reef habitat may be the primary factor controlling the abundance of *P. guttatus*. In our study patches, in addition to *P. guttatus* and *P. argus*, many other crevice-dwelling species (e.g., *Mithrax spinosissimus*, moray eels, groupers, triggerfish, octopuses, etc.) occur. However, some of these species are also potential lobster predators, as well as other species associated to the reef habitat. Predation-induced mortality was high within our traps, where confined lobsters were unable to escape predators, but the true magnitude of this type of mortality in the natural reef, to which we ascribe most of the population losses, will remain undetermined until specific studies on predator-prey dynamics are conducted in these reefs.

Protracted reproductive periods, indicative of repetitive breedings, are common in tropical palinurid species (Quackenbush 1994). In the coral reefs of Puerto Morelos, Briones-Fourzán & Contreras-Ortiz (1999) found that female *P. guttatus* <50 mm CL breed mostly during the winter and spring, whereas females >50 mm CL breed during the four seasons. These authors estimated the index of reproductive potential of *P. guttatus* by conservatively assuming one brood per year for all size classes. But if females >50 mm CL can breed three to four times per year as suggested by our results, their contribution to the overall egg production may be much higher than that estimated by Briones-Fourzán & Contreras-Ortiz (1999).

An effect of repetitive breeding on females is a decrease in their growth rates, due to a reduction in the molt increments and an increase in the intermolt periods (Hunt & Lyons 1986), but our data on female growth were insufficient to explore this assumption. Injuries have no clear effect on growth in some lobster species (Davis 1986; Forcucci et al. 1994), but in others injuries reduce molt increments (Brown & Caputi 1986). In our study, those injured *P. guttatus* that grew showed, in general, lower molt increments than uninjured individuals. Moreover, other injured individuals molted, as evidenced by their regenerated appendages, but without any increase in CL. Injured lobsters were mostly males, and the occurrence of detached appendages in our traps suggests that some were injured while in the traps, perhaps through aggressive interactions (Lozano-Álvarez & Briones-Fourzán 2001) or when trying to deter the predators that entered the traps. The real incidence of injuries among the population is undetermined.

Our male growth data, although scant and inconclusive, suggest that growth rates of male *P. guttatus* decrease as size increases, but few males grew during their time at large. In Martinique, most *P. guttatus* molt at least twice a year (Marfin 1978), whereas the average intermolt periods of our 10 uninjured males that grew (8–12 weeks) suggest the possibility of four to six molts per year. Applying the average growth values of uninjured males in Table 5, it would take a 48 mm CL male about 2.5 years to reach 80 mm CL. However, many individuals did not molt during similar or longer times at large (up to 24 weeks), and if this portion of males is also taken into account in the growth estimates, the results suggest that uninjured adult male *P. guttatus* may molt at least 2–3 times per year. The average molt increment of males over the entire size range of recaptured individuals was 2.8 mm CL. With these values, a 48- mm CL male *P. guttatus* would grow to 80 mm CL in about four years. More data are needed to improve these results, which nevertheless indicate that *P. guttatus* has lower growth rates than *P. argus* of similar sizes, because it would only take one year for a male *P. argus* to grow from 48–80 mm (Lozano-Álvarez et al. 1991b).

Most of the recaptures of *P. argus* were obtained within 15 weeks, suggesting that these patch reefs are a transitory habitat for these subadults, which would presumably emigrate further on to deeper offshore areas where the large adults dwell (Lozano-Álvarez et al 1991a). In contrast, some individuals of *P. guttatus* were recaptured over periods up to 50 weeks, suggesting that these patch reefs are a more permanent site of residence for *P. guttatus*.

Our results show that the coral reef patches at Puerto Morelos support a sizeable population of *P. guttatus* and are also an important habitat to the subadults of *P. argus*. In the future, more refined studies focused on the population dynamics of *P. guttatus* should include sampling by nighttime diving, a direct estimation of tag-induced mortality, tagging in a broader area, and increasing the sampling effort to obtain a larger set on growth data.

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RESPONSES OF MIGRATING WESTERN ROCK LOBSTERS *PANULIRUS CYGNUS* (GEORGE, 1962) TO TWO DIFFERENT TAGGING RELEASE PROCEDURES

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ABSTRACT In the 1997/1998 season, 3,412 migrating 'white' sub-legal sized western rock lobsters were tagged at Jurien Bay (42–49 m depth), and Cervantes (92–133 m depth) on the Western Australian coast. Most lobsters (n = 2,245) were brought ashore to be tagged, held overnight in coffs, and released 18–24 hours after capture within 6 km and ±10 m depth from where they were taken. A smaller number (n = 1,167) were tagged and released at sea immediately after capture. Over three seasons, more animals tagged at sea were recaptured than those tagged ashore ($P < 0.01$). At both sites, lobsters tagged ashore moved significantly further and faster than those tagged at sea ($P < 0.01$). The correlation between the angle of movement and the speed of movement ranged from 0.25 to 0.42 ($P < 0.05$) for the two tag-and-release procedures and two sites. However, all the lobsters moved in a northerly to north-westerly direction. Growth increments in the first moult after release were unaffected by the different tag and release procedures (P = 0.08); loss of a single limb was, however, likely to result in a 22% decrease in the growth increment.

KEY WORDS: *Panulirus cygnus*, tagging, movement, mortality, growth, migration

INTRODUCTION

Over the last three decades, tens of thousands of western rock lobsters (*Panulirus cygnus*, George, 1962) have been tagged to estimate movement, growth, natural mortality and discard mortality. Tag and release programs are generally expensive, as they usually require deploying research vessels, chartering commercial fishing vessels, or purchasing part of a commercial catch. Often only selected size classes are required for tagging, therefore it would be more economical for commercial fishers to retain individuals with the required characteristics and in this way accumulate large numbers of the animals being targeted for tagging.

However, accumulating animals for later tagging would inevitably mean displacing them from their area of capture and previous research suggests that this can affect subsequent growth (Brown & Caputi 1983, 1984), recapture rates (Chittleborough 1974; Brown & Caputi 1983) and movement patterns (Chittleborough 1974) and may lead to nomadism (Herrnkind 1980).

The object of this investigation was to compare the results of two tag-and-release procedures: one where migrating lobsters were released to the waters where they were caught straight after capture; and the second to bring the lobsters ashore to be tagged, and return them to roughly the same area the next day. In both cases recapture rates, distance speed, direction of movement and growth increments have been compared for the two release procedures.

The lobsters tagged in this study were all migrating animals, known colloquially as 'whites' because they are paler than the dark red resident animals. For most of their lives, adult western rock lobsters show only limited movement; only during the white phase when the lobsters are 4–5 years old, do they move for any distance, migrating from the shallow inshore coastal reefs to the offshore breeding grounds (Morgan 1977; Phillips 1983). The migration takes place each year between late November and January. Tagging data have shown that, while most migrating animals move directly offshore, a significant number make more extensive longshore migrations, generally in a north-westerly direction (Phillips 1983; Cheng & Chubb 1998).

METHODS

Sampling

A total of 3,412 migrating sub-legal size "white" lobsters in the size range 65 mm to 77.9 mm carapace length were tagged and released west of Jurien Bay between 29 December 1997 and 4 January 1998 in 43–49 m and south-west of Cervantes between 29 December 1997 and 6 January 1998 in 92–133 m depth (Fig. 1, Table 1). The depths at Jurien Bay and Cervantes were chosen because they represented two different stages of the offshore migration, the animals in 43–49 m range being in the process of moving to deeper depths offshore and those at the Cervantes site being at the offshore limit of the migration run.

All of the lobsters used for tagging were caught during the course of commercial fishing. The experimental lobsters (Cervantes, n = 1,400 and Jurien Bay, n = 845) were kept alive in tanks with flow-through water circulatory systems and brought ashore to be tagged. After tagging, they were kept overnight in plastic fishing baskets tied to the side of the fishing boats. The next day, they were placed in tanks, taken to the fishing grounds and released (18 to 24 hours after capture) together in a single batch on suitable substrate within 6 km and ±10 m depth from the respective sites where they were caught (Table 1). GPS details of their release positions were recorded. The control lobsters (n = 1, 167) were tagged and released at sea within 12 minutes of capture and within 500 m of where they were caught (Table 1).

All lobsters were tagged ventrally with individually numbered Hallprint type TBA-1 internal anchor tags, as described by Melville-Smith and Chubb (1997). The tag number, size, sex, number of appendages missing, dates of capture and release, and position and depth of capture and release were recorded for each tagged lobster. Tagging at Cervantes and Jurien Bay was done by different people, both experienced at tagging lobsters; at each site the same person tagged at sea and ashore.

Tagged lobsters were generally recaptured by commercial fishers, although small numbers were caught by research and recre-

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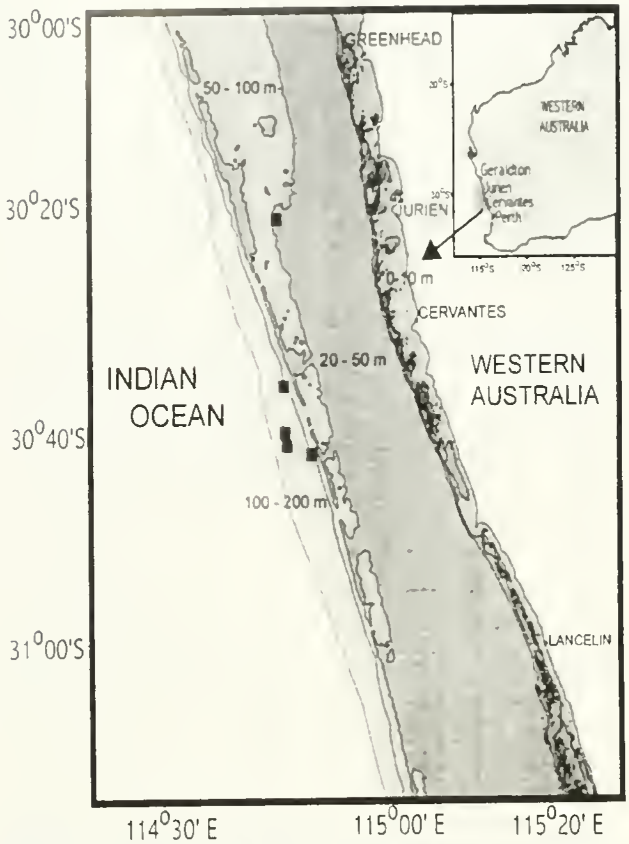

Figure 1. The Western Australian coast, showing where tagged lobsters were released in this study.

ational fishers. All tag recaptures were made during the fishing season, which extends from mid-November to 30 June. Fishers were encouraged to provide details of tagged lobsters by offers of a AUD$2 instant lottery ticket for information relating to any tagged animal recaptured. They were asked to record size sex, number of limbs lost, date, position and depth of capture.

Analysis

The percentage of tag recaptures to numbers tagged were compared for the two tagging sites and their release sites by a generalized linear model with binomial family (logit link). The mean time at large, distance from release sites and speed of movement were calculated. The last two were based on the shortest distance between the point of release and recapture. Animals were grouped by their times (one or two and three seasons) at large and any recaptured within 30 days of release were excluded from analyses calculating the mean time at large and distance from release sites, to avoid biasing the results by including migrating individuals. The second and third seasons were combined instead of being treated separately because published and unpublished data show that once western rock lobsters have undertaken their migration and have settled on the deep water spawning grounds, that thereafter their movements are limited. Combining the second and third years increased the power of the statistical tests.

Recaptured animals were analyzed for defined time periods at large by student-*t* tests to estimate the power of the test. General linear modeling was used to model the size of the first growth increment after release, with distance moved and loss of limbs treated as covariates; and sex, site of release and release procedure as factors.

The uniformity of the directional movement of lobsters tagged at sea and ashore was examined by a Rayleigh test (Zar 1998) and the mean angle of movement of the animals tagged by the two procedures relative to 0° (i.e., True North), was tested by a Watson-Williams test (Zar 1998). Angular-linear correlations for lobsters tagged at sea and ashore were determined by the method described in Mardia (1976) and Johnson and Wehrly (1977). Regression methods were used to establish the relationship of the angular movement to the maximum speed of movement.

RESULTS

Recapture Rate

The mean percentage of tagged rock lobsters that were recaptured from the two release sites and tagging procedures ranged from 7.4% to 12.6% (Table 1).

A generalized linear model showed that recapture rates were unrelated to the two release locations ($P = 0.38$), but that they were significantly correlated with the two tagging procedures ($P = 0.03$). A binomial test on two proportions (Zar 1998) produced a similar outcome. The overall recapture rate of lobsters tagged at sea was about 4% higher than those that were tagged ashore.

Time at Large

The time at large before recapture can influence comparisons of different tag-release procedures. For example, one group of lobsters may move further than another, not because of the experimental procedure, but because they were at large for longer. In the first season (to the end of June 1998) after release, animals that were tagged ashore were recaptured significantly later ($P < 0.03$;

TABLE 1.

Western rock lobsters tagged at sea and on shore at Cervantes and Jurien Bay on respectively 29 December 1997 to 6 January 1998 and 29 December 1997 to 4 January 1998, and recaptured by 30 June 2001.

	Tagging Procedure					
	Ashore			At Sea		
Release Sites and Depths	Release (n)	Recaptured (n)	Percentage Recaptured	Released (n)	Recaptured (n)	Percentage Recaptured
Cervantes (92–133 m)	1400	103	7.36	564	71	12.59
Jurien Bay (43–49 m)	845	79	9.35	603	72	11.94
Total	2245	182	8.11	1167	143	12.25

TABLE 2.

Days at large (Days) of western rock lobsters released at Cervantes and Jurien Bay in 1997/98 after tagging either at sea (n = 1167) or ashore (n = 2245). Results are for the sexes combined, recaptured by the end of the first, and second and third fishing seasons at large.

	Days Before Recapture							
	End of First Season (30 < *days* ≤210 After Release)				Second and Third Seasons (210 < *days* ≤(210 + 730) Days After Release)			
	n	Days (mean)	SE	P Value	n	Days (mean)	SE	P-value
Tagged at sea	81	103.12	2.41		72	419	9.14	
Tagged on shore	50	111.70	2.96	0.03	60	430.4	9.24	0.38

combined sexes) than those that were tagged at sea (Table 2). However, in the second and third seasons at large, there was no significant difference between the two groups (P = 0.38; combined sexes) (Table 2). Although the data have not been presented here, neither the release sites nor the sex of the lobsters produced different results.

Distance Moved

Lobsters that were tagged ashore moved greater distances on the whole than those tagged and released at sea. This difference was significant (P < 0.01) for recaptures at both Cervantes and Jurien Bay made by the end of the first season (Fig. 2). The trend remained clear when only recaptures made in the second and third seasons were considered, but was only statistically significant for the Cervantes data (Fig. 2). The substantial differences in the displacement of recaptured lobsters at the two sites and depths have precluded the data being combined in analyses comparing their movement.

Depending on whether they had been tagged at sea or ashore, lobsters that were tagged at Cervantes and that were at large for two or three seasons had moved about the same distances as those that had been at large for only one season (means ranging from 38.23-45.53 km for those released ashore, compared to 8.98–17.01 km for those released at sea, Fig. 2). This did not apply to lobsters released at Jurien Bay, where the one-season lobsters had moved shorter distances than the two or more season lobsters (means of 10.87 and 5.03 km for those released ashore and at sea respectively by the end of the first season, compared to 30.06 km and 15.78 km for those released ashore and at sea respectively at the end of the combined second and third seasons at large, Fig. 2). However, the proportional differences moved by the at-sea and ashore release groups at Jurien Bay was similar for those at large for one compared to two or more seasons, in all cases those tagged ashore moving two to three times further than those released at sea.

Speed

As there is little movement by western rock lobsters after their 'whites' migration phase which ends by late January, only data for recaptures made from 1 February to 30 June 1998 (i.e., within the first season after release) have been presented in Table 3. Bearing in mind the differences in distance moved between lobsters tagged at sea and ashore (Fig. 2), it is not surprising that the two groups showed significant differences in their speeds of movement at both tagging locations (Table 3).

Direction of Movement

Comparisons using a Rayleigh test of uniformity of movement showed that the lobsters did not migrate uniformly in one direction, regardless of whether they were tagged at sea or ashore. A Watson test to compare the mean angles of movement from the two treatments showed no significant difference for either those animals released in the intermediate depths at Jurien Bay (P = 0.2), or those released in the deep water at Cervantes (P = 0.6). In both cases the mean direction of movement was north to nor-nor-westerly.

Relationships Between Direction, Distance and Speed of Movement

Angular-linear correlations (Mardia 1976; Johnson & Wehrly 1977) used to determine whether distance or speed of movement is correlated with direction of movement, were determined separately for the two tagging sites and for the animals released at sea and ashore. Some, but not all, of the correlations comparing angle of movement to distance moved were significant (Table 4); however, angle of movement was significantly correlated (P < 0.05) with speed of movement in all cases.

A regression analysis comparing speed and angle of movement showed that, while the general direction moved was northerly, recaptured lobsters that had been tagged ashore had a more westerly component to their movement. Since the correlation between angle and speed of movement was higher for lobsters tagged ashore (0.34, 0.42) than at sea (0.25, 0.33), this indicates that migrating western rock lobsters moved fastest when heading in a north-westerly direction.

Growth Increment

The results from a general linear model of the growth increments of lobsters that were likely to have moulted once between release and recapture (i.e., only animals recaptured between February 1998 and July 1998) are shown in Table 5. Distance moved is treated as a covariate (P = 0.15), and sex (P = 0.46) and

TABLE 3.

Mean speed of movement of recaptured western rock lobsters tagged at Cervantes and Jurien Bay in December 1997 and January 1998 and recaptured between 1 February and 30 June 1998 (i.e., within the first season after release).

Release Site and Depth (m)	Tagging Procedure	Recaptured (n)	Speed (m/day)	S.E.	P = value
Cervantes	Ashore	27	392.1	55.64	
92–133 m	At sea	26	142.1	30.35	<0.01
Jurien Bay	Ashore	23	103.7	28.18	
43–49 m	At sea	55	52.52	4.50	0.01

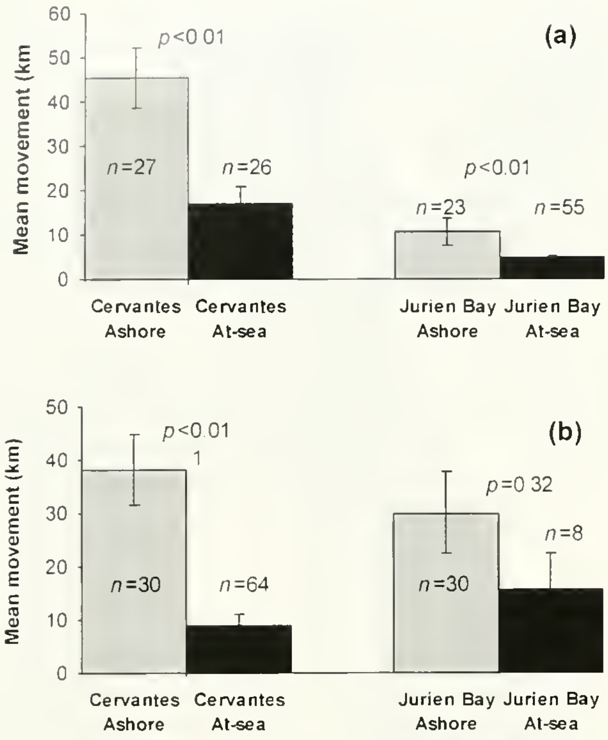

Figure 2. Mean distance western rock lobsters moved after tagging at Cervantes and Jurien Bay in December 1997 and January 1998 (a) by the end of the first season excluding the first month after release and (b) in the second and third seasons.

release procedure ($P = 0.08$) as factors; none had a significant effect on growth increment. However, region of release ($P = 0.03$) and the number of limbs lost at tagging ($P = 0.01$) did have a significant effect on growth increment. As only three recaptured lobsters had more than two lost limbs at tagging, nothing can be inferred about the effect of multiple limb loss on growth. However, it can be seen (Table 5), that the loss of a single limb is likely to result in a 22% decrease in growth increment.

DISCUSSION

The lower recapture rates of lobsters tagged ashore and released a day later, suggest that their mortality rates were higher than lobsters tagged and released at sea within 12 minutes of capture. Brown and Caputi (1983) noted that the length of time between capture and release had a detrimental effect on survival and growth of sub-legal size lobsters. However, in the present study every effort was made to limit the time of exposure out of water. After capture, the animals were stored on board in baskets submerged in dark, aerated wells in the hull as is commercial product. Once ashore, the lobsters were tagged immediately and then kept overnight in plastic baskets hung over the side of the boats in well-aerated surface water. For the return trip to the fishing grounds, they were stored in the same way as after capture.

Displacement of western rock lobsters from their home range probably increases their vulnerability to predation (Chittleborough 1974; Brown & Caputi 1983). Brown and Caputi (1983) further concluded that "when lobsters are migrating they are somehow affected by displacement more than they are affected by it in their non-migratory phase (p. 125)." When western rock lobsters make their offshore migration, they are thought to move in groups or "packs", rather than singularly (Gray 1992). There is some evidence of a survival benefit in groups, rather than single lobsters moving over the sand (Herrnkind 1969; Kelly et al. 1999). Displacing migrating lobsters from their groups by bringing them inshore to be tagged may, therefore, have increased their vulnerability to predation, at least until they could rejoin another group of migrating animals.

Even though a higher percentage of lobsters tagged ashore were recaptured significantly sooner (within the first season) than those tagged at sea, they had moved significantly further from their release site than had those tagged at sea. This group of shore-tagged lobsters had been at liberty for a shorter time, and therefore had less time in which to migrate. There is evidence that *Panulirus argus* and *P. cygnus*, can return to their resident homesite if displaced over a few hundred metres (*P. cygnus*; Chittleborough 1974, and *P. argus*; Herrnkind et al. 1975). Chittleborough (1974) and Herrnkind et al. (1975) both showed that displacing animals from their home range or a familiar region results in abnormal patterns of movement. Although he had no data on relocating migrating lobsters, Herrnkind (1980) speculated that moving them away from their characteristic migratory pathway could conceivably cause disorientation and wandering, which would distort tag-recapture studies.

The present study does not confirm Herrnkind's (1980) expectations: although the animals tagged ashore, that had both a delayed and displaced release, migrated further, their mean direction of movement was the same as that of the lobsters tagged at sea. This observation does not support the suggestion they wandered in a disorientated fashion. Furthermore, rock lobsters have been found to use magnetic fields to orient themselves (Lohmann et al. 1995). It seems that, despite the unusual treatment of most of the animals in this study, they retained their directional orientation during their time in captivity. The question as to why shore-tagged lobsters travelled further remains unresolved.

Previous western rock lobster tagging programs have shown that while most lobsters migrate less than 10 km from their point of release, many migrate up to several hundred kilometers, almost always in a north-westerly direction (Gray 1992). Furthermore, Cheng and Chubb (1998) report that migrating western rock lobsters tagged in deepwater move longer distances than those in shallow water. It is commonly accepted therefore, that most "white" migrating lobsters become resident in 40–90 m depths west or northwest of the shallows from where they started their migration, but that a minority that reaches deep water (>90 m) follows the contours northward, before peeling off inshore along the way to settle on the breeding grounds in depths of around 40–90 m.

The distance and direction moved by animals tagged in the depths reported in this study are consistent with the scenario suggested above. Those tagged in the middle depths (43–47 m) at Jurien Bay generally moved shorter distances than those in the deepwater (>90 m) at Cervantes. These middle depths (40–90 m) is where the resident breeding stock population occurs. Interestingly, lobsters at large for one season at Cervantes, whether tagged at sea or ashore, moved roughly the same distances as those at large for two or three seasons. In contrast, at Jurien Bay, lobsters recaptured in the second and third seasons had moved further than those recaptured in the first season. However, animals tagged at sea and ashore both moved greater distances in the second than in the first. The lack of difference in distances moved once the white migrating lobsters become resident (within the first season) sug-

TABLE 4.

Correlations between displacement and angle of movement, and speed and angle of movement of western rock lobsters tagged either ashore or at sea and released at one of two different sites.

Release Location	Tagging Procedure	Correlation Between Distance Moved and Angle of Movement	*P*-value	Correlation Between Speed and Angle of Movement	*P*-value
Cervantes	Tagged ashore	0.18	0.35	0.34	0.03
92–133 m	Tagged at sea	0.31	0.01	0.25	0.05
Jurien Bay	Tagged ashore	0.33	0.04	0.42	0.01
43–49 m	Tagged at sea	0.27	0.10	0.33	0.03

gests that the tagging-release procedure affects distance only in the first season.

The differences between the distance travelled by displaced lobsters (shore tagged) and those released where they were captured suggests that movement data from animals tagged during their migration phase be interpreted cautiously. The spatial and temporal displacement resulting from bringing the animals ashore to be tagged and releasing them at a distance from their catch site clearly has an effect. Smaller displacements, such as result from the drifting of tagging platforms or being held overnight in a pot when captured, might also affect the movement of lobsters tagged at sea during their migratory phase. Results from this study would therefore suggest that the effects of release procedures on the behavior of migrating lobsters, must be considered before biological conclusions are drawn.

No oceanographic data were collected, either at the tagging locations, or at the anchorages where the animals were held overnight. However, substantial amounts of environmental data have been collected on the western rock lobster grounds in the past, and it can be confidently assumed that differences in the salinity, temperature and oxygen concentrations in the coastal waters where the lobsters were held, compared to the offshore waters where they were caught, would have been well within the seasonal ranges experienced by the species. This does not preclude the possibility that the overnight acclimation of the animals to the changes in water conditions may have contributed to the different behavior of the at-sea and ashore tagged animals.

The mean speeds of movement recorded by the migrating animals tagged in this study are substantially slower than those (1.2 km per day) recorded by Cheng and Chubb (1998). Our speeds have been underestimated because all recaptures over the first season at large were used in the calculation, but western rock lobsters migrate only between late November and late January. The strong correlation between angle of movement, and both distance traveled and speed of movement, is a reflection of the lobsters that moved furthest (and therefore recorded the fastest speed of movement while at large) generally heading in the northwesterly direction almost parallel to the coastline, whereas those that moved least (and therefore recorded the slowest speed of movement while at large) generally heading in a northerly direction.

TABLE 5.

Results of a general linear model of growth increments of lobsters between February 1998 and July 1998 (one moult increment).

Name of Variable	Estimated	*P*-value
Intercept	4.28	0.00
Distance moved	0.00	0.15
Released procedure	−0.45	0.08
Released location	0.61	0.03
Sex	−0.19	0.46
Single limb loss at tagging	−0.94	0.01

Single growth increments of lobsters at the end of the first season (after their first moult) did not suggest that migrating animals tagged at sea grew either faster or slower than those held ashore overnight and displaced from their point of capture ($P > 0.05$). This result differed from that of Brown and Caputi (1984), who found that displaced undersize western rock lobsters of a larger size range than those used in this study, had significantly ($P > 0.05$) smaller growth increments than nondisplaced animals. Although the animals in this study would presumably have been stressed immediately after capture, during tagging and possibly while being held overnight, these affects have previously been shown (Melville-Smith et al. 1997) to be short-term and unlikely to affect the moult increment later in the season. The significant difference ($P = 0.03$) in growth increment between the two tagging sites is likely to be due to regional differences in growth, which are well documented in this fishery (Chittleborough 1976; Joll & Phillips 1984).

This study investigated the effects of different handling methods and, on the basis of the results, drew some conclusions about the scope for possible cost reduction in tagging programs by having fishers accumulate selected size-classes of migratory lobsters for tagging ashore. The result has shown that this approach would not be feasible for studying movement patterns or for purposes where recapture rates are critical to the result, such as estimating mortality and/or population sizes. However, growth increments were not significantly affected; if growth measurements were the only requirement from the data, then this cooperative approach between research and industry could result in affordable and reliable tagging projects. Comparative cost savings of tagging the cumulative catch of a number of fishers ashore, as opposed to a single fisher at sea is not possible, because the result is dependent on catch rates of the animals being selected for tagging and on the number of fishers accumulating that part of their catch.

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MUSHROOM TYROSINASE AS A CONTROL MATERIAL FOR PHENOLOXIDASE ASSAYS USED IN THE ASSESSMENT OF CRUSTACEAN "HEALTH"

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ABSTRACT Mushroom tyrosinase (E.C. 1.14.18.1) was evaluated as a control material for use in a plasma prophenoloxidase assay in the American lobster *Homarus americanus* as part of the assay's validation procedure. Reconstituted, lyophilized tyrosinase solution was stable for 72 h at 2–4°C. Percent recoveries were 94%, 120%, 113%, and 48% for "mid-range" activity dilutions and 93%, 94%, 87%, and 38% for "low-range" activity dilutions at 24, 48, 72, and 96 h, respectively. The activity of the reconstituted tyrosinase solution was stable for 4 wk when stored at −80°C, and percentage recoveries, compared to activity of the freshly reconstituted solution, were generally within 15% for both mid-range and "high"-activity dilutions. The between-run coefficients of variation (CVs) were 10.6% for the mid-range and 10.8% for the high-activity dilution over the 4-wk period. Enzyme activity was unstable when stored at −20°C. Mushroom tyrosinase is an acceptable control material for use in assays of phenoloxidase activity. Inclusion of this control material will provide a means to confidently compare results on a day-to-day or run-to-run basis when phenoloxidase assays are used in the assessment of crustacean "health."

KEY WORDS: prophenoloxidase assay, control, tyrosinase, crustacean, health

INTRODUCTION

The prophenoloxidase activating system (PPAS) and its active enzyme product, phenoloxidase (*o*-diphenol:O_2 oxidoreductase E.C. 1.14.18.1) are integral components of the innate defense system in crustaceans (Söderhäll et al. 1996; Söderhäll & Cerenius 1998; Sritunyalucksana & Söderhäll 2000). Dark pigmentation (melaninization) at sites of injury reflects prior generation of phenoloxidase, followed by the spontaneous formation of melanin (Söderhäll et al. 1996; Söderhäll & Cerenius 1998; Sritunyalucksana & Söderhäll 2000). Both the reactive quinone intermediates and the final melanin products formed during this reaction have antimicrobial and protective properties (Söderhäll et al. 1996; Riley 1997).

Determination of the activity of phenoloxidase in either whole hemolymph or hemocyte lysate supernatants has been used to assess the "health" or "immune" status of crustacean species (Hauton et al. 1997; Sritunyalucksana et al. 1999; Rodríguez & Le Moullac 2000). Commercial test kits[1] are available to detect phenoloxidase activity in whole hemolymph samples from shrimp. It is recognized that assays used in the assessment of crustacean health should be standardized (Bachère 2000).

Evaluation and validation of a laboratory test are required to confirm that an assay is performing within its defined parameters before it can be accepted for use in a diagnostic setting (Bellamy & Olexson 2000). This includes determination of the assay's reportable range, precision, accuracy, and reagent stability. Control materials are essential to monitor the performance of the assay (Westgard & Klee 1999). Inclusion of a control with each group of samples permits reliable comparison of the results obtained from samples analyzed during different runs. Ideally, control materials are derived from the same biological source as the samples that are being evaluated.When this is not possible, materials with similar properties can be substituted (Westgard & Klee 1999).

Most phenoloxidase assays are based on (or are modifications of) the method of Horowitz and Shen (Horowitz & Shen 1952; Aspán & Söderhäll 1995). During development of an assay to detect prophenoloxidase activity in plasma of the American lobster, *Homarus americanus* H. Milne Edwards, a control material was required. A purified source of lobster phenoloxidase having consistent activity was not available. Lyophilized mushroom tyrosinase was evaluated as a control material because of its similarities to phenoloxidase and commercial availability (Aspán & Söderhäll 1995).

MATERIALS AND METHODS

Mushroom tyrosinase (E.C. 1.14.18.1) stock solution was prepared by adding 2.5 mL of sodium phosphate buffer (0.2 M Na_2PO_4, pH 7.5) to 13 mg of lyophilized mushroom tyrosinase (Sigma Chemical Company, St. Louis, MO), giving a final concentration of approximately 10,700 units of tyrosinase activity per mL of solution. Serial dilutions of this reconstituted stock solution were used to determine assay linearity, sensitivity, and within-run precision as determined by calculation of coefficient of variation (%CV). A second stock solution, calculated to have similar activity per unit volume, was prepared from a different lot of lyophilized tyrosinase. This second solution was used to conduct refrigerated and frozen stability trials. Eight 150-µL aliquots were placed in 0.5-mL polypropylene microcentrifuge tubes (Fisher Scientific, Canada). Four aliquots were stored at −20°C, four aliquots were stored at −80°C, and the remaining solution was refrigerated (2–4°C), for stability trials.

To complete linearity and precision assessments, serial dilutions at 1:200, 1:400, 1:800, 1:1600, 1:3200, and 1:6400 of the stock solution were made using a 0.2 M sodium phosphate buffer, pH 7.5. Twenty microliters of each dilution was added to individual wells of a flat-bottomed tissue culture plate (Falcon Microtest 3072, Becton Dickinson and Company, U.S.A.). There were five replicates per dilution. Wells to be used as "substrate-free blanks", one well for each dilution, also received 20 µL of the appropriate tyrosinase stock dilution. Twenty microliters of phosphate buffer was placed in wells used as reagent blanks. Eighty microliters of sterile, distilled water was added to all wells using a multichannel pipette. One hundred microliters of the substrate solution, 3.8 mM dopamine (3-hydroxytyramine) (Sigma Chemical Company), was then added to all wells except for the substrate-free blanks. These wells each received 100 μL of sterile distilled water. Production of the gold-orange quinone intermediate was monitored every 11 sec (wavelength 470 nm) during a 5-min period in an automated spectrophotometer (SpectraMax, Molecular Devices Corporation, U.S.A.) after a 5-sec mixing cycle. The software package SoftMax™ (Molecular Devices Corporation, U.S.A.) was used for calculation of maximum enzyme activity (V_{max}), defined as the rate of maximal change in optical density (OD) of the reaction solution per minute (Δ mOD/min) over a user-defined time interval. The V_{max} used for all further calculations for each dilution was the mean V_{max} of the five replicates. Within-run coefficients of variation [%CV = (standard deviation/mean) × 100] were then calculated (Table 1).

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[1] Spot On, DiagXotics Inc., Wilton, CT 06897.

Stability of the refrigerated stock solution was determined by calculating the percentage recovery for two dilutions, identified as having "low" and "mid-range" activities relative to the linear range of the assay, over a period of 4 days. Percent recovery was calculated as: [(measured activity at time "x"/initial activity at reconstitution) × 100] at 24, 48, 72, and 96 h (Table 2). Four replicate wells were used for each dilution.

Long-term frozen stability of the stock solution, stored at −20°C and −80°C, was evaluated by measuring enzyme activity in serial dilutions of a thawed (room temperature, 20–22°C) aliquot every week for 4 wk. Four replicate wells were used for each dilution. Percent recovery calculations were made for the aliquots stored at −20°C and −80°C (Tables 3a and 3b). Between-run (week to week) %CVs were calculated for each dilution of the aliquots stored at −80°C (Table 4).

The refrigerated stability of thawed stock solution, after storage at −80°C, was also evaluated. Enzyme activity was measured at 48 and 72 h post-thawing for two dilutions (mid-range and high activities). Percentage recoveries were calculated by comparing the activity after 2 and 3 days of refrigeration to the activity obtained immediately after thawing (Table 5).

RESULTS

No significant absorbance was detected in the substrate-free blank wells during the initial assays. Substrate-free blanks were not used for the remaining assays. Reagent blanks were used for all assays and also functioned as the plate blanks. The reaction curve was linear during minutes 2–4 of the 5-min monitoring period. V_{max} was determined from this 2-min period. Initially, a gold-orange product developed in all of the reaction wells containing the dopamine substrate and the mushroom tyrosinase. Subsequently, a black product also appeared in the wells. At first, only small amounts of the black product were present; however, with time, the gold-orange product was no longer visible and only the black product could be seen. The amount of this second product seemed proportional to the concentration of enzyme solution in the wells.

TABLE 1.

Sensitivity, linearity, and precision results for reconstituted mushroom tyrosinase.

Dilution	Expected[a] Activity (mOD/min)	Observed Activity[b] (mOD/min)	SD[c]	CV[d] (%)
1:200	—	41.6	0.53	1.3
1:400	20.8	19.6	0.16	0.8
1:800	10.4	9.3	0.39	5.0
1:1600	5.2	5.0	0.32	6.5
1:3200	2.6	1.4	0.20	14.3
1:6400	1.3	0.4	0.23	60.7

[a] Calculated from the 1:200 dilution.

[b] Represents the mean of five replicates.

[c] Standard deviation.

[d] Coefficient of variation [%CV = (standard deviation/mean) × 100].

TABLE 2.

Refrigerated (2–4°C) stability of reconstituted mushroom tyrosinase.

	Initial Activity[b] (mOD/min)	Percent Recovery (%)[a]			
		1 day	2 days	3 days	4 days
Dilution no. 1	22.4	94	120	113	48
Dilution no. 2	12.3	93	94	87	38

[a] Percent recovery = (measured activity/initial activity) × 100.

[b] Represents the mean of four replicates.

The catechol oxidase activity of mushroom tyrosinase, as measured in this assay system, was linear, and had acceptable precision results, from 5.0 to 41.6 mOD/min when evaluated by serial dilution ($R^2 = 0.99$) (Table 1, Fig. 1). Using the activity of the 1:200 dilution as a starting point, the expected and observed (in parentheses) activities (mOD/min) for each further dilution were 20.8 (19.6) for the 1:400 dilution, 10.4 (9.3) for the 1:800 dilution, 5.2 (5.0) for the 1:1600 dilution, 2.6 (1.4) for the 1:3200 dilution, and 1.3 (0.4) for the 1:6400 dilution. The within-run CVs (n = 5 replicates), for each dilution were 1.3% for the 1:200 dilution, 0.8% for the 1:400 dilution, 5.0% for the 1:800 dilution, 6.5% for the 1:1600 dilution, 14.3% for the 1:3200 dilution, and 60.7% for the 1:6400 dilution (Table 1).

The stability of the freshly reconstituted reagent was acceptable (i.e., generally within 15% of the initial values) for up to 72 h after refrigeration, but decreased to an average of 43% of initial activity by 96 h (Table 2). Percent recoveries for a mid-range dilution at 24, 48, 72, and 96 h were 94%, 120%, 113%, and 48%, respectively. Percent recoveries for a low-activity dilution at 24, 48, 72, and 96 h were 93%, 94%, 87%, and 38%, respectively (Table 2).

The stock solution was unstable when stored at −20°C. The enzyme activity, calculated as a mean of all dilutions, decreased to 37%, 28%, 27%, and 21% of the initial activity of the freshly reconstituted reagent by weeks 1, 2, 3, and 4, respectively (Table 3a).

The stock solution was stable when stored at −80°C. The percent recoveries, average of mid-range (dilution no. 2) and high- (dilution no. 1) activity dilutions, by week, were 87% for week 1, 99% for week 2, 116% for week 3, and 106% for week 4 (Table 3b). The between-run CVs, over the 4-wk period, were 10.6% for the mid-range dilution and 10.8% for the high-activity dilution (Table 4). The refrigerated stability of the thawed stock solution was generally satisfactory for weeks 1–3, but tended to deteriorate by week 4 of storage at −80°C (Table 5).

DISCUSSION

The value of any assay resides in its ability to consistently provide accurate and precise results. An incorrect result can be

TABLE 3a.

Frozen stability of mushroom tyrosinase stock solution stored at −20°C for 4 weeks.

Dilution	Initial Activity[b] (mOD/min)	Percent Recovery (%)[a]			
		1 wk	2 wk	3 wk	4 wk
No. 1	33.4	37	28	26	22
No. 2	17.0	37	28	28	19
Mean		37	28	27	21

[a] Percent recovery = (measured activity/initial activity) × 100.

[b] Represents the mean of four replicates.

TABLE 4.

Weekly between-run coefficients of variation (CV) for mushroom tyrosinase activity when stored at −80°C.

Weeks at −80°C	Mean[a] Activity (mOD/min)	
	Dilution No. 1	Dilution No. 2
0	33.4	17.0
1	28.4	15.1
2	31.1	17.7
3	37.2	20.2
4	35.7	17.9
Mean	33.1	17.6
SD[b]	3.5	1.9
CV[c] (%)	10.6	10.8

[a] n = 4 replicates.

[b] Standard deviation.

[c] Coefficient of variation = (mean/standard deviation) × 100.

worse than no result at all. Assay validation includes quality control steps used to ensure that diagnostic tests perform within predetermined specifications. Control materials are used to monitor the assay's performance on a run-to-run basis. Ideally, such materials are derived from the same species as the samples to be analyzed; however, this is not always possible. Steps in assay validation usually include (but are not limited to): determination of the assay's reportable range; evaluation of assay precision by calculating both within-run and between-run coefficients of variation (%CV = standard deviation/mean × 100); and, determining the stability of the assay reagents and the sample under different storage conditions (Bellamy & Olexson 2000).

Phenoloxidase (*o*-diphenol:O_2 oxidoreductase E.C. 1.14.18.1) assays have been used to investigate and assess the health status of a variety of crustaceans in both research and commercial settings (Sritunyalucksana et al. 1999; Rodríguez & Le Moullac 2000). A stable source of enzyme to use as a control material was required in the development of a plasma prophenoloxidase assay for use in *H. americanus*. A commercial source of purified lobster (*H. americanus*) phenoloxidase is not available. Lobster hemocyte lysate supernatant preparations represented a highly concentrated source of prophenoloxidase; however, these were unsuitable due to their poor long-term storage characteristics and the inherent, inconsistent activity between preparations (unpubl. results, ALB).

Mushroom tyrosinase (E.C. 1.14.18.1) was evaluated as a potential alternative because of its similarity to phenoloxidase. Both enzymes have cresolase (monophenol monooxygenase) and catechol oxidase (polyphenol oxidase) activity (Aspán & Söderhäll 1995). Determination of the enzyme's copper content and location of conserved histidine residues indicates that arthropod prophenoloxidase belongs to the tyrosinase group of enzymes found in bacteria, fungi, and plants (Aspán & Söderhäll 1995). The reported long-term stability of frozen, reconstituted mushroom tyrosinase and its commercial availability made this enzyme an attractive alternative (Kertesz & Zito 1965).

Mushroom tyrosinase was readily soluble in the 0.2 M sodium phosphate buffer (pH 7.5) used in this assay system. Other studies have used a sodium cacodylate-based buffer. Sodium cacodylate is listed as a hazardous material and is a recognized carcinogen (Sigma-Aldrich 2001). The sodium phosphate buffer used herein presents minimal hazards, is readily available, and was compatible with this assay system. L-DOPA (3,4-dihydroxyphenyl-L-alanine), a commonly used substrate in phenoloxidase assays, was not compatible with the phosphate buffer. Dopamine (3-hydroxytyramine) was chosen as an alternate catechol. Dopamine has been identified as a preferred substrate for phenoloxidase from *Manduca sexta* (Sugumaran et al. 1999). This may also be true for other invertebrates. Mushroom tyrosinase converted the dopamine substrate to an orange-gold intermediate product, presumably the *o*-quinone, 4-(2-aminoethyl)-1,2-benzoquinone. This product was eventually replaced by a black precipitate (melanin), which is the expected result of activation of the PPAS *in vivo* (Söderhäll et al. 1996; Söderhäll & Cerenius 1998; Sritunyalucksana & Söderhäll

TABLE 3b.

Frozen stability of mushroom tyrosinase stock solution stored at −80°C for 4 weeks.

Dilution	Initial Activity[b] (mOD/min)	Percent Recovery (%)[a]			
		1 wk	2 wk	3 wk	4 wk
No. 1	33.4	85	93	112	107
No. 2	17.0	89	105	119	105
Mean		87	99	116	106

[a] Percent recovery = (measured activity/initial activity) × 100.

[b] Represents the mean of four replicates.

TABLE 5.

Refrigerated stability of thawed mushroom tyrosinase stock solution stored at −80°C.

Dilution	Weeks at −80°C	Initial activity[b] (mOD/min)	Percent recovery (%)[a]	
			2 days	3 days
No. 1	0	33.4	nd[c]	nd
	1	28.4	110	135
	2	31.1	106	110
	3	37.2	106	102
	4	36.7	85	75
No. 2	0	17.0	nd	nd
	1	15.1	106	139
	2	17.7	86	103
	3	20.2	104	97
	4	17.9	84	89

[a] Percent recovery = (measured activity/initial activity) × 100.

[b] Represents the mean of four replicates.

[c] Not done.

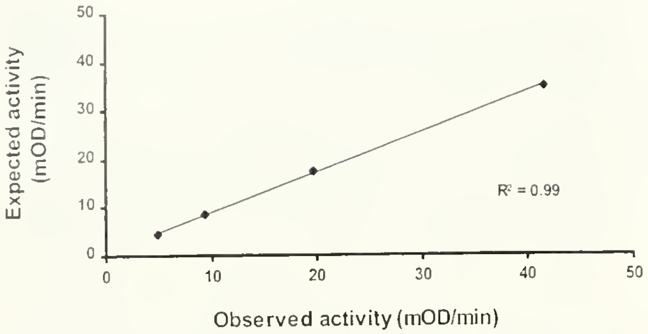

Figure 1. Linearity of catechol oxidase activity of reconstituted mushroom tyrosinase determined by serial dilution.

2000). An identical series of products is observed when either lobster hemocyte lysate supernatant or lobster plasma, after activation with trypsin, is used as the enzyme source for the assay.

The reconstituted tyrosinase solution was stable, when refrigerated, for up to 3 days as indicated by the percentage recovery studies (Table 2). Tyrosinase activity was stable for up to 4 wk when the reconstituted solution was stored at −80°C. Reconstituted mushroom tyrosinase was unstable when stored at −20°C, in contrast to a previous report (Kertesz & Zito 1965). The discrepant results found in this study may reflect differences in the buffers used for reconstitution. Overall, the refrigerated and frozen (−80°C) stability of reconstituted mushroom tyrosinase was considered acceptable for its use as an assay control material.

Ideally, control materials contain a predetermined amount of the enzyme being measured and are incorporated in a material (matrix) similar to the matrix of the sample to be analyzed. In this study, mushroom tyrosinase was reconstituted in a phosphate buffer. This would be the optimal situation when phenoloxidase activity is being measured in hemocyte lysate supernatants where a similar buffer had been used to prepare the lysates. Similarly, incorporation of the mushroom tyrosinase into whole hemolymph or plasma collected from normal, apparently healthy animals would be preferable when phenoloxidase activity is being measured in these types of samples. This was not evaluated in this study. When reconstituted in a sodium phosphate buffer, mushroom tyrosinase is a suitable control material for use in phenoloxidase assays, providing assurance that the assay is working correctly and permitting comparison of within-laboratory sample results on a run-to-run or day-to-day basis.

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ERRATUM

ARTIFICIAL ENVIRONMENTAL CONDITIONS CAN AFFECT ALLOZYME GENETIC STRUCTURE OF THE MARINE GASTROPOD *PATELLA CAERULEA*

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[*Journal of Shellfish Research*, Vol. 20, No. 3, 1059–1063, 2001]

After this article appeared in the December 2001 issue of the *Journal of Shellfish Research*, the following printer's errors were noticed:

The author's name was misspelled in the author line of the title page of the article.

Marculeo was misspelled in the title page footnote.
*Corresponding author: **marculeo@unipa.it**

Enzyme was misspelled in the Table 1 title.

TABLE 1.
Enzyme commission number for the loci analyzed.

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ABSTRACTS OF TECHNICAL PAPERS

Presented at The 5th

INTERNATIONAL CONFERENCE

ON

SHELLFISH RESTORATION

Nanaimo B.C., Canada

September 18–20, 2001

CONTENTS

W. S. Arnold
Bay scallop (*Argopecten irradians*) population restoration in Florida, USA: Culture versus management.. 305
W. S. Arnold
Testing alternative strategies for enhancement of hard clam (*Mercenaria* spp.) population in Florida, USA waters...... 305
Lise M. Auffrey, Shawn M. C. Robinson and Myriam A. Barbeau
Effect of macroalgal mats on burial depth of soft-shell clams............ 305
Leslie Banigan
Protecting and restoring commercial shellfish beds in Kitsap County, Washington............ 305
Helen A. Beadman, M. J. Kaiser and R. I. Willows
Increasing mussel shell thickness by relaying to improve resistance to crab predation 306
Brian F. Beal
Public stock enhancement of soft-shell clams, *Mya arenaria*, in Maine, USA: The role of community-based aquaculture, applied research, and technology transfer in shellfish restoration............ 306
Alan J. Birch, M. W. Luckenbach, P. G. Ross, R. Gammisch, J. Brubaker and J. A. Wesson
Design and construction of subtidal oyster reefs: Evaluating effects of reef scale 306
C. Boldt
The muck stops here a community decides............ 307
William T. Collins
Acoustic seabed classification for shellfish habitat mapping 307
Paul Cormier, T. Landry and J. F. Mallet
Oyster population restoration in Caraquet, N.B.; Phase II, Optimizing productivity 307
A. Craig and C. Downs
Abstract theme area............ 308
Dale Diaz, K. Caevas and W. S. Perret
Side scan sonar as a management tool in Mississippi 308
Craig A. Downes, Charles E. Robinson, Arnold Huang, Gary Shigenaka and John E. Fauth
Cellular-physiological assessment of bivalves after ten years of chronic exposure to spilled Exxon Valdez crude oil using a Noval Molecular Diagnostic biotechnology 308
Brett Dumbault, D. Armstrong, G. Hosack and B. Semens
Oyster aquaculture as fish habitat in Pacific Northwest coastal estuaries 309
John William Ewart, J. Alderman and K. Maull
Demonstration and evaluation of commercially important bivalve culture and stock enhancement methods in Rehoboth and Indian River Bay, Delaware (1998–2001)............ 309
K. G. Field
Molecular detection of fecal *Bacteroides* as source indicators for fecal pollution in water 309
Gef Flimlin and Michael P. Stringer
Habitat restoration and shellfish stock enhancement initiatives in New Jersey............ 309
Stuart D. Glasoe
New directions for shellfish protection in Puget Sound 310
Heather Goble
Semiahmoo Bay Water Quality Project: Phase 1 findings............ 310
William Goldsborough, Stew Harris, D. Jackson, D. W. Meritt and S. Tobash
Remote-set alternative substrate on leased ground: An innovative approach to reef restoration 311
Kimberly A. Hamilton, D. LaDon Swann, Richard K. Wallace, Yolanda J. Brady, David B. Rouse, Scott Rikard and Holly Hall
Restoration of the eastern oyster (*Crassostrea virginica*) through a volunteer program in Mobile Bay, Alabama........ 311
Matthew Hardy, T. Landry and A. Boghen
Reproductive sanctuary for the northern quahaug, *Mercenaria mercenaria*, in Prince Edward Island 311
J. Hetrick
Status of shellfish enhancement in Alaska............ 311

Barbara Joughin and William Heath
Innovative community processes produce integrative management tools 311
Brian Kingzett
Advances in shellfish safety assurance and participation in sanitary growing water monitoring programs by the BC shellfish aquaculture industry 312
Brian Kingzett
Development of intensive deepwater oyster culture systems in British Columbia 312
K. E. Landrum, K. M. St. Pe, B. Ache and F. Kopfler
Using innovative stormwater controls for water quality improvements and oyster habitat restoration in the Barataria-Terrebonne National Estuary 312
Kevin L. LeBlanc
Water quality improvement: A focus on new technologies 313
A. T. Leggett, A. Blaw, W. Goldsborough and R. D. Brumbaugh
Commercial-scale oyster production for reef restoration and stock enhancement 313
Donald Lennartson
Protection and restoration: Washington State shellfish program 313
B. G. Lucas and A. Campbell
Abalone restoration through collaborative partnerships in British Columbia 313
B. G. Lucas, A. Campbell and D. Brouwer
Site assessment, selection and monitoring methods for abalone restoration near Kitkatla, British Columbia 314
Mark W. Luckenbach, J. Nestlerode, P. G. Ross and A. J. Birch
Effects of reef architecture and scale on fish utilization of oyster reefs in Virginia 314
Thomas B. McCormick
The role of aquaculture in the restoration effort to save white abalone (*Haliotis sorenseni*) in California 314
Jim McIsaac
Boat waste and shellfish closures: A comparison of Canadian and United States policies 315
C. W. McKindsey
Succession in mussel communities: The importance of what is measured 315
Eric McLay
An archeological perspective on the culture significance of shellfish resources to First Nations in the Georgia Basin... 315
Donald W. Meritt, S. Tobash, C. S. Frentz, W. Goldsborough and S. Reynolds
Restoring oysters to the Chesapeake Bay: A coordinated effort involving government universities, community groups, and industry 315
Donald W. Meritt, S. Tobash, K. T. Paynter and T. Koles
Oyster restoration in Chesapeake Bay: I. Criteria for site selection 316
C. A. Milbury and P. M. Gaffney
Genetic monitoring of oyster stock enhancement in the Choptank River, Chesapeake Bay, Maryland, USA 316
Rob Miller
Open Saanich Inset shellfish beds: A collaborative approach 316
Marc Ouellete and T. Landry
Hydroacoustic seabed classification technology applied in shellfish productivity research 317
Paynter, Kennedy and Elizabeth Flynn
The living veneer: Characterizing habitat structure created by oysters 317
Paynter, Kennedy, T. Koles, D. Meritt and S. Tabash
Oyster restoration in Chesapeake Bay: II. Impacts of water quality and disease 317
P. G. Ross, M. W. Luckenbach and A. J. Birch
Early community development of oyster reefs in Virginia: Effects of reef scale 318
Rejean Tremblay and T. Landry
Genetic and pathogenic aspects in shellfish restoration of scallop populations 318
Aswani K. Volety, S. Gregory Tolley and James T. Winstead
Water quality and oyster health (*Crassostrea virginica*): An integrated approach to determining habitat restoration potential 318
Pamela Winquist
The nutrient value of shellfish and other traditional foods, their past and current contribution to the diet of First Nations people 319
Melisa C. Wong, M. A. Barbeau, L. A. Grecian, L-A. Davidson, M. Niles and Donna Murray
Predation of juvenile sea scallops (*Placopecten magellanicus*) during seeding trials in the Northumberland Strait 319

BAY SCALLOP (*ARGOPECTEN IRRADIANS*) POPULATION RESTORATION IN FLORIDA, USA: CULTURE VERSUS MANAGEMENT.

W. S. Arnold, Florida Marine Research Institute, Florida Fish and Wildlife Conservation Commission, 100 Eighth Avenue SE, St. Petersburg, FL 33701, USA.

Bay scallops have historically been widely distributed in Florida waters, comprising discrete populations from West Palm Beach on the Atlantic coast to Pensacola in the panhandle. In recent decades, many of those discrete populations have collapsed due to one or a combination of anthropogenic factors. In response to that collapse, recreational and commercial harvest restrictions have been implemented and a culture-based restoration program is underway. The restoration program is experimental in nature and includes a genetic component that is designed to provide information on the relative contribution of cultured scallops to the subsequent year-class.

Preliminary results of this ongoing study suggest that, despite a two-orders-of magnitude increase in mean scallop density within the targeted restoration area, active restoration has contributed little to this recovery. The lineage of fewer than 5% of the subsequent year-class can be traced back to the parental stock that was originally cultured. Instead, it appears that reductions in harvest intensity precipitated by a change in harvest regulations may be the primary contributor to the observed resurgence in scallop populations. Apparently, removal of fishing pressure on already depauperate scallop populations allowed for increased reproductive success and recruitment. If these results are verified through the final year of the study, they indicate that active intervention is not always required to instigate population recovery. Instead, effective and ecologically based management may suffice.

TESTING ALTERNATIVE STRATEGIES FOR ENHANCEMENT OF HARD CLAM (*MERCENARIA* SPP.) POPULATION IN FLORIDA, USA WATERS.

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Although lacking the history of its northern counterparts, the hard clam fishery in Florida became a significant contributor to US clam landings during the 1980s and early 1990s. Most Florida landings have been realized from the Indian River lagoon. In the late 1990s, the clam population in the lagoon almost completely collapsed in response to increased rainfall associated with ENSO. Salinity has since "recovered" to a level suitable for hard clam survival, but the clam population has shown no concomitant recovery. Active intervention may be required to increase clam population abundance to a level at which the fishery is again profitable.

Three methods have been compared for their suitability to increase clam abundance. Spawner transplants involve the harvest of widely dispersed adult clams followed by the release of those clams in a relatively small area. This approach is designed to concentrate reproductively active clams, thereby increasing fertilization efficiency and the production of larval clams. Seeding involves planting various size classes of clams under protected conditions, again with the goal of increasing fertilization efficiency by creating dense clumps of actively spawning clams. Larval injection involves spawning clams in the hatchery, fertilizing the resultant eggs, and releasing the developing embryos within a preselected area of the lagoon. Preliminary results suggest that larval injection can increase abundance of larval clams by several orders of magnitude, but there is as yet no evidence that any of the three strategies has actually led to increased recruitment of hard clams in the lagoon.

EFFECT OF MACROALGAL MATS ON BURIAL DEPTH OF SOFT SHELL CLAMS.

Lise M. Aulfrey,[1] **Shawn M. C. Robinson,**[2] and **Myriam A. Barbeau,**[2] [1]Department of Biology, University of New Brunswick, Bag Service 45111, Fredericton, NB, E3B 6E1, Canada; [2]St Andrews Biological Station, 531 Brandy Cove Road, St Andrews, NB, E5B 2L9, Canada.

Green macroalgal mats are becoming prevalent on important soft-shell clam (*Mya arenaria*) harvesting beaches in southwestern New Brunswick, Canada. We investigated the effect of macroalgal mats on burial depth and body condition of clams in the field (2 sites with high algal cover) and laboratory. In the field, we located patches covered with algae and clear of algae at each site. We measured burial depth by digging trenches and measuring the distance between the anterior edge of the clam and sediment surface. We then collected the clams for body condition analysis. Burial depth was significantly lower for clams under macroalgal mats than for those in clear areas. Clam body condition was generally lower under algae than not under algae. In the lab, clams (4/aquaria) were placed in mud (10 cm deep) and covered either with 0, 2 or 6 cm of macroalgae. Clams under algae de-burrowed after a few days. Clam body condition and various abiotic variables are being measured in the lab and will be analysed in the near future.

PROTECTING AND RESTORING COMMERCIAL SHELLFISH BEDS IN KITSAP COUNTY, WASHINGTON.

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Kitsap County, located in the central Puget Sound area of Washington State, developed a Surface and Storm Water Management (SSWM) Program that successfully identifies and corrects nonpoint pollution affecting shellfish areas. The Program's success is due to:

1. Political Support: In 1992, Washington passed legislation requiring counties to address water pollution sources causing shellfish closures. In 1993, the Health District received local

political support to develop Kitsap County's SSWM Program, a comprehensive four-agency team to address nonpoint pollution.
2. Financial Support: SSWM provides ongoing stable funding for the Health District's Pollution Identification and Correction (PIC) Program.
3. Effective Public Outreach: An effective outreach program was developed to help inspectors gain access to private property.
4. Enforcement Capability: The Health District developed regulations addressing failing on-site sewage systems and improper animal manure management.
5. Standardized Procedures: The Health District uses an objective system of ranking water quality problem areas and has a detailed procedures manual.

In 1996, the State closed a portion of Port Gamble Bay to shellfish harvesting. The Health District completed a PIC project in the watershed. In 1999, the State upgraded the commercial shellfish beds to "approved" status.

Burley Lagoon was closed to shellfish harvesting in early 1999. Due in large part to PIC work conducted by the Health District, one-half of Burley Lagoon was re-opened to shellfish harvest in 2001.

INCREASING MUSSEL SHELL THICKNESS BY RELAYING TO IMPROVE RESISTANCE TO CRAB PREDATION.

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The mussel cultivation industry is currently the fastest expanding and most valuable sector of the shellfish aquaculture industry in the United Kingdom. One of the constraints of the potential continued success and expansion of the industry is an unpredictable supply of seed mussels for relaying. It is suggested that a possible solution to this problem is to lay seed in times of abundant spatfall on high shore areas where they can ongrow. In subsequent years when spatfall is low these mussels can then be transferred further downshore to allow faster growth to marketable size. A further advantage of growth in high shore areas is the development of a thicker shell that aids resistance to crabs predation.

The aim of this study is to determine how the difference in mussel shell thickness achieved by growth at different heights on the shore can be related to resistance to crab predation and how this changes when mussels are relaid at different tidal heights. This has been achieved through an experimental approach relaying mussels from high shore, intertidal and subtidal areas to the intertidal. Shell thickness is determined through direct means and indirectly through crab predation experiments and shell compression. Results show a significant difference between mussels grown at different shore heights and associated differences in shell compression strengths and resistance to crab predation. Mussels grown at lower shore levels initially displayed thinner shells, of lower compression strength and were preferentially predated upon by crabs.

PUBLIC STOCK ENHANCEMENT OF SOFT-SHELL CLAMS, *MYA ARENARIA*, IN MAINE, USA: THE ROLE OF COMMUNITY-BASED AQUACULTURE, APPLIED RESEARCH, AND TECHNOLOGY TRANSFER IN SHELLFISH RESTORATION.

Brian F. Beal, Division of Environmental and Biological Sciences, University of Maine at Machias, Machias, ME 04654, USA.

In Maine, USA, the intertidal zone is legally owned by the coastal community adjacent to it. For 50 years, commercially important soft-shell clams, *Mya arenaria*, residing in these tidal flats have been co-managed by communities and the state's marine resources department. Clam landings have been cyclical varying from 3,100 metric tons (t) in 1950 to 658 t in 1959, to 3,500 t in 1977 to 800 t in 1997. Historically, management has been based on the vagaries of natural recruitment and the strength of a particular 0-year class.

Beginning in 1987, the first-ever soft-shell clam restoration program in the US was established through the Beals Island Regional Shellfish Hatchery (BIRSH) whose mission is to enhance Maine's soft-shell clam and other shellfish resources through aquaculture, applied research, technology transfer and public education. BIRSH produces 5–10 million 8–12 mm seed clams annually, and, over the years, these cultured individuals have been planted in approximately 50 of the state's 100 coastal communities that manage their shellfish beds.

BIRSH has been the focal point of Maine's research and outreach on soft-shell clam ecology during the past 15 years and has been done at the request and in conjunction with coastal communities. Research projects have tested the interactive effects of planting date, seed size and density, type and aperture of protective netting and how these factors vary spatially and temporally. Generally, seed clams (>10 mm) should be planted during April, at the beginning of the growing season, and protected with flexible netting (6.4 mm).

DESIGN AND CONSTRUCTION OF SUBTIDAL OYSTER REEFS: EVALUATING EFFECTS OF REEF SCALE.

Alan J. Birch, M. W. Luckenbach, P. G. Ross, R. Gammisch, and **J. Brubaker,** College of William and Mary, Virginia Institute of Marine Science, and **J. A. Wesson,** Virginia Marine Resources Commission, USA.

Restoration of oyster reef habitat in mid-Atlantic estuaries is requisite for restoring oyster fisheries and recovering lost ecological services. Recent evidence suggests the importance of establishing proper vertical relief and interstitial space in the initial placement of substrate. A third component of reef architecture that has yet to be investigated is the aerial extent or scale of reefs. We are currently evaluating the effects of scale on various components of communities that develop on high relief reef bases ranging in size from 400 m^2 to 8000 ^{2}. As a result, accurate physical descriptions of the constructed reefs are important to meaningfully quantify reef scale.

We present some preliminary design and construction aspects of three dimensional subtidal reefs in the Rappahannock River, a tributary of the Chesapeake Bay. Because of their subtidal nature, describing the reefs physical characteristics becomes difficult. Subsequently, Side-scan sonar mapping, bathymetry surveys and current velocity mapping were conducted during the first year after construction and will be continued in subsequent years as the reefs develop.

From a management aspect, where potential substrate is limiting and expensive, any ecological advantages or disadvantages derived from allocating resources to many small vs. several large reefs can have direct economic impacts on restoration efforts. Furthermore, identification of details of the physical characteristics that may help achieve restoration goals can be extremely important in providing resource managers with the information that can aid future reef design criteria.

THE MUCK STOPS HERE A COMMUNITY DECIDES.

C. Boldt, Union Bay Liquid Waste Management Committee, P.O. Box 32, Union Bay, British Columbia, Canada, V0R 3B0.

What does an ocean side community do when:

- residents cannot safely harvest shellfish from its beaches for over 7 years?
- a quiet, community walk lets you know who is washing clothes, who is having a shower, who has flushed the toilet?
- its ditches are unsafe for child's play
- its ditches are overgrown with water cress costing $1000's of dollars to government to clean out?
- kids swimming on local beaches can be a health issue?
- over 60% of septic fields are failing?

Well, one community said "Enough is Enough!" While its neighbour were saying NO to a 1997 valley wide referendum on a solution to liquid waste problems, Union Bay said "Yes".

Union Bay has worked hard to involve, inform, educate and enlist residents, local credit union, local businesses, politicians and government agencies from all levels to develop a solution.

Working with major funding from Georgia Basin Ecosystem Initiative, Environment Canada and provincial grants, Union Bay has developed a liquid waste treatment solution which will ultimately turn waste to wealth, using liquid waste, including a campus for post-secondary institutions to support their environmental program, enhance salmonid habitat, provide an interpretive centre.

Community process has been the key to significant funding for this project and it has created a climate of acceptance and support for rural grassroots, community-based planning. This process has been accepted by the Regional District of Comox-Strathcona and adopted in at least three other rural communities in the Comox Valley.

ACOUSTIC SEABED CLASSIFICATION FOR SHELLFISH HABITAT MAPPING.

William T. Collins, Quester Tangent Corporation, Sidney, BC V8L 5Y8, Canada.

The amplitude and shape of an acoustic signal reflected from the sea floor is determined mainly by the sea bottom roughness, the density difference between water and the sea floor, and reverberation within the substrate. Remote classification of the sea bottom requires an acoustic data acquisition system and a set of algorithms that analyze the data, determine the seabed type and relate the results of the acoustic classification to the physical properties of the marine sediments. Quester Tangent Corporation has successfully combined high-speed digital signal processing technology with multivariate statistical analysis to create the QTC VIEW seabed classification engine. The QTC VIEW captures and digitizes the seabed echo from a conventional echo sounder, processes, analyses, displays and records acoustic waveform data characterizing the sea floor. The output is a set of point data representing sea floor acoustic diversity. The georeferenced classifications can then be objectively correlated with other data such as sediment properties or directly to fish census.

The technology has been applied world wide to map marine sediments for a variety of applications. It has been used for mapping substrate in support of benthic habitat characterization. The technology can also be configured for use in water depths less than 5 m. Examples of oyster mapping projects in West Galveston Bay, Texas and Chesapeake Bay, Maryland are highlighted. Strategies for the integration of acoustic and nonacoustic data are included.

OYSTER POPULATION RESTORATION IN CARAQUET, N.B.; PHASE II, OPTIMIZING PRODUCTIVITY.

Paul Cormier, T. Landry, and **J. F. Mallet.** Department of Fisheries an Oceans, GFC, P.O. Box 5030, Moncton N.B., E1C 9B6, and Department of Agriculture, Fisheries and Aquaculture, 22 Boul. Saint-Pierre, Caraquet, N.B. E1W 1B6, Canada.

A decrease in the productivity of oysters in Caraquet Bay, N.B. is generating some interest in restoration projects. This bay represents the most northern location with a sustainable oyster (*Crassostrea virginica*) population. The first phase of this initiative provided invaluable information on the distribution, abundance and population structure of oyster beds. Over 60% of the oysters found in this bay are pre-recruits to the fishery (35–75mm). These oysters are mainly found in the northern portion of the main bed, which has a relatively low abundance of commercial size oysters (>75mm) and. is locally renown for its "stunted growth oysters". Samples of oysters from this location where transferred at four stations in the bay and monitored for growth and conditioning. Results are suggesting that the growth limitation of these oysters is associated with the geographical location and even more to the bottom conditions. Management strategies will be developed to optimize the productivity of oyster from this highly successful recruitment location, which offers limited possibility for commercial production.

ABSTRACT THEME AREA: A. Craig, and **C. Downs,** EnVirtue Biotechnologies, Inc. 2255 Ygnacio Valley Rd., Suite H-1, Walnut Creek, CA 94598, USA.

Using the tools of proteomics and molecular diagnostics, one of our primary goals is to determine if environmental factors are affecting shellfish susceptibility to infections. This strategy of research can also aid in answering other important issues to shellfish husbandry such as why some shellfish cultivars or strains are more susceptible to infection than others and the cellular mechanisms responsible for disease resistance? Proteomics and functional genomics can be used to discover novel and appropriate molecular biomarkers that can aid in diagnosing the health status of a crop, develop powerful biomarkers to aid in predicting crop yield, and aid in developing genetic markers for advantageous cultivar traits. Finally, understanding the cellular and biochemical mechanisms of shellfish disease may allow for the development of practical measures or treatments to limit disease.

SIDE SCAN SONAR AS A MANAGEMENT TOOL IN MISSISSIPPI. Dale Diaz, K. Cuevas and **W. S. Perret,** Office of Marine Fisheries, Mississippi Department of Marine Resources, 1141 Bayview Avenue, Suite 101, Biloxi, MS 39530, USA.

Oyster harvest from Mississippi reefs provide jobs for numerous fishermen and contribute substantially to the economy. Proper management of these reefs is vital for continued harvest. Enhanced management techniques (i.e. cultch planting) should help to ensure even greater future production. Cultch planting is a key component of management measures conducted by the Mississippi Department of Marine Resources (MDMR). The use of sounding poles, tongs, dredges or scuba equipment gauged the effectiveness of different cultch planting methods. These methods are not efficient techniques when surveying large areas for coverage rates or distribution of materials.

Using side scan sonar to monitor cultch plants has a number of advantages over these previously used methods. Side scan sonar can cover large areas in less time, provide accurate measurements of coverage, and simultaneously provide differential GPS coordinates. The information acquired from side scan sonar provides a long-term record, which can be used to detect changes due to environmental catastrophes, vessel groundings and harvest pressure. A side scan sonar survey was performed on two cultch plant sites located in the western MS Sound, St. Joe Cultch Plant and Pass Christian Cultch Plant. The St. Joe Cultch Plant site was surveyed before and after cultch planting. On the Pass Christian Cultch Plant only a post side scan sonar survey was conducted. The data was processed and input in a mosaic software program for further analysis. This study will demonstrate the abilities of side scan sonar as a management tool for oyster reef management.

CELLULAR-PHYSIOLOGICAL ASSESSMENT OF BIVALVES AFTER TEN YEARS OF CHRONIC EXPOSURE TO SPILLED EXXON VALDEZ CRUDE OIL USING A NOVAL MOLECULAR DIAGNOSTIC BIOTECHNOLOGY. Craig A. Downes,[1] Charles E. Robinson,[1] Arnold Huang,[1] Gary Shigenaka,[2] and **John E. Fauth,[3]** Envirtue Biotechnologies, Inc., 2255 Ygancio Valley Rd., Suite H-1, Walnut Creek, CA 94598, USA; [2]Hazardous Materials Response Division, US National Oceanic and Atmospheric Administration, 7600 Sand Point Way NE, Seattle, WA 98115, USA; [3]Department of Biology, University of Charleston, 66 George Street, Charleston, SC 29422, USA.

The objective of this study was to determine the cellular physiological status of the bivalves *Mya arenaria* and *Mytilus trossulus* in an area impacted by a ten-year chronic exposure of spilled Exxon Valdez crude oil in Prince William Sound.

Bivalves were collected from a well characterized impacted site on Knight Island and from a nonimpacted site north of Bainbridge Island. We used a novel biotechnology known as the Envirtue Molecular Diagnostic System™ (EMDS) to determine if bivalves were (1) physiologically stressed, (2) the nature of the altered physiological state, and (3) whether the organisms were responding specifically to an exposure to polyaromatic hydrocarbons (PAH). Molecular diagnostic analysis indicated that bivalves at the impacted site were stressed, experiencing both an oxidative and xenobiotic stress, resulting in increased protein turnover and chaperone activity. Further, bivalves from the impacted area were responding specifically to a PAH xenobiotic exposure and accumulating DNA-PAH adducts. Finally, species-specific responses were observed that were related to the spatial-habitation characteristics of each species.

We conclude that bivalves inhabiting an area impacted by crude oil from the Exxon Valdez spill of 1989 are still adversely affected by the spill's remnants.

OYSTER AQUACULTURE AS FISH HABITAT IN PACIFIC NORTHWEST COASTAL ESTUARIES. Brett Dumbault,[1] D. Armstrong,[2] G. Hosack,[2] and B. Semens,[2] [1]Washington State Department of Fish and Wildlife, Willapa Bay Field Station, P.O. Box 190, Ocean Park, WA 98640, USA; [2]School of Aquatic and Fishery Sciences, Box 355020, University of Washington, Seattle, WA 98195, USA.

Increased pressure on traditionally managed stocks of marine and anadromous fish, calls for protection of essential fish habitat under the *Magnuson-Stevens Act,* and recent listings of several salmonid stocks under ESA have brought aquaculture activities that take place in coastal estuaries under increased public scrutiny.

We initiated a study designed to examine the ecological role that oyster aquaculture plays as habitat in coastal estuaries of the Pacific Northwest. The goal of the project is to identify and quantify beneficial and adverse impacts of shellfish farming on eelgrass, juvenile salmonids, and other selected estuarine fauna and flora and to develop farming practices and recommend management protocols that protect or enhance those resources. We present results of initial fish and invertebrate sampling in selected habitats from Willapa Bay during Spring and Summer 2001 and an experiment designed to examine the effects of oyster harvesting on eelgrass habitat. We make a plea to managers to consider oyster aquaculture areas as fish habitat on a broader estuarine scale.

DEMONSTRATION AND EVALUATION OF COMMERCIALLY IMPORTANT BIVALVE CULTURE AND STOCK ENHANCEMENT METHODS IN REHOBOTH AND INDIAN RIVER BAY, DELAWARE (1998–2001). John William Ewart,[1] J. Alderman[2], and K. Maull,[2] [1]Delaware Sea Grant Marine Advisory Service, Graduate College of Marine Studies, University of Delaware, 700 Pilottown Road, Lewes, DE 19958, USA; [2]Delaware Center for the Inland Bays, 467 Highway One, Lewes, DE 19958, USA.

Delaware's coastal lagoons, known locally as "inland" bays, have been experiencing the impacts of chronic eutrophication and sediment erosion resultant from several decades of sustained nutrient input and development from within the surrounding watershed. The Delaware Center for the Inland Bays (CIB) was established as a nonprofit organization in 1994 to oversee the implementation of the Inland Bays Comprehensive Conservation and Management Plan and to facilitate a long-term approach for the wise use and enhancement of the watershed. The James Farm Ecological Preserve, a 150 acre property with frontage on Indian River Bay, was established in 1998 to serve in part as a demonstration site for beneficial land use practices and similar watershed based activities such as shoreline stabilization, habitat creation/protection and shellfish stock enhancement to improve water quality. In 1998, the CIB initiated an ongoing program to evaluate and demonstrate the technical feasibility of using aquaculture methods to maintain or improve populations of commercially viable bivalves and to create additional habitat for shellfish and other species. Current field demonstration activities include low density plantings (1–2/square foot) of hard clams (*Mercenaria mercenaria*), for stock enhancement and recreational fishery improvement; monitoring oyster growth, survival and prevalence of MSX and Dermo disease in eastern oysters (*Crassostrea virginica*) held in off-bottom transient gear, submerged trays and Taylor floats; construction and deployment of a Floating Upweller System (FLUPSY) for nursery culture of bivalve seed; and establishment of a 1/4 acre oyster reef to monitor growth, survival, disease prevalence and habitat development.

MOLECULAR DETECTION OF FECAL *BACTEROIDES* AS SOURCE INDICATORS FOR FECAL POLLUTION IN WATER. K. G. Field, Oregon State University, Department of Microbiology, Corvallis, OR 97331, USA.

Fecal contamination of shellfish environments afflicts many regions worldwide, and carries numerous risks to human health. Fecal bacteria, pathogenic protista and viruses may be highly concentrated in shellfish. Often the problem can't be corrected, because standard methods of measuring fecal pollution in water do not distinguish its source. We have developed a PCR-based alternative method of fecal source discrimination. The method consists of amplifying 16S rRNA gene fragments of the fecal anaerobic *Bacteroides-Prevotella* group with specific primers. Because *Bacteroides* is restricted to gut habitats, its presence in water indicates fecal pollution. Molecular detection circumvents the complexities of growing anaerobic bacteria. We identified *Bacteroides* length-heterogeneity PCR (LH-PCR) and terminal restriction fragment length polymorphism (T-RFLP) ribosomal DNA markers unique to either cow or human feces, and recovered the same unique fecal markers out of polluted natural waters. We cloned and sequenced the markers and used the sequences to design PCR primers that reliably discriminate human and ruminant sources of fecal contamination. New primers under development will distinguish elk/deer, horse, pig, chicken, duck/goose, beaver, and harbor seal fecal pollution. With this approach, sensitivity is greater than fecal coliform assays. The method does not require culturing bacteria, allows for small sample size and easy sample handling, is comparable in complexity to standard tests carried out in food safety and public health diagnostic labs, and lends itself to automation and high-throughput.

HABITAT RESTORATION AND SHELLFISH STOCK ENHANCEMENT INITIATIVES IN NEW JERSEY. Gef Flimlin,[1] and Michael P. Stringer,[2] Rutgers Cooperative Extension, 1623 Whitesville Rd., Toms River, NJ 08755, USA; [2]NY/NJ Baykeeper, Bldg. 18, Sandy Hook, Highlands, NJ 07732, USA.

New Jersey has been a major shellfish production state since colonial times. The Raritan Bay area once boasted a 400-boat oyster fleet from the town of Keyport alone. However, industrial pollution, water quality degradation, and the onset of MSX and Dermo have practically eliminated natural oyster stocks in most areas.

Efforts over the past three years have lead to the establishment of two reefs, one on the Liberty Flats near the Statue of Liberty and one off the shore of Keyport. These shellstocked reefs have had cultured oyster seed placed on them and a monitoring program has begun. Another effort is underway in the Mullica River, the last area on the state's Atlantic Coast side to produce oysters, to restock the public beds with natural oysters from the seedbeds upriver. This area will be proposed for a larger scale project for a Community Based Restoration Program in the year to come.

The Baykeeper and Rutgers Cooperative Extension have been working with the Baymen's Protective Association and the New Jersey Shellfisheries Association, two shellfish industry groups, to propel these initiatives. Primary and secondary school education projects explaining the benefit of oyster restoration and aquaculture are being done to garner more support for the projects. It is envisioned that eventually the industry will reap the benefit of these projects when the reefs may be harvested in the future.

NEW DIRECTIONS FOR SHELLFISH PROTECTION IN PUGET SOUND. Stuart D. Glasoe, Office of the Governor, Puget Sound Water Quality Action Team, PO Box 40900, Olympia, WA 98504, USA.

The Puget Sound Water Quality Management Plan serves as a cornerstone for shellfish protection in Puget Sound. First adopted in 1987, the plan employs a suite of strategies to raise awareness, change behaviors and build and strengthen systems to protect the health of Puget Sound.

Although the plan was designed with the dual goal of protecting and reopening shellfish beds, early trends were daunting. Between 1987 and 1989 alone, nearly 33,000 acres of commercial beds were downgraded as a result of declining water quality and more intensive monitoring. Trends shifted dramatically in the 1990s as restoration efforts successfully upgraded over 13,000 acres, offsetting nearly identical acreages downgraded during the period. These results show that the strategies are paying off even in the face of rapid population growth, but they also reveal an unsustainable emphasis on reactive, restoration techniques.

The region must now shift gears and make better use of proven approaches to more effectively and permanently prevent contamination of shellfish growing areas. These include better land use planning under the Shoreline Management Act and Growth Management Act to preserve shellfish areas as critical areas and natural resource lands; greater use of pollution prevention measures such as low impact development practices and on-site sewage maintenance programs; widespread creation of surface water utilities to fund local services and projects; early detection and rapid response to emerging water quality problems; and better communication with all audiences to raise awareness on the impacts and tradeoffs associated with pollution and urbanization in shellfish watersheds.

SEMIAHMOO BAY WATER QUALITY PROJECT: PHASE 1 FINDINGS. Heather Goble, Shellfish Project Coordinator, Georgia Basin Ecosystem Initiative, Clean Water Action Plan, 224 West Esplanade, North Vancouver, BC, V7M 3H7, Canada.

We have now completed Phase 1 of a three-year action plan to identify the major sources of contamination to Boundary Bay and propose subsequent actions required to mitigate and remediate the prohibited status of the shellfish growing waters in this area. The objective of Phase 1 of the Semiahmoo Bay Water Quality Project was to characterize non-point source contamination patterns within Semiahmoo Bay. We sampled 19 beach locations along the shoreline as well as 32 natural and manmade storm water drainages into Semiahmoo Bay.

Precipitation levels had a positive correlation with bacteriological counts for both outfall and ocean sampling sites. This indicates contamination in storm water outfalls had a direct impact on water quality along the shoreline of Semiahmoo Bay. Ocean sites demonstrated fecal coliform counts that consistently exceeded acceptable standards for shellfish growing waters and occasionally exceeded accepted standards for recreational water quality. The majority of outfalls had fecal coliform counts that occasionally reached values associated with sewage-impacted waters.

Boundary Bay sediments and shellfish were tested for common metals and organics that may adversely affect growing areas. The levels of heavy metal residues and organochlorines in shellfish were found to be within acceptable limits. However the tests for lead and arsenic levels were inconclusive and the shellfish will be retested using different parameters.

Phase 2 of the Semiahmoo Bay Water Quality Project will conduct contaminant investigations to reduce the impact of contaminated drainage systems on the shellfish growing waters of Semiahmoo Bay.

REMOTE-SET ALTERNATIVE SUBSTRATE ON LEASED GROUND: AN INNOVATIVE APPROACH TO REEF RESTORATION. William Goldsborough,[1] Stew Harris,[1] D. Jackson,[1] D. W. Meritt,[2] and S. Tobash,[2] [1]Chesapeake Bay Foundation, 6 Herndon Avenue, Annapolis, MD 21403, USA; University of Maryland, Center for Environmental Science, Box 775, Cambridge, MD 21613, USA.

Oyster reef restoration in Chesapeake Bay faces a variety of challenges that will require new and innovative approaches. Among the challenges are: identifying sanctuary locations, obtaining suitable substrate, establishing oysters on the substrate, and protecting the site from poaching.

Thousands of acres of Bay bottom are currently under lease from the states of Maryland and Virginia. Much of this ground is not being actively farmed and may be available for establishing reef sanctuaries under agreement with leaseholders.

Oyster shell is considered the best reef material, but supplies of shell are dwindling. The scale of reef restoration now anticipated will require development of alternative materials. Marine limestone rock (marl) has been shown to be a good setting substrate and may prove to be a valuable substitute for shell in reef construction. A second valuable function that marl may provide is that of a physical obstruction to poaching with traditional harvest gear.

In a pilot project undertaken in the Nanticoke River, a tributary of Chesapeake Bay in Maryland, marl rocks were set remotely with hatchery-produced larvae and distributed on a one-acre area of leased ground to create a field of mini-reefs. In the process a new and promising approach to reef sanctuary creation was developed.

RESTORATION OF THE EASTERN OYSTER (*CRASSOSTREA VIRGINICA*) THROUGH A VOLUNTEER PROGRAM IN MOBILE BAY, ALABAMA.

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Restoration programs for the eastern oyster, *Crassostrea virginica*, in Mobile Bay, Alabama are used to increase the number of oysters on remaining beds and to renew ecological functions such as providing habitat and water filtration. To educate the general public of the ecological importance of oysters and oyster restoration efforts, an oyster gardening program was created for restoration of natural and constructed reefs. The volunteer program was implemented in Mobile and Baldwin counties along Mobile Bay and monitored for 7 months beginning in May 2001.

A total of 30 volunteers representing sites located in unrestricted oyster harvesting waters were provided a Taylor float and 1,000 oyster spat per volunteer. The 36.2 mm spat were provided to the first 15 volunteers and 12.5 mm spat were provided to a second group of 13 volunteers. Spat were stocked into the Taylor float (surface area of 0.74 m^2) and tied to a pier, piling, or weight. Growth and condition of oysters in Taylor floats were compared and correlated with water quality parameters (temperature, dissolved oxygen, salinity, and water clarity) between each site.

REPRODUCTIVE SANCTUARY FOR THE NORTHERN QUAHAUG, *MERCENARIA MERCENARIA*, IN PRINCE EDWARD ISLAND.

Matthew Hardy,[1] **T. Landry,**[2] and **A. Boghen,**[1] Université de Moncton, Moncton, N.-B., E1A 3E9; [2]Department of Fisheries and Oceans Canada, Science Branch, Maritime Region, Moncton, N.-B., E1C 9B6, Canada.

West River was historically one of the most productive estuaries for quahaugs in PEI. However, due to the construction and subsequent removal of a causeway, in addition to extensive fishing, stocks are now considered to be too low to sustain the present level of commercial harvesting. Density estimates in West River ranged from 1.37 to 2.52 quahaugs · m^{-1}, which are low in relation to other quahaug producing areas.

Several years of research lead to the establishment of a reproductive sanctuary for large quahaug in the subtidal zone of West River. This was based on results showing that reproductive success could be optimized through low-level and inexpensive interventions. Large quahaugs are advantageous as broodstock because they have high fecundities and they are not significantly affected by high stocking densities. *In vitro* fertilization experiments showed that reproductive success increased significantly with higher gamete concentration and lower gamete ages. These related directly to the distance between a spawning pair of adults. The subtidal zone also provides ecological and socio-economic benefits.

This type of enhancement offers certain advantages over hatchery produced seeding programs. Preliminary work tends to indicate that the sanctuary concept could be an effective restoration or enhancement technique for the quahaug. Plankton tows showed a greater presence of quahaug larvae in West River, following the establishment of the sanctuary, compared to previous years and other estuaries. Ultimately, it might be possible to enhance overall productivity to sustainable levels through properly managed broodstock areas.

STATUS OF SHELLFISH ENHANCEMENT IN ALASKA.

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The Alaskan shellfish aquatic farm industry has been growing steadily with an increase in the number of farms and an instate shellfish hatchery for production of indigenous species. Recent attempts by native villages and local communities to enhance beaches for subsistence and recreational groups have been mired in bureaucratic gridlock. At issue is a directed use of a public resource, management of enhanced stocks and their interaction with standing stocks and the lack of enabling legislation for shellfish enhancement. An outline and review of projects completed and underway will be presented.

INNOVATIVE COMMUNITY PROCESSES PRODUCE INTEGRATIVE MANAGEMENT TOOLS.

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Baynes Sound, located in western Canada on the east coast of Vancouver Island, is a highly productive area for shellfish culture, salmon, herring and waterbirds. Over the past decade, Baynes Sound has experienced increasingly degraded water quality from non-point source pollution, as well as loss and degradation of sensitive wild habitat. The Baynes Sound Round Table was formed in 1994 to improve water quality in Baynes Sound, and has guided a series of collaborative community stewardship projects that address threats to environmental and economic health in Baynes Sound. These projects create opportunities for citizens, governments and shellfish growers to work together to identify and remediate non-point source pollution impacts in Baynes Sound. Hundreds of citizen volunteers have participated in recent stewardship programs. Community involvement and stakeholder collaboration have established a strong foundation for proactive and integrated remedial planning and programming. An important tool has evolved from the Baynes Sound process - the development of the 'State of the Sound' Program. The State of the Sound Program is a long term monitoring, reporting and planning process that measures and reports the health of Baynes Sound. The program uses a geographic information system (GIS) to gather and analyse data for water quality and other indicators. Results are used to plan actions, and are communicated to the community to help increase public awareness and involvement. Information becomes accessible, 'crisis control' is replaced with comprehensive management, and an effective tool is available to assist with planning processes such as local liquid waste management programs.

ADVANCES IN SHELLFISH SAFETY ASSURANCE AND PARTICIPATION IN SANITARY GROWING WATER MONITORING PROGRAMS BY THE BC SHELLFISH AQUACULTURE INDUSTRY.

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The BC Shellfish Growers Association (BCSGA) represents the majority of shellfish production in British Columbia. Promoting shellfish safety and involvement in the growing water management have become significant aspects of the work of the association and its members. Decreases in regulatory monitoring budgets and increased demands for service by shellfish aquaculturists have led to proactive industry partnerships and programs. These involve industry participation in monitoring for *Vibrio parahaemolyticus*, an industry driven farm based *Vp* control program, involvement in sanitary growing water monitoring and remediation, and maintenance of marine biotoxin sentinel sites. Currently the BCSGA is participating in a national program developing standard methodologies and curriculum for industry growing water sampling and farm based HACCP for shellfish culturists.

DEVELOPMENT OF INTENSIVE DEEPWATER OYSTER CULTURE SYSTEMS IN BRITISH COLUMBIA.

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Socio-political reasons have restricted the growth of the shellfish culture industry in British Columbia during the last decade. The industry is relatively small and recent growth has primarily come about on small deep-water sites. Shellfish growers in British Columbia have developed a series of approaches to developing intensive oyster production in deep-water (off bottom) culture systems. This has involved adapting international techniques and developing unique technologies, which allow significant production from small areas.

USING INNOVATIVE STORMWATER CONTROLS FOR WATER QUALITY IMPROVEMENTS AND OYSTER HABITAT RESTORATION IN THE BARATARIA-TERREBONNE NATIONAL ESTUARY.

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The loss of nearly 22 square miles of emergent wetlands per year in the Barataria-Terrebonne National Estuary represents the imminent loss of a nationally significant wetland resource and threatens the area's unique culture and local infrastructure. Opportunities exist for rerouting some of the estuary's 260 stormwater pump station outfalls within the estuary to improve the water quality associated with these discharges prior to entering historically productive oyster grounds. Runoff from rural and agricultural areas are collected in borrow canals within the existing levee systems and are generally pumped into large man-made canals to ensure rapid evacuation of stormwater.

The Barataria-Terrebonne National Estuary Program and EPA's Gulf of Mexico Program are spearheading efforts to monitor and assess changes in estuarine vitality near rerouted pump station outfalls to demonstrate the benefits of this unique process. Qualitative evidence of the positive effects of the redistribution of stormwater into adjacent wetland areas is visually evident throughout both basins, although only limited quantitative assessment has occurred. Redirecting discharges so that freshwater is retained in adjacent wetlands rather than moved through them has been demonstrated to maintain lower salinities, promote vigorous plant growth through nutrient uptake, and lead to pathogen degradation. Numerous studies provide evidence of wetland uptake of pollutants and nutrients in constructed wetlands and riparian fringes, and the enhancement of marshes adjacent to hurricane protection levees would provide additional storm surge protection for properties and local infrastructure.

WATER QUALITY IMPROVEMENT: A FOCUS ON NEW TECHNOLOGIES. **Kevin L. LeBlanc,** Fisheries and Oceans Canada, Gulf Fisheries Centre, 343 University Street, Moncton, New Brunswick, E1C 9B6, Canada.

Water quality improvement requires effective monitoring, financial support, partnerships, remediation and bio/socio-economic planning. However, tools that clearly define sources of contamination remain the backbone for effective water quality improvement. Sewage is typically measured through the use of an indicator species such as faecal coliform bacteria, but effective remediation requires a clear link to the source of pollution. The South-western New Brunswick Clam Resource Committee (CRC), New Brunswick, Canada, has improved water quality and monitoring for the reclassification of shellfish growing areas since 1995 using the above mentioned strategies. By 2001, the CRC has increased the overall shellfish growing area by 27%. However, an additional 38% of shellfish growing areas, equivalent to 434 hectares (1,074 acres), could be available for harvest if the identification of the actual source of fecal coliform was possible. Tools such as genetic markers and gene sequencing of bacteria are examples of new approaches in differentiating between different sources of fecal coliform. These innovations will be critical in establishing feasible and effective courses of remediation in shellfish growing areas. Furthermore, such advances could lead to the development of molecular-based kits for monitoring activities, similar to those used for biotoxin monitoring. The continued development of science in these fields is necessary for the continued success of water quality improvement initiatives.

COMMERCIAL-SCALE OYSTER PRODUCTION FOR REEF RESTORATION AND STOCK ENHANCEMENT. **A. T. Leggett, A. Blow, W. Goldsborough, R. D. Brumbaugh,** Chesapeake Bay Foundation, 142 W. York Street, Suite 318, Norfolk, VA 23510, USA.

Oyster restoration in Virginia has focused on the construction of three-dimensional reefs made from oyster shells since the early 1990s. Since 1997, the Chesapeake Bay Foundation (CBF) has assisted with his effort by organizing and training citizens and students to grow oysters (*Crassostrea virginica*) using small-scale aquaculture techniques. To date, approximately 1.1 million oysters have been grown using these small-scale techniques, and stocked on Virginia Sanctuary reefs.

In 2000, CBF initiated the Virginia Oyster Aquaculture Program to complement these volunteer-based efforts by producing an additional one million oysters per year for Virginia reefs. A commercial-scale oyster farm was designed and put into operation growing oysters in ADPI mesh bags contained in commercial oyster trays stacked on PCV racks. The oysters were placed directly in the trays when they reached an appropriate size (>25 mm) with approximately 1,500 oysters per tray. After eleven months, over 930,000 oysters (mean size = 52 mm) were harvested using volunteer labor and transplanted onto eight sanctuary reefs. Survival to transplanting for the three stocks used in the program was approximately 85%, and first year capital expenses were approximately $62,400. The oysters produced through this program were used as in-kind match for newly appropriated federal funds dedicated to Virginia oyster restoration. Future considerations of the program include genetic aspects of the oyster stocks used and the application of new techniques including the use of a floating upweller (FLUPSY) for the nursery phase of the grow-out.

PROTECTION AND RESTORATION: WASHINGTON STATE SHELLFISH PROGRAM. **Donald Lennartson,** Office of Food Safety and Shellfish Programs, Washington State Department of Health, 7171 Cleanwater Lane, Building 4, Olympia, WA 98504, USA.

The Washington State Department of Health oversees 126 shellfish growing areas with over 1,400 marine water sampling stations. The state shellfish industry produces over 80% of the shellfish harvested on the west coast, not including geoducks. This rich shellfish resource faces a range of threats, from rural land use activities to rapid urban growth. Prevention of classification downgrades and the restoration of water quality require careful planning, strong technical skills, tough regulatory tools, and creative partnerships among local, tribal, state, and federal agencies.

The stimulus for shellfish restoration activities was the innovative Puget Sound Water Quality Management Plan, whose purpose is "to restore and protect the biological health and diversity of Puget Sound". A major component of this ongoing Plan is the Shellfish Protection section, which outlines seven action elements, the first two of which address shellfish protection policy and the restoration of commercial shellfish beds.

In keeping with the spirit of the Plan, the Department of Health initiated an Early Warning System, a proactive measure which alerts county governments to the threat of classification downgrades. If preventative measures fail and a downgrade does occur, state law requires the county to establish a Shellfish Protection District, and a Closure Response group is convened to formulate actions in the watershed to identify and correct the pollution sources.

ABALONE RESTORATION THROUGH COLLABORATIVE PARTNERSHIPS IN BRITISH COLUMBIA. **B. G. Lucas,** and **A. Campbell,** Stock Assessment Division, Science Branch, Fisheries and Oceans Canada, Pacific Biological Statio, Nanaimo, BC V9R 5K6, Canada.

Diverse stakeholder groups are collaborating to test potential methods to restore threatened abalone populations in British Columbia. Fisheries and Oceans Canada is working with six groups throughout coastal BC in an attempt to halt further decline of abalone stocks and restore populations to self-sustaining levels.

Five aquaculture projects are underway to develop the culture technology required to provide northern abalone seed for pilot restocking experiments. In addition, four projects are currently promoting local community stewardship of abalone resources through public education, increased awareness and, in some cases, experimental manipulations of wild abalone populations. These projects are providing training and employment in local communities which have been impacted by declining opportunities in the fishing industry.

SITE ASSESSMENT, SELECTION AND MONITORING METHODS FOR ABALONE RESTORATION NEAR KITKATLA, BRITISH COLUMBIA. **B. G. Lucas, A. Campbell,** and **D. Brouwer**, Stock Assessment Division, Science Branch, Fisheries & Oceans Canada, Pacific Biological Station, Nanaimo, BC V9R 5K6, Canada.

Several pilot projects are currently underway to attempt to restore abalone populations in British Columbia (BC). Near Kitkatla, on BC's north coast, an extensive process of assessing, selecting and surveying sites for pilot abalone rebuilding experiments was recently completed. General areas for potential experiments were selected in conjunction with local First Nation advisors and Fisheries & Oceans Canada staff.

In September 2000, five divers spent 7 days conducting preliminary surveys to locate specific areas of suitable habitat that met predetermined site selection criteria. After analysis of the survey results, 16 potential sites with similar characteristics were chosen. In April 2001, three dive teams spent 6 days permanently marking 15 sites and collecting detailed baseline information at those sites.

The proposed experiments will test the survival and recapture rates of seeded juvenile abalone while examining the effects of sea urchin sizes and densities in the experimental plots. Long term monitoring of the sites is expected to continue for at least five to ten years after the experiments begin.

EFFECTS OF REEF ARCHITECTURE AND SCALE ON FISH UTILIZATION OF OYSTER REEFS IN VIRGINIA. **Mark W. Luckenbach, J. Nestlerode, P. G. Ross** and **A. J. Birch**, College of William and Mary, Virginia Institute of Marine Science, Eastern Shore Laboratory, Wachapreague, VA 23480, USA.

Current efforts to restore oyster reefs in the Chesapeake Bay are directed toward establishing self-sustaining reef sanctuaries that provide valuable ecological functions, such as benthic-pelagic coupling and support of increased diversity and production of macrobenthos and finfish. Recent evidence has revealed the importance of two components of reef architecture—vertical relief and interstitial space—on the development of oyster populations on restored reefs. We will present data which show that finfish utilization varies between "reefs" with and without viable oyster populations. A third component of reef architecture—aerial extent or scale—is expected to affect both reef development and utilization by fish. In conservation biology this topic has often been characterized as the SLOSS (Single Large or Several Small) debate, but is more generally about optimizing the scale of a bio-reserve or sanctuary to support the desired species. To investigate this issue for oyster reefs we have initiated a large-scale restoration experiment in the Chesapeake Bay. In a replicated block design, we have constructed high relief reef bases ranging in size from 400 sq m to 8000 sq m and are characterizing the development of resident and transient assemblages of organisms on the reefs. We will present data from the first year of the study on finfish utilization of these reefs.

THE ROLE OF AQUACULTURE IN THE RESTORATION EFFORT TO SAVE WHITE ABALONE (*HALIOTIS SORENSENI*) IN CALIFORNIA. **Thomas B. McCormick**, Channel Islands Marine Resource Institute, 323 E. Matilija Street, Ojai, CA 93023, USA.

Stocks of white abalone (*Haliotis sorenseni*) in southern California declined precipitously from an average of 2,000 to 10,000 abalone per hectare in the to 1.6 per hectare in the 1990s. A short-lived fishery in the 1970s landed 270 metric 1970s tons from depths of 20 to 50 m. The fishery was closed in 1997 and in 2001 the white abalone was listed as an endangered species, the first marine invertebrate to be so classified in the USA.

The White Abalone Working Group was formed by federal and state agencies, scientists, universities, non-profit organizations, and mariculturists as a proactive step towards preventing the extinction of white abalone. The group developed the following four-step plan for recovery of the species: (1) survey historic fishing grounds to locate survivors; (2) collect and hold adults as breeding stock; (3) produce a new generation of young adult abalone in the hatchery; and (4) introduce hatchery grown adult animals into refugia to reestablish self-sustaining wild populations.

Success has been achieved in maintaining and spawning white abalone in captivity. Abalone cultivation systems incorporate the use of the red macrophyte, Pacific dulse (*Palmaria mollis*) as both a nutritious feed and biofilter. Large numbers of juveniles are being raised using commercial techniques. This program differs from other enhancement efforts in that the abalone will be raised to adult size (10 cm shell length) prior to release. Larger animals such as this should be better able to resist predation and are capable of spawning immediately.

BOAT WASTE AND SHELLFISH CLOSURES: A COMPARISON OF CANADIAN AND UNITED STATES POLICIES.

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Diseases such as hepatitis, typhoid and cholera have been transmitted by untreated boat sewage. Popular beaches and shellfish areas attract boaters; the resulting contamination from boat sewage creates health concerns for millions of shellfish lovers. Swimmers that frequent contaminated waters have been known to develop skin rashes, amoeba dysentery, and parastic worm diseases. Boat sewage has closed beaches and shellfish harvesting areas around the world.

In 1991, the Canadian government passed the Pleasure Craft Sewage Prevention Regulations intending to protect the marine environment from the negative impacts of sewage disposal. Since passing the Regulations, nine west coast marine waterbodies have been designated no-discharge zones (NDZ). A further 54 marine sites have been recommended for NDZ and are under review. However, no reduction in breach or shellfish closures has been noted.

In contrast, the United States Congress passed the *Clean Vessel Act* in 1992. Today, all vessels inside US waters are required to have a marine sanitation device. Seven states have all their surface waters designated as NDZ. An additional eleven other states have segments of their waterbodies designated as NDZ. Washington and Massachusetts have already identified a reduction in beach and shellfish closures.

With intentions from both federal governments so evident, why are the results so different? In the United States, the *Clean Water Act* and the *Clean Vessel Act* clearly lay out areas of jurisdictional responsibility and financing arrangements. Implementation plans are in State control where boat waste disposal plans were developed. In Canada, boat waste policies are caught in multi jurisdictional bureaucracies with overlapping acts and conflicting regulations, resulting in no boat waste disposal being developed.

SUCCESSION IN MUSSEL COMMUNITIES: THE IMPORTANCE OF WHAT IS MEASURED.

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This study examines the community of organisms in crevices in the St. Lawrence estuary, Canada, through succession and shows how extrapolations from the study of a limited number of organisms to the entire community of macro-invertebrates may not be valid.

I first examined how varying the lower size limit of the organisms considered in the analyses (organisms >0.5, 1, 2, 4, and 8 mm) alters the observed trends in community structure (richness and diversity) through succession. Diversity was maximal in mid-succession for the >2 and >4 mm size groupings but continued to increase through succession for the >1 and >0.5 mm groupings whereas richness always increased through succession for all groupings. Examining one of four distinct zones in crevices gave the same result as examining the total community, improving sampling efficiency. However, the other three zones did not show this predictive power.

I then examined whether variation in richness and diversity through succession was a function of the structural heterogeneity (SH) provided by the dominant taxa or of the age of the communities *per se*. This was done by comparing control crevice communities at four successional stages to ones that had been modified such that only the dominant species remained following a brief period to allow for colonization (mimic crevices). Within three months, control and mimic communities resembled each other in terms of both diversity and richness and the trends mirrored those observed four months early, thus supporting SH. Nonparametric multivariate analyses supported these conclusions.

AN ARCHAEOLOGICAL PERSPECTIVE ON THE CULTURAL SIGNIFICANCE OF SHELLFISH RESOURCES TO FIRST NATIONS IN THE GEORGIA BASIN.

Eric McLay, Archaeologist, Hul'qumi'num Treaty Group.

Archaeology offers an invaluable perspective toward understanding the fundamental importance of shellfish resources to coastal First Nations in British Columbia. This study examines the relationship between archaeological site locations and shellfish resources on Valdes Island, a large southern Gulf Island in the Georgia Basin, British Columbia. A close association is observed to exist between the location, size and content of archaeological shell deposits and the distribution of local shellfish resources.

It is argued that Central Coast Salish settlement patterns on Valdes Island demonstrate a precontact economic orientation toward exploiting productive coastal resource zones, particularly sandy intertidal habits, where populations aggregated to collect localized, predictable and abundant bivalve shellfish and other sandy foreshore resources. This archaeological study has important implications for modelling strategies of precontact settlement and subsistence in the Georgia Basin, and for considering the cultural significance of shellfish resources to modern First Nations.

RESTORING OYSTERS TO THE CHESAPEAKE BAY: A COORDINATED EFFORT INVOLVING GOVERNMENT, UNIVERSITIES, COMMUNITY GROUPS, AND INDUSTRY.

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Once the world leader Chesapeake Bay oyster harvests are near all-time lows today. Overexploitation, disease and habitat loss have all contributed to this decline. Traditional techniques of spreading shell to collect spat are no longer reliable to produce consistent numbers of high quality oyster seed because, oyster diseases are highest in areas where natural spatfall is most abundant.

Utilizing hatcheries and a strategy designed to minimize the risk of infection, oyster seed have been produced with little or no Dermo. Hatchery spat have been used to accomplish a wide range of objectives including supplying citizens growing oysters for restoration, re-seeding harvest grounds in areas sensitive to disease, involving commercial oystermen in restoration, and establishing ecological sanctuaries. This program has grown dramatically over the past decade and now is responsible for planting tens of millions of oyster spat each year.

The Maryland Oyster Recovery Partnership along with the University of Maryland Center for Environmental Science, the Chesapeake Bay Foundation, Maryland Watermen's Association and local community groups have been successful in producing the seed oysters, preparing the bottom, and planting the seed in a coordinated effort.

OYSTER RESTORATION IN CHESAPEAKE BAY: I CRITERIA FOR SITE SELECTION.

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Interest in restoring historical oyster reefs or in creating new oyster reefs in Chesapeake Bay has heightened in recent years. The state of Maryland has a long history of activities aimed at oyster repletion for the public fishery. Additionally, private oyster culture was once a major producer of market oysters in the Bay region. Disease, overharvest, and habitat loss have all contributed to the decline in oyster populations and the resultant harvests are at near all time lows.

The economic importance of a healthy oyster fishery to waterfront communities is well documented. Of more recent interest is the concern for the ecological role of healthy oyster communities to the overall health of the Chesapeake Bay. Oyster restoration for non-commercial uses is becoming more commonplace while the more traditional repletion program activities are being modified in an attempt to become more efficient.

Many community groups find it desirable to have an oyster reef in their local area. Not every site is suitable for oyster reef construction. Many exhibit some but not all of the characteristics needed for successful oyster growth and survival. Success of any given project depends upon accurate assessment of these characteristics and the use of proper construction techniques.

GENETIC MONITORING OF OYSTER STOCK ENHANCEMENT IN THE CHOPTANK RIVER, CHESAPEAKE BAY, MARYLAND, USA.

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The increased spread of parasitic diseases (primarily MSX and Dermo), in conjunction with overharvesting, has led to the rapid decline of many Eastern oyster (*Crassostrea virginica*) populations, especially in Chesapeake Bay. Regional variation in disease resistance to these parasites may be useful in restoration efforts. In collaboration with the University of Maryland Horn Point Lab, we have proposed to assess the success of recent enhancement efforts within the Chesapeake Bay using molecular genetic markers.

In 1997, oysters propagated from Louisiana broodstock were planted at ten sites within the Choptank River, Maryland. *C. virginica* exhibits regionally diagnostic DNA profiles in the form of North Atlantic, South Atlantic, and Gulf Coast 16s mitochondrial haplotypes. The presence of the Gulf Coast haplotype in newly settled spat confirms the survival and propagation of the Louisiana broodstock. DNA sequencing techniques developed by Pyrosequencing Inc. were used to determine the mitochondrial haplotypes of a large number of oyster spat collected at several bars throughout the Choptank River estuary. This rapid, mass screening method revealed that 94% of spat collected were of the North Atlantic haplotype and approximately 5% were South Atlantic. Of 2,466 spat screened, four possessed the Gulf Coast haplotype (0.2%). Haplotype identifications were confirmed using restriction fragment length polymorphisms in other regions of the mitochondrial genome. The use of these genetic markers has enabled us to assess the survival, propagation, and dispersal of the Louisiana oyster stock within the Choptank River, Chesapeake Bay.

OPEN SAANICH INLET SHELLFISH BEDS: A COLLABORATIVE APPROACH.

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The Saanich Peninsula and Inlet are located on the southern tip of Vancouver Island near Victoria, British Columbia. The Saanich Inlet shellfishery is an important food source to three Saanich Peninsula First Nation Bands and the community. Many of the shellfish beds on the east coast of Saanich Inlet are closed for harvesting due to elevated levels of fecal coliform bacteria. The primary method of fecal coliform transmission from the land to the marine environment is stormwater.

The Capital Regional District (CRD) Stormwater Quality program works to limit the impacts of stormwater runoff to the environment and public health and protect freshwater and nearshore marine ecosystems. In 1999, Environment Canada and the CRD established a project titled *Open Saanich Inlet Shellfish Beds* (*OSISB*). This collaborative project will run until 2003 and involves all levels of government and the community working towards opening shellfish beds.

Under this project, the sources of fecal coliform contamination responsible for shellfish closures are identified. The jurisdiction(s) involved then work toward their reduction/elimination.

Limited nearshore marine sampling is also undertaken to determine fecal coliform levels near shellfish beds and their association with stormwater. The data are particularly relevant to monitoring changes over time and measuring the success of efforts to open shellfish beds.

Since the project began there has been a significant decrease in the number of stormwater discharges with high fecal coliform levels along this 27 kilometer coastline. Limited marine nearshore sampling has also shown a significant reduction in fecal coliform levels.

HYDROACOUSTIC SEABED CLASSIFICATION TECHNOLOGY APPLIED IN SHELLFISH PRODUCTIVITY RESEARCH.

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A good understanding of the relation between shellfish population dynamics and their habitat is essential in order to develop successful enhancement and/or restoration methods for our natural populations through shellfish or habitat management. The physical, chemical and biological characteristics of the seabed are key elements in shellfish productivity. In the past, this element has proven to be difficult and expensive to evaluate in part due to our inability to efficiently classify seabed on a large scale. With the recent development of new data acquisition and analysis tools (Global Positioning System, Geographic Information System, geostatistics and hydroacoustics), however, it is now possible to map seabed areas within a reasonable time and financial framework. Most importantly, these new technologies are capable of providing accurate and repeatable measurements. This will provide us with the ability to measure spatial and temporal variation of the benthic assemblage in relationship to mollusc productivity.

A new shallow water seabed classification system, QTC View (Series V), is being used for the first time to survey sites in the Gulf of St. Lawrence and Fundy Bay were research is presently being carried out on,oyster bed restoration, quahaug population management and mussel farming.

THE LIVING VENEER: CHARACTERIZING HABITAT STRUCTURE CREATED BY OYSTERS.

Paynter, **Kennedy**, and **Elizabeth Flynn**, Department of Biology, University of Maryland, College Park, MD 20742, USA.

Oyster reefs, like coral reefs, are biogenic structures. The settlement and growth of oysters create them. Therefore, the density of settlement, growth rate, and the mortality rate of oysters on a given reef will greatly affect the structure of the reef and the habitat created. Natural densities of oysters up to 1,000 individuals/m^2 have been reported while the mean density of exploited reefs in Maryland is less than 3 oysters/m^2. Although the large-scale, three-dimensional relief aspects of oyster reefs has received much attention, the structure created by oysters themselves—the spaces among and between living oysters—is less well studied.

We have attempted to characterize the habitat created by different densities of oysters both in the lab and in the field. A variety of approaches are available for this kind of measurement including estimates of the "space" created by oyster shells in various assemblages, a chain index, and fractal dimension. While interstitial volume was not significantly different between clumps and loose shell, chain indices and fractal dimension estimates were quite different. Seabed roughness may also be a useful measure as it takes into account shell height and nearest-neighbor distances. Such characterization is important to understand the relationship between physical structure and habitat value. We hope to associate the structural differences between reefs with benthic fauna associated with them as an assessment of habitat value.

OYSTER RESTORATION IN CHESAPEAKE BAY: II. IMPACTS OF WATER QUALITY AND DISEASE.

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The siting of oyster restoration projects is typically guided by several considerations. Physical habitat present (ie., "hardness of the bottom") is typically the first consideration. Local disease prevalence is sometimes assessed and water quality is usually assumed. Dissolved oxygen (DO) levels are typically not measured prior to restoration.

Oxygen dynamics in shallow zones of estuaries are not well understood. While general trends typical of eutrophic systems are apparent including surface overproductivity, stratification, and deep anoxia, relatively little attention has been paid to water quality in shallow (<6m) nearshore, sub-littoral zones. Furthermore, the effects of hypoxia (<4mg O_2/l) on benthic communities and organisms are not well understood. We have observed chronic hypoxia on many oyster reefs in Chesapeake Bay. One reef was exposed to <3mg 0_2/l for nearly 72 h with only 2 or 3 brief (<30 min) episodes of higher DO. DO levels measured by continuous monitoring at this reef were <5 mg/l 90% of the time throughout August and September, 2000, and May and June, 2001. DO levels measured in 2001 at approximately 30 other reefs throughout the Maryland portion of the Bay was typically <5 mg/l and often <3 mg/l. These levels of hypoxia could have important detrimental affects on restoration projects.

EARLY COMMUNITY DEVELOPMENT OF OYSTER REEFS IN VIRGINIA: EFFECTS OF REEF SCALE.

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Traditionally, oyster reef restoration in the Chesapeake Bay region has primarily focused on oyster production. Increasingly, however, efforts have included restoration of associated communities and the overall ecological function of self-sustaining reefs as management goals. The communities that develop in association with oyster reefs in Chesapeake Bay, VA have been shown to be diverse and ecologically important. Additionally, reef architecture, such as vertical relief and interstitial space, has been shown to be important to developing and maintaining community diversity. Another, previously unaddressed architectural component, aerial extent or scale, is also expected to affect community development. In a replicated block design, we have constructed high relief reef bases ranging in size from 400 sq m to 8000 sq m and are characterizing the development of the associated resident and transient assemblages of organisms. We will present data evaluating early community development on these different scale subtidal reefs. We compare reefs of differing scales (i.e., small versus medium versus large size reefs), but also make comparisons within a reef (e.g., inner portions of reef base versus reef edge). Substrate for constructing reefs is often limiting and expensive and resource managers face tough decisions on how to allocate scarce resources (e.g., construct several large or many small reefs). When maximizing diversity and ecological function of reefs is a management objective, knowing the impact of reef scale can be a valuable asset for making such decisions.

GENETIC AND PATHOGENIC ASPECTS IN SHELLFISH RESTORATION OF SCALLOP POPULATIONS.

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With recent declines in the scallop fishery in Atlantic Canada, several projects on stock enhancement are being conducted. The success of shellfish population restoration is directly related to the fitness of the scallop seed. Results obtained from restoration activities of Giant scallops (*Placopecten magellanicus*) populations will be used to discuss of the impacts of genetic and pathogenic aspects on the fitness of scallops and the success of these activities. Factors that will contribute to reduce the overall genetic variability may compromise the capacity of a species to adapt to environmental changes and to resist to pathogens. Thus, the long-term survival of that species may be compromise. Indeed, if genetic variation within individual populations is reduced, there will be less of a basis for future adaptation within a given population. Genetic changes often occur during the hatchery process. In Quebec, scallop enhancement activities are carried out using juveniles produced in hatchery, or collected over scallop beds and that are either re-released in the same area or transfer to other areas. The impacts of all these practices on genetic variability of populations are not known. The genetic of shellfish produced in hatchery are frequently altered through inbreeding, selective breeding or domestication with an overall reduction in genetic variability. This can also be true for seed collected on artificial collectors. These enhancement activities could result in hybridization, with the possibility of reducing the genetic variation and hence, the fitness of the enhanced population.

WATER QUALITY AND OYSTER HEALTH (*CRASSOSTREA VIRGINICA*): AN INTEGRATED APPROACH TO DETERMINING HABITAT RESTORATION POTENTIAL.

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The influence of water quality and season on disease prevalence and intensity, gonadal condition, recruitment potential, and growth of oysters was examined monthly at five locations along the Caloosahatchee River estuary, Florida. Habitat suitability of oyster reefs for fishes and decapod crustaceans was examined at three of these sites. Higher temperatures and salinities favored the parasite *Perkinsus marinus,* and histological analyses revealed the presence of several additional parasitic or commensalistic organisms.

Comparison of mortality among sites indicated that juvenile oysters tolerated salinities of 15–38 ppt. Spat recruitment was higher at sudtidal (1–5 spat/shell) than at intertidal locations, where sparse oyster distribution and swift currents appeared to limit settlement success. The late peak in gametogenesis (August to September) observed at all sites may have resulted from reduced salinities during May to July or may imply that oysters spawn twice per season. Oyster-reef habitat supported a fish and decapod assemblage averaging 77 individuals m^{-2} or 14 individuals l^{-1} oyster cluster.

Reef-resident fishes included *Gobiesox strumosus, Chasmodes saburrae, Gobiosoma robustum,* and *Opsanus beta:* xanthid and porcellanid crabs represented the dominant crustaceans. Species diversity (H′) did not vary among sites; however, significant differences in density (individuals l^{-1} oyster cluster) were found in the fishes *G. strumosus* and *C. saburrae* and in the crabs *Panopeus herbstii* and *Petrolisthes armatus.* Overall, results suggest that periodic freshwater releases may benefit oysters by lowering the salinity and thus the intensity of parasite infection, *Perkinsus marinus.* It should be cautioned that the long-term effects of low salinity on oysters have not been investigated.

THE NUTRIENT VALUE OF SHELLFISH AND OTHER TRADITIONAL FOODS, THEIR PAST AND CURRENT CONTRIBUTION TO THE DIET OF FIRST NATIONS PEOPLE. Pamela Winquist, First Nations and Inuit Health Branch, Pacific Region, Health Canada. #540–757 West Hastings Street, Vancouver, BC V6C 3E6, Canada.

The nutrient value of shellfish and other foods traditionally used by First Nations people in British Columbia, Canada will be reviewed. Information on past and current consumption, and factors affecting use, will be discussed.

Shellfish and other traditional foods offer superior nutrient value compared to commercial alternatives. These foods have contributed significantly to the overall nutrient intake of First Nations people, in the past. Seafood and other traditional foods continue to contribute to the overall nutrient quality of the diet of First Nations people. Traditional foods provide nutrients that are often low in the commercial food diets of First Nations people, such as vitamin A, calcium, iron, polyunsaturated oils and omega-3 fatty acids and folic acid. When these foods are replaced by commercial alternatives, fat, sugar and salt intakes increase. Diets high in saturated fat, sugar and salt are one of the risk factors in the development of chronic diseases such as obesity, diabetes and coronary heart disease. Higher rates of these illnesses are seen in First Nations people, compared to the general population.

Factors influencing present use of traditional foods include access to a commercial food source, knowledge and skills of family members to fish, hunt and/or trap, amount of traditional food available, fish and wildlife regulations, contamination of traditional foods, and household income.

In conclusion, shellfish and other traditional foods are of high nutrient value. These foods should continue to be consumed as part of a nutritious diet, when they are available for safe harvest and preparation.

PREDATION OF JUVENILE SEA SCALLOPS (*PLACOPECTEN MAGELLANICUS*) DURING SEEDING TRIALS IN THE NORTHUMBERLAND STRAIT. Melisa C. Wong,[1] M. A. Barbeau,[1] L. A. Grecian,[1] L-A. Davidson,[2] M. Niles,[2] and Donna Murray,[3] [1]Department of Biology, University of New Brunswick, Fredericton, NB, E3B 6E1, Canada; [2]Department of Fisheries and Oceans, Gulf Fisheries Centre, Moncton, NB, E1C 9B6, Canada; [3]Botsford Professional Fishermen's Association Inc., 1696 Route 955, Little Shemogue, NB, E4M 3M6, Canada.

The Botsford Professional Fishermen's Association Inc. and the Department of Fisheries and Oceans (Moncton, NB) have conducted a scallop enhancement project in the Northumberland Strait since 1998. Predation of juvenile sea scallops by sea stars (*Asterias vulgaris*) and rock crabs (*Cancer irroratus*) is a concern in the Northumberland Strait. Our goal was to quantify predator-induced mortality of seeded scallops and to determine how quickly mortality occurs after seeding. We deployed assays (consisting of scallops tethered to lead-weighted lines) to monitor scallop mortality at seeded and non-seeded sites, after seedings in October 1999 and October 2000. In both years, shell remains on the tethered lines indicated the presence of both sea stars (empty intact shells) and rock crabs (shell fragments) at both sites. In October 1999, scallop mortality did not differ between the seeded site and the non-seeded site or show a significant change over time. In October 2000, scallop mortality did not differ between sites, while scallop mortality was significantly higher 10 days after seeding than 2 days after.

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ABSTRACTS OF TECHNICAL PAPERS

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CONTENTS

Jeremy M. Breach and Jennifer L. Ruesink
Population and behavioral characteristics of introduced oyster drills, *Ceratostoma inornatum*, in Willapa Bay 325
Amilee Caffey and Are Strom
Juvenile growth study on the geoduck clam, *Panopea abrupta*.......... 325
Hakan Calik, Michael T. Morrissey, Paul W. Reno, and Haejung An
Effect of high-pressure processing on *Vibrio parahaemolyticus* strains in pure culture and Pacific oysters.......... 325
Elyse K. Cronin and Brent Vadopalas
Age determination in geoduck clams (*Panopea abrupta*) utilizing patterns in the shell annuli.......... 325
Rabyn M. Estes, Russell P. Herwig, and Ralph A. Elston
Characterization of pathogenic and nonpathogenic bacteria associated with bivalve larvae and shellfish hatcheries...... 326
Stephen P. Ferraro and F. A. Cole
Oyster grounds: A superior habitat for small, sediment-dwelling invertebrates.......... 326
Graham E. Gillespie
Status of the Olympia oyster, *Ostrea conchaphila*, in Canada.......... 326
Geoff Hosak, David Armstrong, Bryce Semens, Brett Dumbauld, and Steven Rumrill
Oyster aquaculture as fish habitat in Pacific northwest coastal Estuaries.......... 327
Chris Langdon, Ford Evans, John Brake, and Sean Matson
Survival and growth effects on yields of Pacific oysters, *Crassostrea gigas*.......... 327
Cynthia S. Marshall and Russell P. Herwig
Research to examine use of high hydrostatic pressure to inactivate human enteric viruses in oysters 327
Sean E. Matson, Chris Langdon, Ford Evans, John Brake, and Dave Jacobson
Predictability of grow-out performance from nursery performance of Pacific oyster, *Crassostrea gigas* 327
Daphne M. Munroe and Leah Bendell-Young
The effects of clam aquaculture on intertidal ecosystem structure and function.......... 328
Paul G. Olin and Jim Hobbs
Habitat value of commercial oyster culture gear.......... 328

POPULATION AND BEHAVIORAL CHARACTERISTICS OF INTRODUCED OYSTER DRILLS, *CERATOSTOMA INORNATUM*, IN WILLAPA BAY.

Jeremy M. Breach and **Jennifer L. Ruesink**, Department of Zoology, University of Washington, Seattle, WA 98195-1800.

Japanese oyster drills in Willapa Bay were studied to discern population parameters including density, size-frequency distribution, food preference, and movement rates. Drills were collected during the summer of 2001 from 0.0625 and 1 m^2 quadrats near the Washington Department of Fish and Wildlife lab at Nahcotta, Washington. Drill density averaged 31 m^{-2}, and the size distribution included a peak of small individuals suggestive of this year's recruitment. Drills showed a preference for feeding on smaller oysters, and oysters appeared to escape drill predation at shell lengths exceeding 2 cm. Drills were labeled and released into three different habitats to study drill movement. Significant differences were found between movement rates on eelgrass, oyster bed, and mudflat, with drills traveling up to 1 m/day on mudflat and eelgrass until reaching small patches of oysters. Risks of drill impacts are highest for seed oysters placed on the bottom in infested areas, particularly if few oysters are currently present.

JUVENILE GROWTH STUDY ON THE GEODUCK CLAM, *PANOPEA ABRUPTA*.

Amilee Caffey, Washington Department of Fish & Wildlife, Point Whitney Laboratory, Brinnon, WA 98320; **Are Strom**, University of Washington, WA 98062.

The geoduck clam has proven to be a challenging species to culture in the hatchery setting. Water temperature, salinity, sunlight, food concentrations, and handling all affect levels of growth. The goal of this study was to determine what effect temperature and varying food concentration levels in the surrounding water had on the juvenile geoduck clam.

The statistical analysis method was a two-way ANOVA in which juvenile clams were introduced to four varying environments where temperature and food concentrations were aberrant. Clams were placed in 1-in. square, plastic trays, layered with permeable fabric and sand. The stocking density was 100 juveniles per tray. Smaller juveniles were chosen, ranging between 14 and 19 mm in shell length in order to determine rapid growth rates. Three identical trays were subjected to each of the four treatments, with a total of 12 trays. The first and second treatments were subjected to a higher temperature, between 15 and 16°C. The third and fourth treatments were at a lower temperature, 10–11°C. The first and fourth treatment were held at a higher concentration of food, 45 k/mL. The second and third treatments were held at a lower food concentration of 15 k/mL. Flow rate was 6 L/min.

Shell length did not vary significantly between treatments, however, juveniles exposed to higher temperature (15–16°C), at higher food concentration (45 k/mL algal cells), demonstrated the most growth.

EFFECT OF HIGH-PRESSURE PROCESSING ON *VIBRIO PARAHAEMOLYTICUS* STRAINS IN PURE CULTURE AND PACIFIC OYSTERS.

Hakan Calik, **Michael T. Morrissey**, **Paul W. Reno**, and **Haejung An**, Department of Food Science & Technology, Oregon State University Seafood Laboratory, 2001 Marine Drive, Room 253, Astoria, OR 97103.

Several cases of *Vibrio parahaemolyticus* (Vp)-induced gastroenteritis occurred in the Pacific Northwest due to consumption of raw oysters. High-pressure process (HPP) technology has shown good potential in reducing pathogens. Environmental and clinical strains of Vp in broth cultures and Vp-inoculated live Pacific oysters (*Crassostrea gigas*) were subjected to HPP at different treatment settings (1–10 min at 241 MPa; 1–5 min at 276 MPa; 30–120 s at 310 MPa; 10–90 s at 345 MPa). Results showed that Vp numbers were reduced by HPP in both pure culture and whole oysters. Vp inactivation was dependant on treatment time and pressure. Optimum conditions for reducing Vp in pure culture and oysters from 109 to 101 CFU/mL were achieved at 345 MPa for 30 and 90 s, respectively. Resistance variations were detected between Vp in pure culture and in oysters. Further high-pressure tests with clinical 03:K6 Vp strain isolated from an outbreak in Texas (1998) showed that 5 min at 310 MPa was necessary for complete elimination, making the isolate the most baro-resistant of all strains used in the study. HPP proved to be an efficient means of reducing Vp in oysters.

AGE DETERMINATION IN GEODUCK CLAMS (*PANOPEA ABRUPTA*) UTILIZING PATTERNS IN SHELL ANNULI.

Elyse K. Cronin and **Brent Vadopalas**, School of Aquatic and Fishery Sciences, University of Washington, Seattle, WA 98105.

Geoducks (*Panopea abrupta*) are deep-burrowing bivalves distributed from Southeast Alaska to Baja, California. This extremely long-lived species supports a lucrative fishery in Washington state, yet little is known of geoduck population dynamics. Age data are being collected from various sites in Puget Sound to investigate both population dynamics and temporal genetic structure in geoducks, via collaborations between the Washington Department of Fish and Wildlife (WDFW), Washington Sea Grant, and the University of Washington School of Aquatic and Fishery Sciences.

To age geoducks, the hinge plate of the right valve was thin-sectioned using a diamond saw, polished, and finally etched with 1% HCl. Annual growth rings (annuli) were visualized via light microscopy, and two independent observer counts were made on three thin-sections for each clam. Annuli revealed year-specific width variation consistent across samples. These patterns may correlate with localized environmental factors or regional oceanographic conditions, and are used to establish year reference points in series of annuli.

Ages determined from 200 geoducks collected from a Hood Canal site sampled in 2000 ($N = 1010$) ranged from 5 to 136 y. When complete, this age data set will be used in analyses of genetic population structure and population dynamics of the species in Puget Sound.

CHARACTERIZATION OF PATHOGENIC AND NONPATHOGENIC BACTERIA ASSOCIATED WITH BIVALVE LARVAE AND SHELLFISH HATCHERIES.

Robyn M. Estes, Russell P. Herwig, School of Aquatic and Fishery Sciences, Box 355020, University of Washington, Seattle, WA 98195-5020; **Ralph A. Elston,** AquaTechniques/Pacific Shellfish Institute, P.O. Box 687, Carlsborg, WA 98324.

Bacterial diseases are a major cause of larval mortality in shellfish hatcheries. Even with proper sanitation measures, bacterial pathogens cannot be eliminated. The proper identification of pathogens and the application of probiotics may help control disease outbreaks.

More than 100 bacterial isolates were collected from larval Pacific oysters, larval geoducks, and locations within hatcheries in the Pacific Northwest and Hawaii. Isolates were characterized by whole cell analysis and restriction fragment length polymorphism (RFLP) of 16S rDNA using three restriction enzymes. Both of these methods show similar relationships between the isolates. Pathogenicity tests of isolates collected from Pacific oyster larvae from Washington and Oregon showed that 3 out of 33 isolates were highly pathogenic. These assays examined larval mortality and the ability of larvae to remain suspended in a water column. Pathogenic and nonpathogenic bacteria strains appear closely related to each other. These results could provide information for the development of probiotics in shellfish aquaculture.

Future research includes sequencing the 16S rDNA of pathogenic bacteria, declaring new species if they cannot be identified as known species, and developing polymerase chain reaction (PCR) primers for rapid detection of pathogenic bacteria. This will help in early detection of pathogenic bacteria and in determining the source and ecology of the pathogenic organisms in hatcheries.

OYSTER GROUNDS: A SUPERIOR HABITAT FOR SMALL, SEDIMENT-DWELLING INVERTEBRATES.

Steven P. Ferraro and **F. A. Cole,** U.S. Environmental Protection Agency, 2111 S.E. Marine Science Drive, Newport, OR 97365-5260.

As part of a programmatic effort to determine estuarine habitat values for ecological risk assessments, quantitative field studies of small, sediment-dwelling invertebrates were conducted in Willapa Bay, Washington, in July 1998 and Tillamook Bay, Oregon, in July 1999. The six habitats included in both studies were (1) "grow-out" (2–3 y old) oyster ground culture, (2) eelgrass, *Zostera marina*, (3) mudshrimp, *Upogebia pugettensis*, (4) ghost shrimp, *Neotrypaea californiensis*, (5) bare mud, and (6) subtidal, undredged. About fifteen 0.01 $m^2 \times 5$ cm deep, 0.5 mm mesh samples were collected randomly in each habitat throughout both estuaries.

Multivariate analyses of the data revealed that the invertebrate fauna on oyster grounds was much more similar to that in eelgrass and mud shrimp habitat than that in ghost shrimp, bare mud, and subtidal habitat. Among the six habitats studied, oyster grounds consistently ranked either first or second in terms of the number of species, abundance, and total biomass of invertebrates. Oyster grounds, which have high economic value in terms of oyster production, are also ecologically valuable because they provide a superior habitat for small invertebrates upon which many larger animals (e.g., fish, crabs, waterfowl) feed.

STATUS OF THE OLYMPIA OYSTER, *OSTREA CONCHAPHILA*, IN CANADA.

Graham E. Gillespie, Fisheries and Oceans Canada, Pacific Biological Station, Nanaimo, British Columbia, V9R 5K6.

The Olympia oyster, *Ostrea conchaphila*, is the only oyster native to the Canadian Pacific coast. Olympias supported commercial fisheries in British Columbia from the late 1800s to about 1930, when focus of the oyster industry shifted to Pacific oysters, *Crassostrea gigas*.

Olympia oyster distribution in British Columbia is limited by specialized habitat requirements, and relatively low fecundity and dispersal. Olympias are vulnerable to temperature extremes, and are not resistant to harvests on a commercial scale. Habitats that once supported large aggregations in Georgia Strait no longer do, in part because of historic overharvests and environmental stresses, and because development of large oyster reefs may require centuries without disturbance. Small relict populations survive at low tide levels and under floating structures. Olympias are locally common at sites on the west coast of Vancouver Island, and little information exists on populations in Johnstone Strait or in the Central Coast. They do not occur in the Queen Charlotte Islands.

Olympia oysters are not likely facing imminent danger of extinction or extirpation in Canada. Limiting factors have led to significant reductions to population levels in the past. From the limited data available, the Committee on the Status of Endangered Wildlife in Canada (COSEWIC) assigned a status of Special Concern in November 2000. Proposed federal legislation, the Species At Risk Act (SARA), will require development of an Olympia oyster management plan within 3 y.

OYSTER AQUACULTURE AS FISH HABITAT IN PACIFIC NORTHWEST COASTAL ESTUARIES. Geoff Hosack, David Armstrong, Bryce Semens, School of Aquatic and Fishery Sciences, Box 355020, University of Washington, Seattle, WA 98195; **Brett Dumbauld,** Washington State Department of Fish and Wildlife, Willapa Bay Field Station, P.O. Box 190, Ocean Park, WA 98640; **Steven Rumrill,** South Slough Estuarine Research Reserve, P.O. Box 5417, Charleston, OR 97420.

Increased pressure on traditionally managed stocks of marine and anadromous fish, calls for protection of essential fish habitat under the Magnuson-Stevens Act, and recent listings of several salmonid stocks under ESA, have brought aquaculture activities that take place in coastal estuaries under increased public scrutiny.

We initiated a study designed to examine the ecological role that oyster aquaculture plays as habitat in coastal estuaries of the Pacific Northwest. The goal of the project is to identify and quantify beneficial and adverse impacts of shellfish farming on eelgrass, juvenile salmonids, and other selected estuarine fauna and flora, and to develop farming practices and recommend management protocols that protect or enhance those resources. We present results of initial fish and invertebrate sampling in selected habitats from Willapa Bay during spring and summer 2001, and an experiment designed to examine the effects of oyster harvesting on eelgrass habitat. We make a plea to managers to consider oyster aquaculture areas as fish habitat on a broader estuarine scale.

SURVIVAL AND GROWTH EFFECTS ON YIELDS OF PACIFIC OYSTERS, *CRASSOSTREA GIGAS*. Chris Langdon, Ford Evans, John Brake, and **Sean Matson,** Coastal Oregon Marine Experiment Station and Department of Fisheries and Wildlife, Oregon State University, Newport, OR 97365.

In Spring 1999, 29 full-sib families derived from crossing nonselected "wild" oysters were planted at a subtidal site in Yaquina Bay, Oregon. In addition, in Fall 1999, 32 full-sib, families from crosses within two groups of six selected families were planted at a an intertidal site in Tomales Bay, California. After about 1 y of growth, each cohort was harvested and average yields, survival, and individual growth rates determined for each family. These parameters were adjusted for the effects of differences in average initial weights of planted spat per family if a significant ($P < 0.05$) effect was detected.

Adjusted survival explained 57% and 68%, and growth explained 25% and 21% of the variation in adjusted family yields at the Yaquina and Tomales sites, respectively. Furthermore, up to 79% of the variation in family yields at the Tomales site was explained by differences in survival among families, if survival was not adjusted for a significant (regression, $r^2 = 0.38$; $P < 0.0001$) positive effect of initial spat weight. These results indicate that both growth and, perhaps to a greater degree, survival should be considered when designing breeding programs to increase oyster yields.

RESEARCH TO EXAMINE USE OF HIGH HYDROSTATIC PRESSURE TO INACTIVATE HUMAN ENTERIC VIRUSES IN OYSTERS. Cynthia S. Marshall and **Russell P. Herwig,** School of Aquatic and Fishery Sciences, Box 355020, University of Washington, Seattle, WA 98195-5020.

The desire for safe shellfish products in the retail market and food service establishments is paramount to the success and growth of the shellfish industry. Unfortunately, bacterial and viral pathogenic organisms may be associated with fresh and processed shellfish. These organisms can lead to seafood-borne illness that may result in severe economic impacts on the shellfish industry, causing shellfish bed closures, product recalls, and lost consumer confidence. Human enteric viruses are the causative agents associated with a large, but poorly understood, number of seafood-borne illnesses per year. The principle viruses associated with seafood include hepatitis A, Norwalk virus, Norwalk-like viruses, and astrovirus. Viruses are typically not monitored in seafood products and in shellfish growing waters because of the difficulty and expense of quantifying active virus particles.

There is a large demand for raw oysters in the market. Depuration or relaying may be used to reduce the number of pathogenic organisms present in live shellfish before they are harvested. In recent years, high hydrostatic pressure (HHP) treatment has been shown to reduce or eliminate bacterial pathogens from shellfish while retaining sensory properties of the raw product. We are beginning a new research project that will examine the effects of HHP on human enteric viruses. Poliovirus will be used as a model virus in our experiments. This virus was chosen because it can be safely used in the laboratory and can be cultured using standard protocols. In laboratory experiments, we will vary hydrostatic pressure, time of exposure, pH, and temperature on preparations containing poliovirus.

PREDICTABILITY OF GROW-OUT PERFORMANCE FROM NURSERY PERFORMANCE OF PACIFIC OYSTER, *CRASSOSTREA GIGAS*. Sean E. Matson, Chris Langdon, Ford Evans, John Brake, and **Dave Jacobson,** Hatfield Marine Science Center, Oregon State University, 2030 S. Marine Science Dr. Newport, OR 97365-5296.

The predictability of Pacific oyster (*Crassostrea gigas*) grow-out performance from nursery performance was investigated by measuring yield, survival, and growth of pedigreed families of Pacific oysters, in the nursery and at a grow-out site in Totten Inlet, Washington. Early prediction of grow-out performance could significantly reduce labor, materials, and space required to select pedigreed families on the basis of performance in an oyster breeding program. This research was conducted as part of the Molluscan

Broodstock Program, a breeding program for the sustainable increase in Pacific oyster yields through genetic selection. Yield was measured as oyster bag weight in grams and is a function of both survival and growth.

Unadjusted yield, yield adjusted for initial size, and unadjusted individual growth of Pacific oyster seed in the nursery were used as predictors of unadjusted yield, yield adjusted for initial weight, and unadjusted individual growth in the field. Unadjusted nursery yield was found to be a significant predictor of unadjusted grow-out yield ($P = 0.0009$, $r^2 = 0.250$). Initial planting weight, however, was also a significant predictor of unadjusted grow-out yield ($P < 0.0001$, $r^2 = 0.283$). Similarly, initial nursery weight was a highly significant predictor of nursery yield ($P < 0.0001$, $r^2 = 0.703$). Thus, adjustments were employed to both nursery and grow-out yield, to remove the effect of initial weight. Adjusted nursery yield was not a significant predictor of adjusted grow-out yield ($P = 0.9975$, $r^2 = 2.475 \times 10^{-7}$). These data indicate that heavy spat grew into heavy oysters because they were planted as heavy spat. They also indicate that Pacific oyster families with high yields at grow-out (adjusted for initial weight) are not easily identified in the nursery using the methods described here.

THE EFFECTS OF CLAM AQUACULTURE ON INTERTIDAL ECOSYSTEM STRUCTURE AND FUNCTION.

Daphne M. Munroe and **Leah Bendell-Young,** Department of Environmental Science, Simon Fraser University, 8888 University Drive, Burnaby, BC V5A 1S6.

Quadrat and core sampling was carried out to gather baseline data regarding ecosystem structure and function from three beaches on Denman Island, British Columbia. Ecosystem structure was examined through biodiversity measures (species richness, evenness, and heterogeneity), community composition, and species distribution. Ecosystem function was evaluated using percent silt and percent organic matter from core samples. The three study sites experienced different levels of commercial aquaculture. One site was a recreational harvest beach and the other two were leased for commercial aquaculture: one was 75% covered with nets, the other was only 20% covered.

We observed differences in ecosystem structure and function among the three sites. First, species richness was higher on the beach where no commercial practices occurred. Second, there were higher numbers of organisms per quadrat in the upper areas of the recreational beach and considerably more surface species on that beach. Third, we saw that the distribution of the most abundant clam species was limited to the upper half of the beach where no commercial clam culture was conducted; however, on the other two beaches, the distribution of this clam species extended the length of the study area.

This research indicates a high probability that commercial clam culture causes changes in intertidal ecosystem structural and functional components. Further research is imperative to provide managers with the scientific information needed to develop sustainable and environmentally sound management protocols.

HABITAT VALUE OF COMMERCIAL OYSTER CULTURE GEAR.

Paul G. Olin, University of California Sea Grant, Davis, CA 95616; **Jim Hobbs,** University of California, Davis, CA 95616.

Oyster growers in Tomales Bay, California, produce deeply cupped single oysters for the half-shell market using plastic mesh socking attached to stakes, and plastic mesh bags on the bottom, on racks or floating attached to longlines. This oyster culture gear and the oysters that are grown form a complex three-dimensional habitat that interacts in a variety of ways with the biological and physical components of the estuarine ecosystem. This habitat is utilized by a myriad of fish and invertebrates, which are often prey for larger commercially important species such as halibut or dungeness crab.

To document the habitat value of cultured oysters and gear, fish and invertebrates from 36 culture bags were collected and all macroorganisms were enumerated and identified to species. Ten phyla and 11 taxonomic classes were represented by the 51 different species identified. One oyster culture grow-out bag held more than 5,000 organisms, although more typically between 600 and 1,000 individuals were found in each bag.

In Tomales Bay approximately 8% of the intertidal and subtidal bottom lands are leased for shellfish culture by the state. Of this 8%, around 2% is actively farmed. Although this represents a small portion of the Bay, it is highly productive and provides complex intertidal habitat that has been lost in many areas due to erosion and resulting sedimentation.

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ABSTRACTS OF TECHNICAL PAPERS

Presented at the 22nd Annual

MILFORD AQUACULTURE SEMINAR

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CONTENTS

Walter Blogoslawski
Overview, 22nd Milford Aquaculture Seminar 335
Peter Adamik, Roxanna Andersen, Murray Crous, Graham Hains, Vicke Starczak, Isabelle Williams, Diane Murphy, and Dale Leavitt
Influence of quahog grow-out nets on benthic diversity in Wellfleet, MA 335
John Aldred, Inke Sunila, and Christopher Martin
Possible larval mycosis as a cause of bivalve seed mortality in a production hatchery 335
Standish K. Allen, Jr.
New developments with nonnative shellfish species in the Chesapeake Bay 336
Brian J. Ball, Stephanie T. Rutkowski, Emily T. Griffiths, and Kim W. Tetrault
The effects of varying cold water temperatures, size and population density on the growth and mortality rates of bay scallops, *Argopecten irradians irradians,* in an upweller 336
Amber L. Beitler and John J. Roy
Catch statistics of *Homarus americanus,* the American lobster, from a 3-wk study conducted by students from the Sound School Regional Aquaculture Center aboard a commercial lobster vessel in the waters off Fishers Island, New York 337
David A. Bengtson, Stephen Willey, Erin McCaffrey, and David Alves
Effects of water velocity on conditioning of summer flounder *Paralichthys dentatus* for net pens 337
Luther Blount
Reminiscences of early pioneers in oyster culture 338
Diane J. Brousseau and Ronald Goldberg
"Crab sightings" in Long Island Sound during 2001 338
Walter J. Canzonier
Some classical personalities in the shellfish research arena: Eclectic observations of an erstwhile observer 339
Diane Carle
Massachusetts Ocean Resource Information System (MORIS) 339
Joseph Choromanski, Sheila Stiles, Mark Dixon, and Christopher Cooper
Habitat suitability ascertained by growth and survival of bay scallops in tiered cages 340
Carmela Cuomo, Paul R. Bartholomew, Leslie Angelini, Brian King, and Jeffrey Byczko
Horseshoe crab aquaculture: Preliminary results from hatching and rearing studies 340
John J. Curtis, Sherry W. Lonergan, Paul J. Trupp, Peimin He, Raquel Carmona, Charles Yarish, George P. Kraemer, Christopher D. Neefus, Thierry Chopin, and George Nardi
A cooperative study on the aquaculture of *Porphyra leucosticta* (Rhodophyta) for an integrated finfish/seaweed recirculating aquaculture system in an urban application 341
Gef Flimlin
Update on shellfish restoration and review of local hatcheries in New Jersey 341
Michael J. Goedken and Sylvain De Guise
Flow cytometry as a tool to quantify the oyster phagocytosis, respiratory burst, and apoptosis 342
Josh Goldman
Cobia culture 342
Eric Gaodman, Colleen Cook, and Michael Weiss
Creating opportunities for student aquaculture projects 342
Hélène Hégaret, Gary H. Wikfors, Philippe Soudant, Maryse Delaporte, Jeanne Moal, and Jean-François Samain
An experimental investigation of dietary fatty acids and sterols and the immunology of the American oyster, *Crassostrea virginica*: A well-fed oyster is a healthy oyster, n'est-çe pas? 342
Andrea Hsu, Erin Summers, James Estrada, and Roxanna Smolowitz
The role of three bacteria in shell disease of the American lobster (*Homarus americanus*) 343
Richard C. Karney, Amandine Surier, David W. Grunden, and Thomas E. Berry
Dermo investigations, razor clam nursery trials, and preliminary bay scallop adhesion culture efforts 343
Robin Katersky, Barry Smith, Dean Perry, and David Nelson
Some culture strategies for growing rotifers (*Brachionus plicatilis*) as feed for aquaculture applications 344
Brian Kilpatrick, Joseph DeAlteris, and Robert Rheault
Assessing habitat value of modified rack and bag aquaculture gear in comparison with submerged aquatic vegetation, in particular, an eelgrass (*Zostera marina*) bed 344

Gordon King
The culture of black pearl oysters on subsurface longlines in Savu Savu, Fiji 345
Gordon King
Urban community meets aquaculture: A case study in the Northwest 345
Dale Leavitt, William Burt, Diane Murphy, and Rebecca Hanson
Progress with culturing the razor clam (*Ensis directus*).......... 345
Kathryn R. Markey and John J. Roy
A comparison of survival in juvenile *Argopecten irradians irradians* using various culture techniques at the Sound School Regional Aquaculture Center.......... 346
Paul D. Maugle
Suspended aquaculture development in Connecticut.......... 346
Mary F. Morgan, Kathleen K. Becker, and Kim Tetrault
It takes a community to grow a scallop.......... 347
Diane Murphy, Dale Leavitt, Bill Burt, and Bill Clark
Bay scallop (*Argopecten irradians irradians*) restoration on Cape Cod 347
Jennifer Mugg-Pietros and Michael A. Rice
Effects of *Crassostrea virginica* populations on sedimentation, phytoplankton species composition, and ammonia cycling in experimental mesocosms.......... 348
Steven Pitchford and Richard Robohm
A review of diseases in the bay scallop (*Argopecten irradians irradians*) and some observations on mortalities at the Milford Laboratory 348
Perry Raso and Michael A. Rice
Shellfish aquaculture's effect on total organic carbon (TOC) in the benthos.......... 349
Edwin Rhodes
Industrial-scale scallop culture in Chile—the C.M.I. experience.......... 349
Karen Rivara, Amber L. Beitler, and John J. Roy
The development of an instructional shellfish hatchery: A collaborative effort between Aeros Cultured Oyster Co. and the Sound School Regional Aquaculture Center 349
Karen Rivara
The East Coast Shellfish Growers Association: A work in progress 350
René Sanz, Sherry Lonergan, Jennifer Sutorius, and Dania Lieberthal
Heavy metal survey of *Fucus spiralis* collected from southwestern Long Island Sound 350
Martin P. Schreibman, Chester Zarnoch, John T. Tanacredi, Lucia Magliulo-Cepriano, Jacob Raz, and Stefano Diomede
Aquaculture activities in Brooklyn, New York? 351
Sandra E. Shumway
Shellfish aquaculture: Good for the economy, good for the environment, good for you! 351
Roxanna Smolowitz, Susan Ford, and Lisa Ragone-Calvo
Health management guidelines for shellfish culture in the northeastern United States 351
Roxanna Smolawitz, Inke Sunila, Nancy Stokes, and Lisa Ragone-Calvo
Prevalence and mortality associated with SSO and SSO-like infections of *Crassostrea virginica* in the Northeast.......... 352
Karin A. Tammi, Najih Lazar, Arthur Ganz, James G. Turek, and John G. Catena
Rhode Island's Shellfish Restoration Program in response to the *North Cape* oil spill.......... 352
Kim Tetrault, R. Michael Patricio, and Mary Morgan
SPAT (Special Programs in Aquaculture Training) update, 2002. Establishing a model for community-based shellfish culture and restoration.......... 353
John Wadsworth, Tessa Simlick, and Nancy Balcom
A new clam for Connecticut 353
Bethany A. Walton
Life in a trailer—development of a new shellfish hatchery at the Massachusetts Maritime Academy 354
Donald Webster
The new oyster wars: Policy perspectives on the introduction of *Crassostrea ariakensis* in the Chesapeake Bay 354

Scott Weston, Mark Fregeau, and Joe Buttner
Developments in softshell clam hatchery and nursery production on Massachusetts' North Shore........................ 354
James C. Widman, Jr. and David J. Veilleux
Rapid growth of bay scallops, *Argopecten irradians irradians*, in Long Island Sound.................................... 355
Gary H. Wikfors
Livestock domestication in the third millennium: All wet?... 355
Lawrence Williams and Tessa Simlick
Blue mussel aquaculture in Long Island Sound.. 356
Stephen Willey, David A. Bengtson, and Moti Harel
Arachidonic acid requirements in larval summer flounder, *Paralichthys dentatus* 356

OVERVIEW, 22nd 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 more than 193 registrants, the 22nd Annual Milford Aquaculture Seminar was our largest gathering of industry, research, and academic interests.

By blending both the theoretical and practical aspects of aquaculture, the meeting permitted attendees an exchange of technology in aquaculture methods outside their own expertise and provided a forum where the latest innovations were introduced and discussed.

Fifty formal papers and posters were presented by attendees from 10 U.S. coastal states and France. Meeting attendees represented 3 vocational aquaculture high schools, 16 universities, 6 marine labs, and several state and federal institutions involved in shellfish and finfish aquaculture. A highlight of the meeting was a set of papers reviewing the northeastern aquaculture pioneers, making us aware of the difficulties and successes of those who laid the foundation for our aquaculture ventures and studies. Other topics included information on new hatcheries, education, disease, nutrition, and culture techniques.

The seminar has developed a tradition of offering the latest information available in the field in an informal atmosphere. This has successfully promoted a free exchange among all with an interest in the success and future of aquaculture. This seminar continued that approach which allowed all attendees to enjoy and learn from the formal presentations, and afforded informal opportunities to discuss the latest developments pertinent to this important expanding field.

At this year's seminar, 45 separate aquaculture companies met in an evening session to discuss the feasibility of forming an industry group tentatively titled "The East Coast Shellfish Growers Association." The proposed Association's goals are to promote and protect association members' needs in state and regional contexts and involve all stakeholders in the task of enhancing the shellfish aquaculture industry. The main reason for joining together is to promote industry unity and to counteract recent anti-aquaculture litigation and publicity.

The meeting was sponsored by the National Marine Fisheries Service, Northeast Fisheries Science Center, Milford Laboratory, Milford, Connecticut.

INFLUENCE OF QUAHOG GROW-OUT NETS ON BENTHIC DIVERSITY IN WELLFLEET, MA.

Peter Adamik, Roxanna Andersen, Murray Crous, Graham Hains, Vicke Starczak, Isabelle Williams, Diane Murphy, and **Dale Leavitt,** SouthEastern Massachusetts Aquaculture Center, Massachusetts Maritime Academy, 101 Academy Dr., Buzzards Bay, MA 02532.

The question of environmental impacts from shellfish aquaculture has been posed recently both locally and at an international level. An often-used indicator of environmental health is the diversity of species within the ecosystem in question. The prevailing assumption is that a decrease in biodiversity suggests a decrease in the quality of the environment. To begin addressing the issue of environmental impacts of shellfish aquaculture on local intertidal flats, we initiated a study investigating benthic diversity associated with netted raceways used for quahog (*Mercenaria mercenaria*) grow-out as compared to uncovered, unimpacted intertidal flats. The specific question asked was, "Does the presence of aquaculture netting structures and high quahog planting densities in the intertidal marine habitat alter the diversity of macrobenthic species in proximity to the nets?"

The study plan was to visit two sites having a history of quahog aquaculture and collect replicated core samples within and outside of the netted raceway. The samples were preserved, stained, and returned to the laboratory. In the laboratory, the samples were sieved to 500 μm and all living tissue, as stained with rose bengal, was sorted and identified to the lowest taxonomic level possible.

A total of 39 macrobenthic species were identified in the core samples from sample site 1 (Town Landing, Wellfleet, MA) and 27 from site 2 (Old Wharf, Wellfleet, MA). The dominant species in all samples at Town Landing was the glassy tube worm, *Spiochaetopterus oculatus*; at Old Wharf, the dominant species were the capitellid worm, *Heteromastus filiformus* and the mud snail, *Ilyanassa obsoleta*. The impact of shellfish aquaculture on environmental quality was analyzed in terms of the species richness, species diversity, and distribution of dominant species between sites and treatments.

In general, there were minor or no differences between netted and nonnetted treatments at the two sites investigated. Many other factors can influence diversity, species richness, and evenness of the intertidal macrofauna, including hydrography and fluid interaction under the netted area, human activities related to shellfish farming, and the historical background of the netted site (long-term culture, mid-term culture, or short-term culture). It was concluded that further study was warranted to include a seasonal and long-term component to the study.

POSSIBLE LARVAL MYCOSIS AS A CAUSE OF BIVALVE SEED MORTALITY IN A PRODUCTION HATCHERY.

John Aldred, Town of East Hampton, Shellfish Hatchery, 159 Pantigo Road, East Hampton, NY 11937; **Inke Sunila,** State of Connecticut, Department of Agriculture, Bureau of Aquaculture, P.O. Box 97, Milford, CT 06460; **Christopher Martin,** USDOC, NOAA, National Marine Fisheries Service, Northeast Fisheries Science Center, Milford Laboratory, Milford, CT 06460.

Since 1996, the shellfish hatchery at East Hampton has experienced sporadic unexplained mortalities in cultures of hard clams (*Mercenaria mercenaria*) and bay scallops (*Argopecten irradians irradians*). Typically, a population of larvae progressed normally through metamorphosis and then, early in the juvenile stage (post-set) stopped feeding. In such cultures, examination revealed many dead or dying individuals. Within 2–4 days of the first indication of the problem most animals were dead. Affected juveniles ranged in shell length from 0.2 to 1.5 mm. Eastern oysters (*Crassostrea virginica*) reared at the same hatchery have not been affected. Preventative measures have been unsuccessful. These included switching algal diets, careful monitoring of pH, changing water more frequently, and giving special attention to cleaning and disinfecting the system. On occasion, it has been possible to rescue some juveniles from affected cultures by moving them to a flowing water system, with clams surviving better than scallops.

During the 2001 growing season, similar mortalities were observed. Samples of juvenile clams (approximately 1.0 mm in shell length) from two cohorts were preserved in Davidson's fixative. Scallop juveniles also from two cohorts (ranging from approximately 0.2 to 0.5 mm in shell length) were similarly fixed. Following decalcification, the specimens were stabilized in agarose, embedded in paraffin, sectioned to 6 μm, and stained with hematoxylin-eosin according to standard histopathological procedures.

Microscopic examination revealed invasion of most tissues and the shell by fungal mycelia. Mycelia were detected in the gill filaments where they often completely obstructed the hemolymph canals. Fungal hyphae were observed within the mantle cavity, with invasion of all epithelia (mantle, gill, and foot). The hyphae appeared to have some affinity to mucus. Cilia were entrapped. All layers of shell were invaded, with penetration to the exterior. The hyphae stained dark blue with hematoxylin, measured approximately 2–3 μm in diameter, and were rarely branched and septate.

Evidence of starvation was apparent. In most instances, food particles were absent from the digestive system and adductor muscles were atrophied. In advanced stages of invasion, tissues were disintegrated, with complete loss of vesicular connective tissue in some specimens. We concluded that larval mycosis was the cause of the observed mortalities. The fungus appears to be an opportunistic pathogen causing significant morbidity and mortality when conditions are optimal for its pathogenic development. Bivalve cultures in hatcheries often collapse and new cultures are initiated. The cause or causes of mortality are rarely determined. We suggest that larval mycosis may be a common, underrecognized cause of such events.

NEW DEVELOPMENTS WITH NONNATIVE SHELLFISH SPECIES IN THE CHESAPEAKE BAY.

Standish K. Allen, Jr., Aquaculture Genetics and Breeding Technology Center, Virginia Institute of Marine Science, Gloucester Point, VA 23062.

VIMS has been systematically examining the potential of several nonnative species to alleviate the serious decline of oyster stocks in the Virginia portion of the Chesapeake Bay. One that has emerged as quite promising is the Suminoe oyster, *Crassostrea ariakensis*. Field trials with sterile triploids have shown a general resistance to disease, rapid growth rate, and high survival. Formal and informal test marketing of the product has been similarly successful. As with any nonnative species, serious concerns exist about the long-term implications of introduction. Some of these issues are associated with simultaneous introduction of pests or pathogens, and some are associated with the ecological effects of sustained population growth in the Bay. By culturing this species in the hatchery, pest and pathogen issues are largely, but not wholly, addressed. Aquaculture of triploid-only individuals mitigates, but does not eliminate, most of the ecological concerns. Aquaculture of hatchery-raised sterile triploid seed represents an intermediate solution to assisting the industry between abandoning research on nonnative species and wholesale introduction of diploids. The industry potential is enormous, but there are lingering questions about how precisely this "revolution" will or won't proceed.

THE EFFECTS OF VARYING COLD WATER TEMPERATURES, SIZE AND POPULATION DENSITY ON THE GROWTH AND MORTALITY RATES OF BAY SCALLOPS, *ARGOPECTEN IRRADIANS IRRADIANS*, IN AN UPWELLER.

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The growth of scallops has been known to slow in cold temperatures. The objective of this experiment was to observe the growth and mortality rates of larger and smaller sized bay scallops sorted into silos of varying densities in an upweller with decreasing coldwater temperatures.

To set up the experiment, five silos were filled with smaller scallops (10–20 mm) and five silos were filled with larger scallops (21–30 mm). Each silo of larger and smaller scallops varied in population density. The five silos of smaller scallops had 300, 700, 800, 900, and 1,200 scallops per silo, respectively. The five silos of larger scallops had 150, 180, 250, 330, and 350 scallops per silo, respectively.

Once a week, data were collected from a sample size of 30 scallops from each silo. Using a caliper, the size of the scallops was recorded to observe any growth. Any mortality within the sample population was also noted. Eight weeks into the experiment, January 10, 2002, all of the scallops were counted in each silo and all of the deaths were recorded. Using the data, mortality ratios of the entire population for each silo were calculated. A total count was done to verify the accuracy of our sample populations. The scallops were then returned to the silos for further study.

The results of this experiment show that for the varying population densities that we measured, the larger scallops continued to grow and had a low mortality rate in coldwater temperatures ranging from 13.21 to 0.27°C. The population density of each silo for the larger scallops had little effect on the growth and mortality rates. The growth and mortality rates in the smaller scallops were greatly affected by the population densities in our experiment. The silos with the fewest scallops (296 and 659) showed the most growth, whereas the silos with the highest density of scallops (1,162 and 844) showed less growth. For the smaller scallops, the silos with the highest density had the greatest mortality rate. The silo containing 1,200 scallops had a mortality rate of 10.4%, whereas the mortality rates for the remaining smaller groups of scallops had a mortality rate of approximately 5%. Experimental testing will continue through April in order to reach valid conclusions from this study.

CATCH STATISTICS OF *HOMARUS AMERICANUS*, THE AMERICAN LOBSTER, FROM A 3-WK STUDY CONDUCTED BY STUDENTS FROM THE SOUND SCHOOL REGIONAL AQUACULTURE CENTER ABOARD A COMMERCIAL LOBSTER VESSEL IN THE WATERS OFF FISHERS ISLAND, NEW YORK.

Amber L. Beitler and **John J. Roy**, The Sound School, 60 South Water St., New Haven, CT 06519

During the summer of 2001, from July 9 to August 1, six students from the Sound School Regional Aquaculture Center kept catch statistics on *Homarus americanus*, the American lobster, while interning as deck hands aboard the *UP FOR GRABS*, a commercial lobster vessel berthed in Noank, Connecticut. The student time aboard the vessel was credited toward their Supervised Occupational Experience (SOE) compliance requirements. The *UP FOR GRABS* fishes out of Noank and possesses both a Connecticut and a New York State Commercial Lobstering License. Eighty-five percent of the gear was fished on grounds that lie between an east-west line that can be drawn through Ram Island and Sea Flower Reef on the northern (Connecticut) side and a line running east to west 0.5 miles north of Fishers Island on the southern (New York) side of Fishers Island Sound. The remaining 15% of the gear was fished in an area just north of Ram Island. No differentiation was made between the areas being fished. Trawls were at times moved within this zone from areas of low productivity to areas where the catch was found to be higher, during a given week.

The time of the students' internship corresponded with the peak season for lobster capture in that area. Traditionally termed "the run" in the commercial lobster community, the month of July is considered to be the peak month for lobstering in the entire year. Forty-seven pot trawls were fished each week. All of the pots that were fished were wire. An average of 10 trawls, or 70 traps, were hauled each day, 4 days each week. Each trawl was hauled once each week. The students recorded the catch statistics from the 280 traps as each trawl was landed. The data that were taken included: the number of legal lobsters that were caught in each trawl; the number of sub-legal lobsters (or shorts) that were returned to the water from each trawl; the number of male and female legal lobsters found in each trawl; the number of dead lobsters found in each trawl; and the number of lobsters that exhibited shell-rot disease in each trawl. All categories were totaled daily. The daily totals were then compiled by week for 4 wk. A total of 2,018 lobsters were caught during the study.

Forty-five percent of the lobsters (191) landed in the first week were of legal size. The number of legal lobsters (341) that was landed in the second week increased by 56%. The third week of the study had a 4% drop in the number of legal lobsters (322) taken. There was a 38% drop in the number of legal lobsters (201) landed in the final week of the study. The percentage of sub-legal lobsters varied between 44% and 54% of the total number of lobsters taken each week during the study. More legal male lobsters were caught in the first and second week, whereas legal female lobsters appeared in greater numbers during the final 2 wk of the program. The number of dead lobsters found in the trawls ranged from 0.65% to 1.44%. All deaths were attributed to cannibalism, predation, or physical impact of the trap or trap components. The number of lobsters exhibiting shell-rot disease was less than 1% throughout the entire study. However, because of the proximity to the molt, this percentage may become greater later in the season.

The students at the Sound School had first hand experience with the dramatic declines in the lobster populations in western Long Island Sound during the late 1990's. We believe that it is becoming increasingly important to monitor accurately the existing stocks of *Homarus americanus* at all levels of the fishery, as well as in the scientific community. A concerted effort will be required to ensure the survival of this important natural resource.

EFFECTS OF WATER VELOCITY ON CONDITIONING OF SUMMER FLOUNDER *PARALICHTHYS DENTATUS* FOR NET PENS.

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Attempts in the late 1990s to rear summer flounder in net pens suffered from high mortality during the month after fish were transferred from the nursery facility to pens. As part of the University of New Hampshire's Open Ocean Aquaculture Demonstration Project (OOADP), we investigated whether exposing summer flounder to increased (and constant) current velocity in the nursery stage would condition them for better performance in cages placed in Narragansett Bay, Rhode Island (not the OOADP site).

Three experiments, two of 60-day duration and one of 30-day duration, were conducted with different water velocities in tanks, using fish of 124 ± 4 g (exposed to 0, 15, or 30 cm/sec for 60 days), 257 ± 12 g (exposed to 0, 20, or 40 cm/sec for 60 days), and 387 ± 13 g (exposed to 0, 15, or 30 cm/sec for 30 days), in a raceway system with adjustable paddlewheels. For all of the size groups of fish, survival was significantly reduced at the highest current velocity. For the 124-g fish, survival in high velocity (26 ± 1%) was significantly lower than that in medium velocity (57 ± 7%) and in low velocity (67 ± 6%). For the 257-g fish, survival in high velocity (35 ± 19%) was significantly lower than that in medium and low velocities (100% in both cases). For the 387-g fish, survival in high velocity (50 ± 11%) was significantly lower than that in medium velocity (98 ± 2%); all 387-g fish in the low-velocity treatment were lost due to a system malfunction. For both 124- and 257-g fish, growth in the medium-velocity treatment was significantly better than that in the low-velocity treatment, which in turn was better than that in the high velocity treatment. For 124-g fish, growth was 76 ± 12 g, 49 ± 8 g, and 39 ± 0 g in the medium, low, and high velocities, respectively. For 257-g fish, growth was 47 ± 10 g, 25 ± 4 g, and −7 ± 17 g (weight loss) in the medium, low, and high velocities, respectively. For 387-g fish, growth at medium velocity (26 ± 6 g) was significantly greater than that at high velocity (−22 ± 8 g). Food consumption data from the 257-g fish showed that the fish in medium velocity grew most because they consumed significantly more food during the experiment (1,622 ± 128 g per tank) than did fish in low velocity (915 ± 65 g), which in turn consumed significantly more than fish in high velocity (640 ± 90 g). Nevertheless, there was no significant difference in food conversion ratio (FCR) between fish at low velocity (1.54 ± 0.37) and those at medium velocity (1.37 ± 0.23).

At the end of the experiment with 124-g fish, fish from the low-velocity and medium-velocity treatments were moved to cages in Narragansett Bay, where currents of about 1 knot (approx. 55 cm/sec) are routine. After 3 wk in the cages, no significant differences in survival were observed (low velocity = 83% ± 12%; medium velocity = 81% ± 2%). Subsequent damage to some of the cages and escapement of the fish precluded further statistical analysis of survival, as well as any growth measurements. We conclude that current velocities of 15–20 cm/sec in the nursery improve growth of juvenile summer flounder, that current velocities of 30–40 cm/sec are excessive, but that increased current velocity in the nursery does not improve fish survival upon transfer to cages.

REMINISCENCES OF EARLY PIONEERS IN OYSTER CULTURE. **Luther Blount,** Blount Shipyards, Warren, RI 02885.

In the late 1920s and early 1930s, there were about six oyster companies in Warren, Rhode Island, which not only worked closely with the Connecticut oyster people but also had connections in the nearby Taunton River and Assonet Bay areas in Massachusetts, where there was always a good oyster set.

In those days, oyster fishermen in Rhode Island were really into summer clam bakes, where all the oystermen gathered. My uncle, Byron Blount, of E.B. Blount and Sons, often invited Dr. Paul Galtsoff there. In fact, Dr. Galtsoff would drive over from Woods Hole to talk oysters with my uncle. So I knew him as a white-haired scientist discussing good and bad oyster sets and this always was his primary subject. Dr. Victor Loosanoff of the Milford Laboratory, Milford, CT followed Dr. Galtsoff's visits to my uncle and I happened to be there the day he showed us a paper egg crate coated not only with cement, but also literally loaded with thousands of oyster set. For the past half century I found myself working with these shellfish pioneers and learning from them as I built Prudence Island Farms.

Because we had previously bought spat on scallop shells from Dr. George Matthiessen on Fishers Island and knew how he got them, we looked for a marine biologist to help us get a set from Green Hill Pond on the Rhode Island south shore. We hired a young man who just graduated from the University of Rhode Island, called him "John Oyster," and began to buy scallop shells and get rafts made, which are used today in my salt water oyster pond on Prudence Island.

"CRAB SIGHTINGS" IN LONG ISLAND SOUND DURING 2001. **Diane J. Brousseau,** Biology Department, Fairfield University, Fairfield, CT 06430; **Ronald Goldberg,** USDOC, NOAA, National Marine Fisheries Service, Northeast Fisheries Science Center, Milford Laboratory, Milford, CT 06460.

During summer and fall of 2001, there were a number of inquiries to the Milford Laboratory about observations of crustaceans in Long Island Sound. In July, swarms of megalopae of the Asian shore crab, *Hemigrapsus sanguineus*, were seen both in the water column and rafting on floating rockweed near Charles Island in Milford, Connecticut. These larvae were likely the result of an early summer spawning. Recent increases in population size of *Hemigrapsus* may account for greater abundance of the larvae than in the past. In early fall, reports were again made of dense aggregations of small crabs swimming close to the surface. Initially mistaken for Asian shore crabs by many observers, they were later identified by Nizinski (pers. comm.) as sub-adult pinnotherids, *Pinnixa chaetopterana*. This small crab is often found living as a commensal within the tube of the polychaete worm *Chaetopterus*. *Pinnixa* sightings were widespread, occurring in the Thimble Islands, Milford Harbor, and Housatonic River in Connecticut, and near Orient Shoals off Long Island. Fall swarming of *Pinnixa* prior to settlement has been observed previously by Mroczka (pers. comm.), but the early life history of this species has not been described fully. In October, an adult male shame-faced crab, *Calappa flammea*, was collected in shallow water by a scuba diver in Stonington, Connecticut. *Calappa* is predominantly tropical to subtropical, but larvae can drift as far north as southern New England, accounting for occasional occurrence of adults in this region. This molluscivorous crab spends much of its time buried in the sand, making brief excursions to search for food. The observations described above are not unique, but may have been more apparent during 2001 because of interannual variability in climate or the population dynamics of the species involved.

SOME CLASSICAL PERSONALITIES IN THE SHELLFISH RESEARCH ARENA: ECLECTIC OBSERVATIONS OF AN ERSTWHILE OBSERVER. Walter J. Canzonier, M.R.O.C.F., P. O. Box 662, Port Norris, NJ 08349.

The personalities of many of the scientists associated in the past with the shellfish research and development community might be of general interest to those currently engaged in this field. The personalities of these individuals, other than making them colorful and intriguing characters, probably also had a considerable influence on the approaches they used in addressing problems requiring scientific or technological intervention. Ergo, to better appreciate the contributions of some of the members of the community involved with the recent, and not-so-recent evolution of shellfish research, it has been suggested that it might be both entertaining and educational to have a glimpse at their personae. Using a capsule review of some of the key players in the shellfish research and development saga, I will attempt to share with the more recent entrants in the game a few anecdotal sketches of some of those individuals with whom I have had contact in my 45 years of involvement with the academic and the industry elements of molluscadom on the East Coast. This informal documentary, as distorted as it might be as a result of the undoubtedly biased perspective of the reporter, is offered to the curious in hope that it will help them gain a better insight into the personalities of those whom they have encountered merely as names in the nondigitized literature. Some of those mentioned were colleagues, others mentors, still others known to me primarily through accounts by relatives or close associates. Interest in documentation of personalities was first stimulated by the efforts of Sewell Hopkins, who prepared a set of three unpublished but extremely interesting and informative memoirs, two of which (University of Illinois and Project Nine) present enlightening and amusing sketches of his teachers and colleagues.

In this rambling account of the members of the "Old Guard," I will attempt to reconstruct the images of some of the well-recognized, as well as the lesser-known individuals. Certainly the names of Julius Nelson (known to me through the stories from his son); Galtsoff and Loosanoff, who worked in this immediate area; T. C. Nelson; J. G. Mackin and Sammy Ray of dermo fame; J. D. Andrews; and H. H. Haskin will probably be familiar to many currently working in this field. Other, less commonly recognized names include W. F. Wells, known to me only from exchanges of vitriolic correspondence with T. C. Nelson; L. A. Stauber, a polyvalent researcher more noted in the field of parasites of vertebrates, but always available to his students and colleagues as a valid anchor in the basic principles of shellfish pathology and physiology; his students S. Y. Feng and M. R. Tripp, among others; Joe Glancy, who doggedly pursued development of commercial shellfish hatchery technology despite strident attempts to rectify his errant behavior by his neighbor across the Sound; Mike Castagna who pursued a similar course in more southern waters; and M. R. Carriker, Carl Shuster, Herb Hidu, Tom Cheng, and others who at some point in their careers had close ties with the Department of Oyster Culture of the New Jersey Agricultural Experiment Station.

If asked what might be a common trait of these individuals, my observations would lead me to conclude that it was a capacity to mobilize their imagination to devise a plan of action that efficaciously utilized the often very limited resources available to them—whether this be in the laboratory or in the field. This intrinsic ability to adapt their efforts to the resources at hand enabled them to make significant contributions in the realm of academia, and to cost-effectively assist the shellfish industry in its quest for more productive culture practices and strategies. For this vanishing breed, a lack of sophisticated facilities and equipment was never considered to be an insurmountable limitation, but merely a challenging impediment to be circumvented by innovative tactics.

MASSACHUSETTS OCEAN RESOURCE INFORMATION SYSTEM (MORIS). Diane Carle, Office of Coastal Zone Management, Executive Office of Environmental Affairs, 251 Causeway Street, Suite 900, Boston, MA 02114.

Massachusetts Coastal Zone Management (CZM) has embarked on a long-term project to develop the Massachusetts Ocean Resource Information System (MORIS). MORIS will be a comprehensive database and ArcView GIS extension providing access to the broad range of information necessary to carry out the CZM mission. The first phases of the MORIS project were completed in 2001 and focus on information and tools useful for screening for potential aquaculture sites.

CZM contracted EVS Environmental Consultants to conduct the initial data mining and GIS application development. CZM also teamed with NOAA's Coastal Services Center (CSC), Massachusetts Division of Marine Fisheries (DMF), and Massachusetts Department of Environmental Protection (DEP) to add a "georegulation" tool that allows users to query an area for aquaculture-related regulations. Users are presented with a list of relevant regulations and can view summaries as well as the actual legislation. Users can also view the web sites of the relevant regulatory agencies. The CSC is also creating new data layers for the project, including benthic maps of selected areas of the Massachusetts coast and maps of areas suitable for sustaining shellfish growth. CZM is completing maps of existing aquaculture leases in Massachusetts. Future phases of the project will develop new water quality data layers and enhance the applications' water quality mapping capabilities. CZM is also pursuing funding to create an Internet mapping site for the project.

The MORIS CD containing the ArcView extension and database is available at no cost from CZM.

HABITAT SUITABILITY ASCERTAINED BY GROWTH AND SURVIVAL OF BAY SCALLOPS IN TIERED CAGES.

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A laboratory-spawned line of bay scallops (*Argopecten irradians irradians*) was used to evaluate the effectiveness of commercial three-tier, rigid-mesh cages for growing scallops in two proximate but dynamically different sites in eastern Long Island Sound. With permission of the Groton Shellfish Commission and the State of Connecticut Aquaculture Division, we established comparative field sites on the western side of Ram Island (low dynamic, gentle tides), and the southern end of the island (high dynamic, tidal current of up to 2 knots).

The scallops were spawned in March and held in temperature-controlled tanks in the Milford Laboratory at 22°C until May when ambient seawater temperature reached about 15°C. The scallops were then acclimated to the lower temperature and distributed in outdoor raceway tanks using densities determined as optimal—5 L biomass per tank. In late July, scallops with a mean size of 25 mm were deployed at each site, with a starting biomass of 2 L (approximately 400 scallops) in the top tiers of a set of two cages at each site. The cages were made of plastic-coated wire with a 7.5-cm mesh. Each cage measured 56 × 56 × 94 cm and was divided horizontally into three sections or tiers. Two ballast areas below the bottom tier provided an offset from the sea floor of approximately 15 cm. Cage inserts of smaller mesh (10 and 17.5 mm) measuring 41 × 10 × 81 cm were used to hold the scallops. After 1 month, the scallops were divided into three densities of 50, 100, and greater than 150 scallops per tier in each of the cages. The cages were checked monthly to the end of the experiment to determine survival and growth, with the added attention to shell indentations that might indicate density problems, and to check and remove fouling organisms.

Results from the field experiment indicated that satisfactory growth of scallops occurred at both sites. The initial growth rate was rapid, but then leveled off as the water temperature decreased. Growth of scallops was slightly better at the western side of the island (mean size of 51 mm) than at the southern end (mean size of 48 mm). There was no difference in the mean size of scallops in the top and middle tiers, with 50 and 100 scallops, respectively. Bottom tiers of all cages, stocked with greater than 150 scallops, did show slightly slower growth and higher mortality. Scallops in the cages from the southern, high-dynamic area exhibited more shell indentations; this could be attributable to the strong currents pushing the scallops together for periods of time, thereby causing a temporary decrease in available cage area that may mimic effects of higher shelf densities. Further studies are warranted in similar habitats to corroborate these results.

HORSESHOE CRAB AQUACULTURE: PRELIMINARY RESULTS FROM HATCHING AND REARING STUDIES.

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Previous studies undertaken by the two senior authors during the summer of 2000 at the National Marine Fisheries Laboratory in Milford, CT resulted in the successful spawning of captive adult horseshoe crabs, *Limulus polyphemus*. The fertilized eggs were allowed to develop under conditions approximating field conditions, although predators were kept to a minimum. The eggs had a hatching rate of 60% over an initial 2-mo period, followed by a 98% hatching rate over the course of 10 mo. Evaluation of the long-term survival (greater than 1 y) of *Limulus* was not completed successfully because of a malfunction in the rearing tanks.

This study was undertaken in an effort to evaluate the role of different food sources on the growth and survival of post-hatch horseshoe crabs. Approximately 4,500 eggs were kept in small, experimental bowls filled with Long Island Sound (LIS) seawater and aerated. Initial egg densities varied per bowl, with a maximum density of 1,500 and a minimum density of 50. All bowls were checked daily for hatchlings. Upon hatching, all hatchlings from an individual bowl were removed to a separate bowl, with no more than 50 per bowl. Hatchlings were kept in aerated LIS seawater and a series of behavioral observations were made on them. Hatchlings in bowls were checked daily for molting.

Molted individuals were removed from hatchling bowls and placed in individual bowls of aerated seawater. The number of post-hatch molted individuals per bowl ranged between 10 and 30. Horseshoe crabs at this stage were separated into three groups for preliminary experiments examining the role of diet on growth and survival. They were fed one of three food items: newly hatched brine shrimp, rotifers, or concentrated dried food flakes. The juveniles were checked every day and any secondary molts or deaths were noted. When an organism underwent a secondary molt, that organism was transferred to a new bowl and fed one of the three food choices. This same procedure was repeated for every new molt stage reached. At the conclusion of the experiment, all surviving post-hatch molts (second and upward) were removed and placed in aerated, 10-gal aquaria containing artificial seawater and a bottom covered with 3 cm of fine sand.

The results suggest that diet may play a supporting role in the growth and survival of post-hatch *Limulus* juveniles, although this factor needs further investigation. In general, all juveniles reached their first post-hatch molt between 5 and 12 days. The time to second post-hatch molt varied with diet. There was a slight difference in molt timing among the three test groups. Juveniles (first post-hatch molt) reared on a diet of rotifers underwent a second post-hatch molt within 7–12 days, and those reared on a diet of brine shrimp generally molted between 10 and 14 days. Juveniles reared on flaked food generally molted between 9 and 12 days. Of these juveniles, those fed on a diet of brine shrimp experienced a mortality rate of up to 5%, whereas those fed on rotifers or flaked food experienced a mortality rate of <1%. Time to third post-hatch molt varied, with juveniles reared on flaked food molting sooner than juveniles reared on either brine shrimp or rotifers. Those fed flaked food were significantly more likely to molt (>90%) and survive (>85%) than those fed either rotifers or brine shrimp.

The results from these initial studies suggest that food quality and type has only a slight effect on the two earliest post-hatch molt stages of *Limulus polyphemus* juveniles, but has a strong effect upon the third post-hatch molt. It appears likely that this effect is related not only to the food type, but also to the life habit of the horseshoe crab, and that any rearing plan for this species must include at least a two-phase food supply consisting initially of zooplankton, followed by food that is mixed in with the sediments. Research on the effects of food quality on the growth of *Limulus* juveniles continues at this time.

A COOPERATIVE STUDY ON THE AQUACULTURE OF *PORPHYRA LEUCOSTICTA* (RHODOPHYTA) FOR AN INTEGRATED FINFISH/SEAWEED RECIRCULATING AQUACULTURE SYSTEM IN AN URBAN APPLICATION.

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Aquaculture represents an excellent opportunity to help rejuvenate blighted coastal urban areas on the Northeast coast. On land, aquaculture requires a relatively small amount of space; building space can often be acquired at reduced cost in ungentrified city areas and aquaculture can represent an attractive, environmentally benign form of commerce. However, finfish and shellfish aquaculture operations are a source of an effluent with high concentrations of dissolved inorganic nutrients (N, P). To prevent eutrophication, the EPA is developing stringent guidelines for the release of N and P into coastal waters. An integrated recirculating aquaculture system, coupling the growth of seaweed and fish, can solve these problems for urban aquaculture facilities. Not only is the effluent remediated (the seaweed requires for growth the waste products produced by the fish), but an additional multiproduct, high-value crop can be generated. Marine aquaculture in the Northeast consists primarily of coastal pen-based salmon production and shallow benthic production of shellfish (mussels, clams, and oysters). There are very few tank-based (on land) marine aquaculture operations in the Northeast. One operation is GreatBay Aquaculture LLC (Portsmouth, NH), a land-based hatchery and grow-out facility for summer flounder and cod, high-value fish that are being sold to the U.S. and Japanese sushi and sashimi markets and to "white tablecloth" restaurants.

Our work is part of a multi-university effort (University of Connecticut, State University of New York at Purchase, University of New Hampshire, University of New Brunswick, and Shanghai Fisheries University) with GreatBay Aquaculture LLC to develop an integrated finfish/seaweed recirculating aquaculture system (RAS) suitable for urban aquaculture. The unique aspect of our RAS system is that it will be integrating the culture of finfish (i.e., summer flounder) and native species of seaweed (i.e., *Porphyra*). The red alga, "*Porphyra*", is the most valuable maricultured seaweed in the world, with an annual value of more than $1.8 billion. *Porphyra* (nori) is primarily used for food as the wrapping around sushi rolls; it is a major dietary source of taurine (controls blood cholesterol levels) and is rich in proteins, vitamins, trace minerals, and dietary fiber. On a dry weight basis, *Porphyra* can be worth more as a source of biochemicals than as food. It is the preferred source of the pigment, r-phycoerythrin, utilized as a fluorescent tag for biotechnological applications. The United States is dependent primarily upon foreign sources (i.e., China, Japan, and Korea) for nori via coastal net culture. BRVAS students are working alongside undergraduate and graduate students in the construction and operation of these systems.

The life history of *Porphyra* includes a microscopic filamentous stage (the conchocelis stage) and the more conspicuous macroscopic blade stage that grows attached to intertidal and shallow subtidal substrate. There are at least seven recognized species of *Porphyra* in the Northeast. We have begun evaluating one of these native species (*P. leucosticta*) as a candidate for the integrated RAS, because it may be one of the best sources for the sushi food industry and for r-phycoerythrin. We are developing the mass culture techniques (in both free culture or attached to nets) for this native species of *Porphyra*. We will report on the mass seeding technologies that we have developed and the specific growth rates of *P. leucosticta* at the BRVAS culture facilities.

UPDATE ON SHELLFISH RESTORATION AND REVIEW OF LOCAL HATCHERIES IN NEW JERSEY.

Gef Flimlin, Rutgers Cooperative Extension, Toms River, NJ 08755.

Cooperation among several sectors that work with water and shellfishery issues has led to two new accomplishments that combine an interest in improving both the ecology of the waterways and the potential economy of the shellfish industry in New Jersey.

In the summer of 2001, 10,000 bushels of washed oyster shell were deposited to construct a new reef in a near shore area in Keyport Harbor in western Raritan Bay. The project was spearheaded by the New York/New Jersey Baykeeper, with assistance from the National Marine Fisheries Service, Rutgers Cooperative Extension, local shellfishermen, and the NJ Department of Environmental Protection's Bureau of Shellfisheries. In a community-supported effort, 10,000 oysters, which had been raised in Taylor Floats by volunteers from the area, were deposited on the reef to establish a community. This effort of growing oysters by the volunteers continues.

Later that year in the fall, with the support from the South Jersey Economic Development District, the last oyster boat on the New Jersey Atlantic Coast moved 2,000 bushels of oyster seed to an old bed at the mouth of the Mullica River. The Fitney Bit bed will have been closed by the Bureau of Shellfisheries for a year when it is opened for public harvest. Industry representatives and Rutgers Cooperative Extension, who acquired the funds to do this work, are presently looking for more funds to expand this work.

Shellfish hatcheries in New Jersey have been reevaluating their production in light of challenges from Brown Tide blooms and unexplained hatchery mortalities.

FLOW CYTOMETRY AS A TOOL TO QUANTIFY THE OYSTER PHAGOCYTOSIS, RESPIRATORY BURST, AND APOPTOSIS. **Michael J. Goedken** and **Sylvain De Guise**, Department of Pathobiology, University of Connecticut, 61 N Eagleville Road, U-89, Storrs, CT 06269.

Infectious diseases are a significant problem in oyster aquaculture and cause immense production losses. The protozoan parasites *Perkinsus marinus* and *Haplosporidium nelsoni* have generated losses estimated in the hundreds of millions of dollars over the last 35–45 y in the Middle Atlantic states.

The relationship between parasites and oyster defense mechanisms is unclear. A better understanding of the immunopathologic association may reduce these economic losses. 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. Apoptosis was evaluated with TUNEL assay. Three subpopulations of hemocytes (granulocytes, hyalinocytes, and intermediate cells) were identified with unique functional characteristics. Granulocytes were most active at phagocytosis and peroxide production, whereas hyalinocytes were relatively inactive. TUNEL assay application allowed quantification of hemocyte programmed cell death with temperature-dependant changes. 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 bivalve immune system.

COBIA CULTURE. **Josh Goldman**, Fins Technology, Inc., 15 Industrial Rd., Turners Fall, MA 01376.

Cobia (*Rachycentron canadum*), is considered a prime candidate for culture, given its extremely fast growth rate and excellent flesh quality. Interest in cobia culture has grown substantially in the last 5 y, fueled by reported successes in extensive, net-pen operations in Taiwan, and as U.S. researchers have gained some familiarity with captive fish. Fins Technology, with support from NOAA's National Sea Grant, has investigated a variety of aspects of intensive cobia culture in an effort to develop protocols for commercial production. We have evaluated growth and feed conversion efficiency at 10, 20, and 50 ppt salinity in artificial seawater, and have instituted routine bacterial and histological monitoring of our populations to assess the risks and develop appropriate health management strategies needed to support commercial production. During the last 2 y, we and our collaborators hope to demonstrate commercial feasibility of this promising species.

CREATING OPPORTUNITIES FOR STUDENT AQUACULTURE PROJECTS. **Eric Goodman, Colleen Cook,** and **Michael Weiss,** Friends Academy, Duck Pond Rd., Locust Valley, NY 11560.

The goal of our ongoing project is to increase awareness and participation in shellfish biology, habitat preservation, and aquaculture techniques among our peers. This group currently includes seven students in Friends Academy, our high school, as well as five students from Locust Valley and Oyster Bay, two nearby public schools on the North Shore of Long Island. The project began with a program designed as a basic introduction to shellfish aquaculture for local educators to motivate them to start similar projects in their schools. We held a series of seminars for students who showed an interest in our work and who wished to design an aquaculture project of their own. The topics of these seminars included working in the lab with algae and constructing grow-out equipment. Our next initiative was to construct a facility that was open to the public to increase the number of educational and research opportunities in the community. This facility, which is under development, is a hands-on center that includes a classroom as well as a fully functioning shellfish hatchery. We will be growing the Atlantic bay scallop (*Argopecten irradians irradians*) as well as the eastern oyster (*Crassostrea virginica*). Lessons in hatchery operation and maintenance, shellfish life cycles and anatomy, and the use of a FLUPSY are also taught in the hatchery. We hope that through our work in the center, students will use the hatchery to conduct their research relating to shellfish life cycles during the school year.

AN EXPERIMENTAL INVESTIGATION OF DIETARY FATTY ACIDS AND STEROLS AND THE IMMUNOLOGY OF THE AMERICAN OYSTER, *CRASSOSTREA VIRGINICA*: A WELL-FED OYSTER IS A HEALTHY OYSTER, N'EST-ÇE PAS? **Hélène Hégaret,** École Nationale Supérieure Agronomique de Rennes, France; **Gary H. Wikfors,** NOAA Fisheries, NEFSC, Milford, CT 06460; **Philippe Soudant,** Universite de Brest, France; **Maryse Delaporte, Jeanne Moal,** and **Jean-François Samain,** Laboratoire de Physiologie des Invertébrés, IFREMER, Brest, France.

The lipid composition of the algae fed to oysters is very important because fatty acids and sterols in the membranes of oyster cells to some extent have a dietary origin. The fatty acid and sterol composition of hemocyte membranes is thought to affect immune function, thereby linking nutritional status with ability to respond to environmental and health stresses. To investigate this linkage, we designed an experiment in which two replicates of seven feeding treatments were applied to the oyster, *Crassostrea virginica*, and hemocyte function was evaluated before and after a subsequent high-temperature stress. Twelve oysters (ca. 50 mm) were put into each of 14 computer-controlled feeding chambers. Algal diets fed at 10% and 50% daily rations (dry wt/dry wt) were LB 1077/1B (*Skeletonema costatum*), PLY429 (*Tetraselmis chui*), and a 50/50 (dry wt) mix; we also included two unfed controls. These two algae have different lipids: LB1077/1B contains the fatty acids C20:5(n-3) and C22:6(n-3) and cholesterol; whereas, PLY429 contains C20:5(n-3), but no C22:6(n-3), and 24-methyl and 24-methylene cholesterol. After 5 wk of feeding at 20°C, we sampled half of the oysters for hemocyte analyses and gill-membrane lipid composition, subjected the remaining oysters to 1 wk of high-temperature (28°C) stress, and sampled as previously. Gill membrane lipids will be analyzed by GLC (work in progress). Hemocyte function was evaluated by five methods employing the FACSCAN flow cytometer: identification of hemocytes by SYBR green fluorescence, viability by propidium iodide fluorescence, adherence and aggregation by forward- and side-scatter, phagocytosis using fluorescent plastic beads, and respiratory burst by a newly modified method using Zymosan A as the activator. These response variables were tested statistically, first using a nested ANOVA model, with feeding ration and regime as independent variables, and in second way using discriminant analysis.

Individual hemocyte functions were not affected significantly by feeding ration or regime; however, a consistent trend relating the higher ration with enhanced hemocyte function was apparent. By contrast, differences between unfed and fed oysters were, in many cases, highly significant and appreciable, with unfed oysters showing decreased function in most subpopulations of hemocytes (granulocytes, hyalinocytes, and intermediate cells). Discriminant analysis, using data from multiple hemocyte measurements, was able to differentiate between high- and low-quantity feeding treatments, as well as between the different algal diets. These findings indicate that nutrition does affect immune function in oysters, thereby mediating their response to stress.

THE ROLE OF THREE BACTERIA IN SHELL DISEASE OF THE AMERICAN LOBSTER (*HOMARUS AMERICANUS*).

Andrea Hsu, Erin Summers, and **James Estrada,** Boston University Marine Program, Marine Biological Laboratory, 7 MBL St., Woods Hole, MA, 02543; **Roxanna Smolowitz,** Marine Biological Laboratory, 7 MBL St., Woods Hole, MA 02543.

Although typically infecting impounded lobsters, lobster shell disease is becoming increasingly more prevalent in wild populations throughout the New England region. This study utilizes scanning electron microscopy (SEM) and histological analyses to describe the morphology and prevalence of bacterial cells present on carapace samples taken from two wild-caught lobsters with shell disease and one lacking any noticeable infection. SEM analysis revealed and statistical tests verified three separate morphological types of bacteria present on both carapace samples and cultures taken from shell lesions. Results from bacterial identification suggest two bacteria to be of the genus *Vibrio*, whereas the third is a *Pasteurella*-like organism. Bacteria on infected carapace were seen to concentrate on intact epicuticle, the edge of lesions, microscopic cracks and holes, and setal pores. Halo-like holes surrounded all bacterial types, closely matching the shape of the bacteria, suggesting that each bacterium is boring into the epicuticle. Healthy carapace showed substantial bacterial concentrations present only around carapace setae. Several of these bacteria were seen with small-bore holes surrounding them, but active boring and degradation of the epicuticle was minimal in contrast to the infected carapace. Histological observations show that there is a difference between the breakdown of wild and impoundment lesions. Shell lesions of wild lobsters show lattice-like cuticular remnants attached to underlying less degraded cuticle in several eroded foci. This study documents not only the presence of three bacterial species in shell disease of wild-caught New England lobsters, but also illustrates their role in the degradation of the carapace. This is the first evidence of the mechanism that is used to break down the epicuticle of the lobster shell, and suggests that different organisms are involved in causing wild and impoundment shell disease.

DERMO INVESTIGATIONS, RAZOR CLAM NURSERY TRIALS, AND PRELIMINARY BAY SCALLOP ADHESION CULTURE EFFORTS.

Richard C. Karney and **Amandine Surier,** Martha's Vineyard Shellfish Group, Inc., Box 1552, Oak Bluffs, MA 02557; **David W. Grunden,** Town of Oak Bluffs, Box 1327, Oak Bluffs, MA 02557; **Thomas E. Berry,** Martha's Vineyard Shellfish, Box 1660, Edgartown, MA 02539.

To compare the infection patterns of dermo disease (*Perkinsus marinus*) in Edgartown Great Pond and Tisbury Great Pond, cages of wild and disease-free cultured oysters were deployed in both ponds on June 20 and 21 and sampled monthly for mortality and dermo infection progression. Man-made breaches of the salt ponds' barrier beaches allowed for some manipulation of water chemistry within the ponds. Edgartown Great Pond was managed to maximize its exchange with the sea to reduce eutrophic conditions believed to be stressing its oyster population. Management in Tisbury Great Pond sought to limit exchange with the ocean in hopes of achieving salinities unfavorable to the dermo parasite. By October, infection rates in both ponds for all of the wild and two of the three groups of cultured oysters were 100%. The infection rate of one group of the cultured oysters in Tisbury Great Pond did not rise above 76%. By November, cumulative mortality of the wild oysters was 19.8% in Tisbury Great Pond and 29.5% in

Edgartown Great Pond. Mortality of the cultured oysters was 4.4% in Edgartown. The mortalities of cultured oysters at two sites in Tisbury Great Pond were 5.5% and 3.4%. The variation in mortality of the cultured oysters appeared to correlate with the densities of natural oyster beds at the sites.

In late August, seed razor clams (*Ensis directus*) with an average length of 20.1 mm were planted at two densities (54.5/ft^2 and 163.5/ft^2) in four different nursery systems—a tidal upweller, mesh-covered bottom boxes, and mesh-covered and open floating sand boxes. Final growth measurements taken in early November were poorest for the high-density tidal upweller (28.8 mm) and best for the low-density open floating sandbox (54.1 mm). In November, clam survival in the sandbox nurseries was as follows: low density/no mesh 56%; high density/no mesh 50%; high density/mesh 48%; low density/mesh 39%.

Several adhesives have been tested in efforts to develop a culture technology based on gluing bay scallops to plastic nets. A suitable adhesive has yet to be located.

SOME CULTURE STRATEGIES FOR GROWING ROTIFERS (*BRACHIONUS PLICATILIS*) AS FEED FOR AQUACULTURE APPLICATIONS. Robin Katersky, Barry Smith, Dean Perry, and **David Nelson,** USDOC, NOAA, National Marine Fisheries Service, Northeast Fisheries Science Center, Milford Laboratory, Milford, CT 06460.

Rotifers are provided as first feed to larval finfish when they are absorbing their yolk sacs and their digestive systems have developed sufficiently to consume food. Marine finfish aquaculture, therefore, requires them in large numbers at these times. Rotifers can be fed marine microalgae for growth and reproduction as well as for enrichment just before they are fed to the fish.

Rotifers typically are grown in large tanks with algal culture added to the volume to replace the algal biomass previously consumed by the rotifers. Culture water is usually changed by draining the tank (and remaining algae), catching the rotifers on a screen, and resuspending them in clean water. Rotifers can consume large quantities of algae. Finfish studies at the Milford Laboratory require production of millions of rotifers. The finfish are fed daily at a density of 10 rotifers/mL in 893-L tanks. With an algal supply of 1 × 10^6 cells/mL, how does one get enough algal biomass for maximal rotifer growth in an economical space and time? We investigated two culture methods to achieve our requirements.

The first culture method investigated was to concentrate the rotifer tank using a hollow-fiber filter to remove 40 L/day of water only. The rotifer tank then was refilled with algal culture. This procedure was repeated daily, providing an average feeding of 200,000 algal cells/mL in 200- to 240-L rotifer tanks. Rotifer tanks were started with 46 rotifers/mL and reached 233 rotifers/mL in 4.8 days.

The second culture method used a different strategy. Rotifers were introduced into a full tank volume of algal culture (150 L) at a density of ~300,000 cells/mL. The rotifers then were left to grow for 4 days with only experimental samples removed. Rotifer densities consistently went from 59/mL to 232/mL in 3 days.

In all cases algae was consumed to below countable levels. The second culture strategy was much less labor intensive than both the traditional and the first method. The second, or all-algae method, reduced labor by over 50% and required three culture vessels to meet demand. For the remainder of the larval rearing period (summer 2001), the all-algae system was used for rotifer production because of its reliability and ease of use. This system produced an average of 30 × 10^6 rotifers/day.

These experiments, together with past work, indicate that there is much more potential for optimizing the culture of rotifers.

ASSESSING HABITAT VALUE OF MODIFIED RACK AND BAG AQUACULTURE GEAR IN COMPARISON WITH SUBMERGED AQUATIC VEGETATION, IN PARTICULAR, AN EELGRASS (*ZOSTERA MARINA*) BED. Brian Kilpatrick, Joseph DeAlteris, and **Robert Rheault,** Department of Fisheries, Animal, and Veterinary Science, University of Rhode Island, Kingston, RI 02882.

Submerged aquatic vegetation (SAV) has attracted considerable attention in recent years because of its role as an essential fish habitat necessary for sustainable fish production. Recent regulations that protect SAV have been critical in conserving and restoring this resource. Current policies that serve to protect SAV have affected applications for construction, docks, dredging, and aquaculture. Opponents of shellfish aquaculture operations argue that the gear used for the grow-out phase of the shellfish reduces the potential for SAV restoration, and may negatively impact estuarine ecosystems.

This study was conducted to compare and contrast habitat value of modified rack and bag aquaculture gear (MRBAG), submerged aquatic vegetation (SAV), and a nonvegetated shallow seabed (NVSB). Habitat value is defined herein by descriptive species diversity statistics and each habitat's ability to support an abundance of organisms throughout the year. The study took place over the course of 1 y, in which each of the three habitats was studied at the end of each season (three replicate samples per habitat). Sampling was performed in Pt. Judith Pond, an estuarine pond in Rhode Island that discharges directly into Block Island Sound. Specially constructed lift nets were used to sample the aquaculture gear, whereas the remaining two habitats were sampled using a drop-net of identical size and a venturi-driven suction sampler. Organisms (>5 mm) in each sample were identified, enumerated, and measured to the nearest millimeter. Environmental data were collected at the time of each sampling period to discern similarities and/or differences among the three habitats. Biofouling organisms were measured in terms of percent cover, and an average surface area (square centimeters per square meter of seabed) of each biofouling phyla and/or class was estimated for each habitat and season. Structural components, in particular, emergent surface area, were measured to determine their role in providing habitat.

Environmental parameters (temperature, salinity, and dissolved oxygen) were not significant between habitats but were significantly different between seasons. Sediment type was primarily sand and was not found to be significantly different ($P > 0.05$) between habitats when the gravel size component (shell hash due to the aquaculture operation) was removed from the MRBAG habitat. The MRBAG habitat supports a significantly higher abundance of organisms per unit area (m^2) of seabed throughout the year. Total numbers of specimens collected are as follows: MRBAG, 6,777 individuals; SAV, 577 individuals; NVSB, 211 individuals. Species richness is consistently higher throughout the sampling periods in the MRBAG habitat as compared to the SAV and NVSB habitats. A two-way ANOVA using the richness estimates generated by the jackknife procedure using quadrat samples (Heltshe and Forrester 1983) indicated significant differences between all three habitats ($P < 0.001$). A Tukey HSD test indicated that MRBAG > SAV > NVSB in terms of species richness values. The species diversity of each habitat was computed using the Shannon-Weiner Index and showed a significant difference ($P < 0.001$) using a two-way ANOVA between habitats. A Tukey HSD test indicated that there were no differences between MRBAG and SAV habitats, but there were differences between MRBAG and NVSB habitats. The Smith and Wilson measure of evenness was used to describe how each habitat was organized in terms of species abundance proportions. MRBAG habitat had a consistently lower evenness value because the MRBAG habitat was dominated by a few species (*Neopanopeus sayi, Tautogolabrus adspersus, Mytilus edulis).* Habitat structural complexity was determined using emergent surface area of the structures found within each habitat. A two-way ANOVA indicated a significant difference ($P < 0.001$) between the MRBAG and SAV habitats in terms of surface area of structure (MRBAG or SAV) per square meter of seabed. These findings suggest that shellfish aquaculture gear provides habitat to many organisms throughout the year and may be ecologically beneficial to many native species of recreationally and commercially important fish and invertebrates, especially in the early life stages of these resources. This research is the first step in determining habitat value for shellfish aquaculture gear.

THE CULTURE OF BLACK PEARL OYSTERS ON SUBSURFACE LONGLINES IN SAVU SAVU, FIJI. **Gordon King,** Taylor Resources, Inc., 130 SE Lynch Rd., Shelton, WA 98584.

Continuing an informal company policy to diversify into new species and geographical areas, Taylor United, Inc., used family knowledge and contacts to develop a Black Pearl Oyster Farm in Savu Savu Bay on the Island of Vanua Levu in the island nation of Fiji.

The subsurface longline system as promoted by Dr. John Bonardelli was adapted for grow-out of the black pearl oyster, *Pinctada margaritifera.* The installation and development of the farm was complicated by political instability and an armed coup in Fiji. Despite these difficulties, the farm now has more than 30 longlines and 30,000 oysters, with several hundred pearls already harvested and several thousand due for harvest; many more are being implanted in 2002. Taylor Resources is employing a three-pronged approach for production of oysters for implantation: spat collection, buying mature oysters, and a joint-venture hatchery. It is believed that the farm will be a solid source of income for the company and is already providing important employment and revenue for the local Fijian population.

URBAN COMMUNITY MEETS AQUACULTURE: A CASE STUDY IN THE NORTHWEST. **Gordon King,** Taylor Resources, Inc., 130 SE Lynch Rd., Shelton, WA 98584.

Taylor United, Inc., an established shellfish farming company in Washington State, sought to expand their suspension mussel farming operation beginning in 1995. This led to a series of events including the formation of a local association to stop further aquaculture development; the production of a sophisticated anti-aquaculture propaganda video; many hundreds of protest letters to the local politicians, the press, and agencies; the requirement by Thurston County for Taylor United to complete an EIS; and a citizen law suit under the Clean Water Act claiming that the company should be required to get an NPDES permit.

Seven years and many tens of thousands of dollars later, the expansion is still on hold. The presentation documents this series of events and explores some of the various parties' attitudes, and asks how much of the friction is due to *bona fide* environmental concerns and how much is due to the gulf in knowledge and understanding between urban Americans and the industries that produce their food?

PROGRESS WITH CULTURING THE RAZOR CLAM (*ENSIS DIRECTUS*). **Dale Leavitt, William Burt, Diane Murphy,** and **Rebecca Hanson,** SouthEastern Massachusetts Aquaculture Center, Massachusetts Maritime Academy, Buzzards Bay, MA 02532.

As reported in 2001, SEMAC has embarked on a study to assess the feasibility of farming the razor clam (*Ensis directus*) in the northeast. Operating on research funds from the Northeast Regional Aquaculture Center (USDA-CSREES), we have contracted with a commercial shellfish hatchery (Aquaculture Research Corporation, Dennis, MA) to produce 1-cm seed razor clams for distribution to commercial shellfish growers throughout the northeast. The growers' role is to test existing or new technology for grow-out of a product for market. This presentation will provide an update of the results of this study after year 1.

Approximately 4 million razor clam larvae were spawned in the hatchery and reared through the nursery stage using conventional hatchery technology similar to that used for the surf clam (*Spisula solidissima*). Roughly 150,000 1-cm juveniles were delivered to SEMAC in July for distribution to the growers. Although the hatchery process needs refinement to increase the larval and early juvenile survival rate, we demonstrated that the hatchery stage of razor clam production is feasible and can potentially produce enough juveniles to sustain a commercial effort in razor clam farming. Adjustments to the hatchery procedures are currently being made as we enter our second year of hatchery production under this project.

The 2-cm juvenile razor clams were distributed to 10 commercial shellfish growers, ranging from New Jersey to Massachusetts, who had been selected through a competitive proposal process. Each grower received an allotment of seed with funds to construct their proposed culture system. Technology being tested ranges from conventional quahog culture techniques using netted raceways to floating culture trays and containment systems deployed on the bottom. The seed were distributed late in the growing season this year, but it allowed the growers to field test their proposed grow-out system for modification and improvement in anticipation of next year's larger scale seed distribution.

The two parameters of interest during this preliminary stage of the study were recovery of planted individuals and their growth as measured by a change in length. Recovery of juvenile razor clams using a variety of field grow-out technologies varied from very poor, where intensive digging within the grow-out area uncovered few individuals using a conventional quahog netted raceway, to very high, where two 4-in. cores produced more than 50 living individuals in a boarded and netted raceway. Growth rate also varied from very little increase in seed size during the 11-wk test period to significant growth where the 2-cm seed had grown to more than 5.4 cm in length. Given these preliminary results, the prospect of farming razor clams seems to be achievable and the improvement of hatchery and rearing technology will continue through the next growing season.

A COMPARISON OF SURVIVAL IN JUVENILE *ARGOPECTEN IRRADIANS IRRADIANS* USING VARIOUS CULTURE TECHNIQUES AT THE SOUND SCHOOL REGIONAL AQUACULTURE CENTER. **Kathryn R. Markey** and **John J. Roy,** The Sound School, 60 South Water St., New Haven, CT 06519.

Shellfish aquaculture is expanding in the northeastern United States. With the increased interest in commercial aquaculture has come the advancement of the techniques employed by the growers. As such, The Sound School Regional Aquaculture Center is rapidly expanding the portion of the school's curriculum that deals with shellfish aquaculture. We have successfully sought assistance from both commercial growers and scientists dedicated to the advancement of aquaculture. They have supported us in our endeavors to provide our students with a state-of-the-art education whenever possible. We have been involved in the culture of both oysters and hard clams for several years and are currently engaged in our second year of working with *Argopecten irradians irradians*, the bay scallop, at the school.

This year, in August, the National Marine Fisheries Service in Milford, Connecticut, donated approximately 3,000 *Argopecten irradians irradians* to the school. The Groton Shellfish Commission made arrangements with our students to over-winter 6,000 >20 mm and 26,000 10- to 15-mm bay scallops at the school. In addition, the Noank Aquaculture Cooperative made 20,000 4- to 6-mm bay scallops available to our program for a nominal charge. In our previous attempts to work with bay scallops, we concerned ourselves with monitoring growth as well as survival. We have learned, as have many in the industry, that when mortality in a scallop crop reaches 100%, most concerns with growth are unfounded. This year we have refocused our experimental regimes to deal with the issues of survival of the scallops over winter.

A variety of culture techniques have been employed to overwinter the juvenile bay scallops. Cages and ADPI bags, holding scallops at +25 mm in low densities, have been successful to date, with a 91% survival rate. Three upwellers have been designed. One is located on a fixed pier outside the school. The other two were built inside a wet lab in the school. All three upwellers have unique design modifications. The outside upweller holds approximately 1,200 +25-mm scallops and mortalities have been <3% of the total. More than 40,000 bay scallops of various sizes have been successfully held in the inside upwellers, where we attempt to maintain water temperature between 13 and 18°C and provide the juvenile bay scallops with supplemental feedings of microalgae. To date, mortalities in these systems have been <10%. Experience has shown that increased mortalities in the juvenile bay scallops are to be expected during the winter months. Complicating matters further are the issues that cold weather creates with the mechanical equipment. Pump failure or frozen pipes will dramatically increase mortality. However, there is no substitute for experience. With each setback, we learn, and each time we learn, we advance our techniques so that the next year's students have an increased likelihood of success.

SUSPENDED AQUACULTURE DEVELOPMENT IN CONNECTICUT. **Paul D. Maugle,** Mohegan Aquaculture LLC, 5 Crow Hill Road, Uncasville, CT 06382.

Aquaculture in Connecticut has for the last 150 y traditionally harvested native set shellfish from the bottom. Connecticut's oysters are the most valued oysters reared in the United States. Knowing that this approach is not inherently sustainable in eastern Connecticut waters, Mohegan Aquaculture LLC has chosen to have at its core the production of oysters in suspended longlines and floating cage systems (FADPI) culture systems.

It is not only necessary to treat the natural resource as a renewable resource, it is also necessary for the business to set up systems that can provide a sustained income. Traditional approaches to nursery rearing seed stock in the Long Island Sound, and in fact the approach taken along the entire east coast of the United States, is small scale. East coast floating upwelling systems (FLUPSY) development has been a bottleneck to furthering successful aquaculture of culchless oysters. In a FLUPSY, shellfish are nursery-reared from 1.5 to 25 mm. Current east coast FLUPSY technology produces about 800,000 to 1 million seed stock per FLUPSY and uses the labor of two personnel. West coast technology produces 8 to 12 million stockable seed per FLUPSY with two personnel.

The grow-out process plans will initially focus on producing hard clams for shellfish habitat restoration and individual culchless oysters. Hatchery-produced hard clams are reared in FLUPSY until they reach 8–12 mm, and are then seeded into our nursery areas. Oysters reared in the Stonington hatchery are transferred to the FLUPSY at 2.5–3 mm and reared until they reach 20–25 mm. Seed oysters are grown in floating cage systems or in trays along submerged longlines. Once the shellfish reach >50 mm, they are tumbled and transferred to suspended oyster rearing trays for rearing to market size.

To accommodate sail boating and other recreational activities, these tray units hang at regular intervals from a submerged longline 10 ft below the surface in waters that range from 18 to 28 ft at MLT.

Sustained rearing operation will enhance and sustain recreational shellfish activities because each spawn will bring new shellfish seed into the environment. These operations will increase essential fish nursery habitat, remove bacterial and nitrogen from coastal waters, mitigate coastal eutrophication, and serve as an environmental sentinel.

IT TAKES A COMMUNITY TO GROW A SCALLOP. Mary F. Morgan, Kathleen K. Becker, and **Kim Tetrault,** Cornell Cooperative Extension of Suffolk County, Marine Environmental Learning Center, Southold, NY 11791.

Cornell Cooperative Extension of Suffolk County, New York, is in the second year of an expansion of its Marine Program to include a community-based shellfish restoration model to foster stewardship of the marine environment. The project, called SPAT (Special Projects in Aquaculture Training) is based on the understanding that enhancement of shellfish beds contributes greatly to the health of estuarine ecosystems, and that local communities can play a significant role in stewardship and restoration. Bay scallops, *Argopecten irradians irradians*, hard clams, *Mercenaria mercenaria notata*, and eastern oysters, *Crassostrea virginica*, are commercially, recreationally, ecologically, and historically important species to the Peconic Estuary. However, currently the estuary supports only 1% of its historic oyster stocks and less than 1% of its historic scallop stocks, due in large part to the occurrence of brown tide algal blooms and its effects on habitat and shellfish health.

The SPAT project incorporates a community-based shellfish enhancement effort with intensive aquaculture training and information gathering. The active membership is currently 191 families from 41 different communities throughout Suffolk County. The families are currently in training to become Master Shellfish Gardeners, learning the process of growing local shellfish species in containment and collecting data on growth rates and survival. Each participant has been licensed by the NYS Department of Environmental Conservation and local townships to possess shellfish under a scientific collector's permit. In addition, more than 2000 volunteer hours have been donated to date to assist in culturing and planting millions of seed clams, oysters, and scallops in local waterways and select test plots. Dozens of participants assisted in a 250,000 bay scallop relay effort in March at Goose Creek, Southold. A planting of millions of seed clams took place with more than 30 community members at Cedar Beach Creek, in Southold, in August. Thousand of eastern oysters are being grown in containment to adult size in 48 local creeks and embayments. From June to December, two dozen participants have lent their time, effort, and expertise to build a community-operated shellfish hatchery on location, which will concentrate on the culture of bay scallops. The hands-on transfer of techniques that enable community participants, be they high school students, local business owners, or retired professionals, to restore locally important marine resources goes a long way toward raising awareness and developing a stewardship ethic within communities.

BAY SCALLOP (*ARGOPECTEN IRRADIANS IRRADIANS*) RESTORATION ON CAPE COD. Diane Murphy, Dale Leavitt, Bill Burt, and **Bill Clark,** Cape Cod Cooperative Extension, P.O. Box 367, Deeds & Probate Building, Barnstable, MA 02630.

The bay scallop (*Argopecten irradians irradians*) fishery has long held a historic supplemental niche for Cape Cod and southeastern Massachusetts fishermen. However, within the last 15–20 y, bay scallop populations have experienced a dramatic decline, with some localized extirpations. Some suggested causes for this decline include habitat degradation in the form of nutrient enrichment and loss of eelgrass beds as well as increased predation from introduced species such as green crabs (*Carcinus maenus*).

In 1999, in response to the diminished stocks of wild scallops on Cape Cod, a collaborative restoration effort was initiated between Cape Cod Cooperative Extension, SouthEastern Massachusetts Aquaculture Center (SEMAC), and selected Barnstable County municipalities. Why bay scallops? Rapid growth rate, high market value, unstable supply, coupled with the evidence of declining and/or absent natural populations from historically productive areas suggest that bay scallops would be viable candidates for enhancement purposes. Pursuant to an exhaustive review of current information available on the bay scallop, a regional workshop was held to design an effective restoration program. It was concluded that spawning sanctuaries would provide the best means of creating self-sustaining scallop stocks for commercial and recreational fishing purposes.

Now in its third year, the Barnstable County restoration program consists of purchasing small scallop seed derived from local broodstock in late summer to over-winter in off-bottom cages. These cages make optimal use of the water column for filter-feeding, as well as reducing predation and siltation. The following spring, scallop survival is assessed and scallops are redeployed into floating spawning sanctuaries sited in historically productive areas. Embayments are chosen with the greatest potential of larval entrainment—avoiding sites with extreme tidal fluxes to ensure localized settlement of seed. Optimal stocking densities also increase the likelihood of successful spawning. Spat bags are deployed in close proximity to the spawning sanctuaries in order to monitor recruitment. In addition, genetic fingerprinting (using RAPD-PCR) is being used to evaluate the success of the bay scallop restoration program.

EFFECTS OF *CRASSOSTREA VIRGINICA* POPULATIONS ON SEDIMENTATION, PHYTOPLANKTON SPECIES COMPOSITION, AND AMMONIA CYCLING IN EXPERIMENTAL MESOCOSMS.

Jennifer Mugg-Pietros and **Michael A. Rice,** Department of Fisheries, Animal and Veterinary Science, University of Rhode Island, Kingston, RI 02881.

To determine the effects of oyster populations on water quality, a mesocosm study was performed from June to October 2000. Mesocosms with a volume of 13,000 L were used, in which there were triplicate control tanks without oysters and triplicate experimental tanks each with 200 oysters (≈35 mm in valve height; nominally filtering about 55 L day^{-1} ind^{-1}). Experiments were run sequentially in time for 3-wk periods, with water exchange rates ranging from 0% to 100% per day (0–13,000 L day^{-1}). Several parameters were measured and compared between the control and experimental tanks, including chlorophyll-a, particulate organic and inorganic matter, sedimentation rates, nitrate, ammonia, phytoplankton species and numbers, and oyster growth rates. There were no significant ($P < 0.05$) differences between tanks for most parameters, with the exceptions of rates of sedimentation and species composition of phytoplankton in the water column. Diatoms of the genus *Nitzschia* were predominant in mesocosms with oysters; in control tanks, *Skeletonema* were dominant. Rates of ammonia excretion by oysters of various sizes were determined by the sensitive salicylate-hypochlorite method, allowing for rapid determination of excretion rates to minimize biases introduced by volatilization or transformation of the ammonia over time. The excretion of ammonia by oysters can be described by the allometric equation $E = 50.65w^{0.6992}$, when E is the excretion rate in μg NH_3-N hr^{-1} and w is the dry soft tissue weight in g. On the basis of these data, it would be expected in the 3-wk experimental period that an additional 470 μg L^{-1} ammonia above the average control concentrations of about 40 μg L^{-1} would be present in each mesocosm with oysters, but no significant difference was noted between the experimental and control tanks. This suggests that there is rapid cycling of ammonia, perhaps by uptake by the rapidly regenerating phytoplankton populations.

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A REVIEW OF DISEASES IN THE BAY SCALLOP (*ARGOPECTEN IRRADIANS IRRADIANS*) AND SOME OBSERVATIONS ON MORTALITIES AT THE MILFORD LABORATORY.

Steven Pitchford and **Richard Robohm,** USDOC, NOAA, National Marine Fisheries Service, Northeast Fisheries Science Center, Milford Laboratory, Milford, CT 06460.

Various diseases and other causes of mortality in the bay scallop, *Argopecten irradians irradians*, will be reviewed, especially those that have been encountered historically and reported during hatchery and grow-out in the northeast Atlantic region. Unlike some of the other cultured bivalve species such as the eastern oyster, *Crassostrea virginica*, which is affected by MSX and dermo, the adult bay scallop does not appear to be susceptible to specific pathogenic agents that can cause widespread epizootics. Examples of infections in bay scallops to be discussed briefly include those caused by prokaryotes (rickettsia, chlamydia, *Vibrio* ssp.), protozoans, algae, and fungi.

As part of larger studies relating to immune system function and disease resistance in bay scallops, a program to screen for potential pathogenic targets was started in 1994. More than 70 bacterial isolates, the majority of which were *Vibrio* sp., were recovered from dead and moribund larval and adult bay scallops. High-concentration (approximately 10^6 bacteria/larvae) screening assays were conducted with 46 of the strains. Seven of these caused >80% mortality. Next, 1,000 2-day-old larvae/L were exposed in 48 h, using serial dilution challenges with the seven bacteria. Only two isolates were considered to be pathogenic for larvae, with median lethal concentrations (LC_{50}) of 8.65×10^2 and 1.98×10^3 colony forming units (cfu)/mL after 48 h. Following the crash of a bay scallop larval culture in 1998 at the Milford laboratory, monitoring of the cultures, the ambient seawater, and algal food sources for potential bacterial targets also was initiated.

Highlights of the monitoring program include consistent high counts of *Vibrio* sp. for 1 wk following periodic, hot, freshwater flushing of the seawater lines. These high bacterial loads may have resulted from the large amount of dissolved and undissolved organic matter that remained in the piping system. In addition, newly established mass algal cultures also would invariably show very high numbers of *Vibrio* sp. (>10^5 cfu/mL) soon after being started; however, the counts from these cultures would decrease to near 0 cfu/mL, usually after 4–8 wk.

Other occurrences of high mortality at the Milford laboratory also will be discussed; these include events with a possible infectious disease etiology and those where increased mortality resulted from biotic and abiotic causes, such as overcrowding and fouling. Several episodes of gas bubble trauma have resulted in mass mortalities, killing 75%–100% of adult scallops in very short periods. Over-wintering mortalities routinely reach 40% or even higher in cold winters, when water temperatures approach 0°C.

SHELLFISH AQUACULTURE'S EFFECT ON TOTAL ORGANIC CARBON (TOC) IN THE BENTHOS.

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Rhode Island waters provide a suitable habitat for shellfish aquaculture. Rhode Island waters are also highly valued by both local residents and tourists. If shellfish aquaculture is going to expand as a successful industry in Rhode Island, it must progress in a manner that does not negatively affect the estuary or bay in which it is conducted. To examine the effects of shellfish aquaculture on the benthos, sediment samples were tested for total organic carbon (TOC). Fifteen 0.5-L sediment samples were taken at each of 14 different shellfish aquaculture leases in Rhode Island waters. Five samples were taken within the lease; five within 10 m of the lease, and five no less than 100 m from the lease in a similar water body (depth, flow, distance from shore). Results showed normal variance between TOC levels of samples taken within, around, and away from aquaculture leases. Mean TOC of all samples away from the lease was 1.48%, 1.49% near the lease, and 1.21% inside the lease, with confidence intervals overlapping. This study provides data showing that shellfish aquaculture, at the scale that it is currently practiced in Rhode Island, does not significantly affect TOC levels in the benthos.

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INDUSTRIAL-SCALE SCALLOP CULTURE IN CHILE—THE C.M.I. EXPERIENCE.

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Cultivos Marinos Internacionales, S.A. (CMI) is the largest scallop aquaculture company in the world. CMI grows *Argopecten purpuratus*, native to Chile and Peru. Seed for the operation are primarily hatchery-produced, but seed collection is also employed. The original concept of producing 5-mm seed from a land-based hatchery and nursery did not work reliably, but settling hatchery-produced larvae on plastic mesh and putting them directly into the sea suspended from longlines proved to be successful. The scallops are subsequently grown in two pearl net stages, and three lantern net stages, all on longlines. CMI leases more than 1100 hectares of growing area, split about evenly between two bays about 300 miles apart, and uses a total of about 1,500 longlines, each 200 m long. Net changes to control fouling and to adjust scallop density are done on land in one bay, and from barges in the other. Scallops reach the market size of 80 mm shell height in 18 mo, and have an adductor muscle plus roe weight yield of 20 g each. CMI's production in the mid 1990s exceeded 5 million scallops/mo. Final product form is usually individually quick frozen (IQF), and virtually all of the scallops are exported from Chile to France.

THE DEVELOPMENT OF AN INSTRUCTIONAL SHELLFISH HATCHERY: A COLLABORATIVE EFFORT BETWEEN AEROS CULTURED OYSTER CO. AND THE SOUND SCHOOL REGIONAL AQUACULTURE CENTER.

Karen Rivara, Aeros Cultured Oyster Company, 100 Main St., Noank, CT 06340; **Amber L. Beitler** and **John J. Roy,** The Sound School, 60 South Water St., New Haven, CT 06519.

The Sound School Regional Aquaculture Center, in cooperation with Aeros Cultured Oyster Company, designed the Interdistrict Marine Educational Program's Instructional Shellfish Hatchery (IMEP/ISH). The Instructional Shellfish Hatchery program is an Interdistrict Cooperative Grant and is funded by the Office of Urban and Priority School Districts of the Connecticut State Department of Education. The IMEP/ISH project was formed to promote outcome-based learning between science classes from several coastal Connecticut high schools. The IMEP/ISH incorporates the vocational agriculture attitude of "learning through doing" as the method of education that best enables young researchers to conceptualize abstract points in their investigations as well as providing them with the techniques necessary to achieve the definable outcomes being sought.

The IMEP/ISH was constructed in the Purity Processed Seafood building at the Noank Shipyard in Noank, Connecticut. The program began on May 7 and finished on May 31, 2001. During that time period, Karen Rivara of Aeros, in conjunction with staff from The Sound School and instructors from the participating schools successfully implemented 10 laboratory lessons that had been designed for the IMEP/ISH curriculum. The laboratory lessons each considered an aspect of shellfish husbandry. The lessons included the following topics: water quality, shellfish biology, bivalve reproduction and spawning techniques, microalgae culture, hatchery technology, nursery systems, grow-out technology, shellfish health, harvest and handling methods, and depuration techniques.

The Sound School acted as host school for the project. Ella T. Grasso/Southeastern Vocational Technical School, Groton, Connecticut, functioned as a partner school in the program, providing the necessary benchtop space (in the school's BET lab facilities) for the shellfish dissection and the shellfish health laboratories. Students from Ledyard High School's aquaculture classes (VoAg Department) participated in the program as well. When the program was completed, 55 different students had participated in 199 student days of laboratory activities involving the major aspects of shellfish culture as practiced by professional aquaculturalists in Long Island Sound.

THE EAST COAST SHELLFISH GROWERS ASSOCIATION: A WORK IN PROGRESS.

Karen Rivara, East End Marine Farmers Association, Southold, NY 11971.

During the last 25 y, shellfish aquaculture has grown on the East Coast of the United States despite many obstacles. Newcomers in the industry come from many sectors of the economy, from retired schoolteachers and recent college graduates, to members of the traditional fisheries who look to aquaculture to maintain a sustainable living on the water. Established shellfish companies continue to use and improve various culture methods to meet the challenges of consistent production presented by losses due to disease and the loss of good cultivation areas.

Other stakeholders in the marine environment have challenged the growth of this industry. This impediment to the growth and survival of shellfish aquaculture stems predominantly from a lack of understanding regarding the actual impacts of the shellfish aquaculture industry. Those who are opposed to the growth of this industry overlook many of the benefits of shellfish aquaculture. In other cases, shellfish aquaculture is viewed as an impediment to the development of other industries in the marine environment. Unfortunately, those who are against the growth of the industry have in many cases taken their grievances to legislators and the press, further fostering a lack of understanding and animosity toward the industry. Too often aquaculturalists are put in the position of having to take time from growing their businesses to defend them against unreasonable public opposition.

The shellfish aquaculture industry clearly needs to become organized. We need to establish an East Coast Shellfish Growers Association (ECSGA) that will identify socio-political obstacles and work to remove them, will work in a proactive way with other stakeholders, and will develop a public outreach/public awareness protocol that will prevent the industry from constantly having to defend itself. The ECSGA would also take the lead to implement best management practices to guide individuals involved in shellfish cultivation as well as to inform legislators and other policy makers.

It is important that this association be industry-driven, but involve participation from related areas of the industry such as public shellfish aquaculture facilities, state extension programs, academia, government, and regulatory agencies. This year presents a unique opportunity to organize an East Coast Shellfish Growers Association. A roundtable discussion at the 22nd Milford Aquaculture Seminar will be devoted to the formulation of this association. We will discuss organizational considerations such as geographic scope, types of membership, mission statement, and the concerns of the industry that this association will address. In April, the National Shellfisheries Association annual meeting will be held in Mystic, Connecticut. This affords the shellfish aquaculture industry another opportunity to continue the process of forming an association. The goal of the ECSGA meeting at NSA in Mystic would be to continue discussion regarding the formation of the association and establish a committee of interested individuals that would also serve as the first Board of Directors. These individuals would complete the legal process of forming an association, establishing an office, setting meeting dates, and establishing membership.

There are many reasons why the East Coast shellfish aquaculture industry should begin to organize. We need to take advantage of the opportunity to do so this year.

HEAVY METAL SURVEY OF *FUCUS SPIRALIS* COLLECTED FROM SOUTHWESTERN LONG ISLAND SOUND.

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Fucus spiralis, commonly known as rockweed, is a brown alga found in the upper intertidal and midtidal zones of the North Atlantic. Similar to red algae, *Fucus spiralis* contains phycocolloids, which are valuable emulsifiers used in the manufacture of food, pharmaceutical, and healthcare products. In general, macroalgae take in heavy metals, some of which are beneficial for nutrition and others that are possibly harmful to algae and humans in high levels. These heavy metals accumulate in algae, which makes them useful in biomonitoring, marine pollution assessment, and phytoremediation. Because algae are becoming more popular for use in food, industry, and environmental applications, determining heavy metal concentrations is important to a wide range of audiences.

Samples from five sites known as, sites A, B, C, D, and E, located in southwestern Long Island Sound, were surveyed to determine the concentration of heavy metal intake by *Fucus spiralis*. The heavy metals being tested include: cadmium (Cd), chromium (Ca), copper (Cu), lead (Pb), and zinc (Zn). Samples were collected within 1 h of low tide. After samples were collected, the alga was washed, oven-dried, ground, and digested using a microwave lab station. Samples were then analyzed using atomic absorption spectroscopy. Some samples were analyzed in replicate to ensure quality control. Collection for test samples began on October 26, 2001, with the most current being December 19, 2001. Results of the sampling were as follows:

- Cadmium (Cd) at site A ranged from below detectable limits (n/d) to 0.04 μg/g; site B value was 0.04 μg/g; values at sites C and D were all n/d; and site E value ranged from n/d to 0.04 μg/g.
- Copper (Cu) at site A was 0.18 μg/g; site B value was 0.23 μg/g; site C values ranged from 0.036 to 0.0582 μg/g; site D values ranged from 0.048 to 0.1104 μg/g; and site E values ranged from 0.048 to 0.26 μg/g.
- Chromium (Ca) at site A was 0.02 μg/g; site B value was 0.03 μg/g; site E values ranged from n/d to 0.026 μg/g; site D values ranged from n/d to 0.082 μg/g; and site D values ranged from n/d to 0.083 μg/g.
- Lead (Pb) at site A was 0.48 μg/g; site B value was 0.5 μg/g; site C values ranged from n/d to 0.134 μg/g; site D values ranged from 0.022 to 0.14 μg/g; and site E values ranged from 0.02 to 0.51 μg/g.

- Zinc (Zn) at site A was 0.64 μg/g; site B value was 0.7 μg/g; site C values ranged from 0.1882 to 0.2332 μg/g; site D ranged from 0.06 to 0.214 μg/g; and site E ranged from 0.136 to 0.86 μg/g.

Analysis of these metals will continue at additional sites in Long Island Sound. Baseline data will be used in future comparative research of this and other algal species.

AQUACULTURE ACTIVITIES IN BROOKLYN, NEW YORK?

Martin P. Schreibman, Chester Zarnoch, John T. Tanacredi, Lucia Magliulo-Cepriano, Jacob Raz, and **Stefano Diomede,** Aquatic Research and Environmental Assessment Center (AREAC), Brooklyn College, 2900 Bedford Avenue, Brooklyn, NY 11210.

In Brooklyn College's Aquatic Research and Environmental Assessment Center (AREAC), state-of-the-art recirculating aquaculture systems (RAS) have been used to conduct a number of diverse aquaculture and environmental assessment/restoration programs. These projects include the following topics.

Finfish aquaculture: Our program consists of induction of spawning and/or grow-out of commercially important species. Our achievements include production of market-size walleye and tilapia, as well as the culture of winter and summer flounder.

Bivalve aquaculture: Recirculating systems for downwelling and broodstock conditioning were utilized in the culture of hard clams (*Mercenaria mercenaria*). Seawater used in the static algal and larval cultures was biologically and chemically treated and then reused. In its first season of operation, the hatchery produced 2 million hard clams; when 2.0 mm in shell length, they were moved out to a field site in Jamaica Bay, New York. An intensive study is underway to measure the survival and physiological condition of juvenile hard clams during the winter period. In addition, oyster seed were cultured in Taylor float systems at two sites in Jamaica Bay to monitor growth and water quality.

Ornamental and research aquaculture: AREAC has more than 600 aquaria dedicated to the culture of freshwater species for fish hobbyists and scientific research. Soft coral is also being propagated.

Captive breeding of horseshoe crabs: Adult horseshoe crabs were spawned in AREAC in July 2001. Developing animals were cultured to determine optimum temperature, medium, and nutritional parameters in recirculating systems. Currently, we have 2,500 horseshoe crabs with carapace widths ranging between 2 and 35 mm.

Environmental assessment and restoration: The impact of endocrine-disrupting chemical pollutants on freshwater and saltwater fishes has been studied for a number of years in AREAC. A program of field and laboratory experiments has begun to examine the effects of nonylphenols on winter flounder reproduction (in collaboration with Dr. Anne McElroy, SUNYSB). In another project, we have simulated the Hudson River by creating microcosms in our recirculating aquaculture systems in order to study the incidence of liver tumors and mortality in Atlantic tomcods.

Educational outreach programs: AREAC is developing curricula for pre-K–12th grade students, teachers, and their families to enhance their knowledge and teaching ability on issues relating to aquaculture and the environment.

Our programs have been funded by NPS, USACE, DEC, Con Edison, and CUNY.

SHELLFISH AQUACULTURE: GOOD FOR THE ECONOMY, GOOD FOR THE ENVIRONMENT, GOOD FOR YOU!

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The United States ranks 3rd worldwide in the consumption of seafood, yet it ranks 11th in aquaculture production, with just 1.1% of global production by weight. World production of bivalve mollusks has increased steadily over the past decade, and 80% of world bivalve production is cultured product. Oysters comprise 35% of that total; clams and arkshells combined constitute about 33%, with China producing approximately 64% of the world total. The United States ranks 3rd among bivalve-producing nations and only 19th in production via aquaculture. Possible reasons for these discrepancies and the benefits of shellfish aquaculture will be delineated and discussed.

HEALTH MANAGEMENT GUIDELINES FOR SHELLFISH CULTURE IN THE NORTHEASTERN UNITED STATES.

Roxanna Smolowitz, Marine Biological Laboratory, Woods Hole, MA 02543; **Susan Ford,** Rutgers University, Port Norris, NJ 08349; **Lisa Ragone-Calvo,** Virginia Institute of Marine Science, Gloucester Point, VA 23062.

Bivalve diseases can cause significant losses of both cultured and wild stocks. To prevent the spread of disease among populations, most states generally do not allow the importation of bivalve shellfish unless the animals are considered to be "disease free." As a result, examinations for disease have become a necessary prerequisite for determining health status. However, shellfish transfer regulations vary by state, often appear capricious, and complicate the sale and transport of commercially important seed. Furthermore, both regulators and producers have few guidelines that would help them respond to and manage disease outbreaks, when they occur. Lines of communication need to be developed between diverse but knowledgeable groups to produce a set of standardized monitoring/management guidelines for use by state regulators. We describe a project intended to provide a set of uniform, scientifically based recommendations for the health management of commercially important bivalve species in the northeastern United States.

The guidelines will be produced as a result of a series of workshops and meetings proposed for the next 2 y that will include scientists, industry, regulators, and extension agents in the northeastern United States. This interchange will first identify the strengths and weaknesses of, as well as alternatives to, current approaches to bivalve health management in the region, and investigate those in use or proposed by other states, by the federal government, and by other countries. The guidelines that follow from these discussions will be published in booklet form and will be posted on the Web.

In addition to the guidelines, the document will also contain sections pertaining to (1) concepts in disease spread, control, and diagnosis; (2) descriptions of standard diagnostic methods; (3) an examination of the potential application of novel molecular-based diagnostics, including an assessment of their accuracy and sensitivity, and how their outcome should be interpreted; (4) a fact sheet for each disease or disease agent; and (5) a glossary of general terms. The fact sheet will include the common and scientific names of the disease agent and its host or hosts, known geographic distribution, known environmental limitations, life cycle, method of transmission, recommended diagnostic procedures, and treatment or amelioration methods. Each disease agent will be classified according to its potential danger to bivalve stocks. Although the guidelines will have no force of law, we anticipate that the inclusion of all interested parties in their development should lead to their use by all northeastern states because the recommendations are reasonable, scientifically based, and because they will benefit the shellfish industries in the region.

PREVALENCE AND MORTALITY ASSOCIATED WITH SSO AND SSO-LIKE INFECTIONS OF *CRASSOSTREA VIRGINICA* IN THE NORTHEAST.

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Haplosporidium costale (seaside organism, SSO) was identified as a cause of mortality in the eastern oyster (*Crassostrea virginica*) on the Atlantic coast of Maryland and Virginia in 1962 and is now endemic there. Early (1962) investigations in Virginia showed mortality associated with SSO could reach 60% in some years. SSO plasmodia are first identified in tissue sections in early spring. Mortality resulting from synchronous sporulation in the connective tissues occurs in May–June each year. Until recently, post-sporulation SSO plasmodia have not been positively identified in oyster tissues until the following spring.

SSO-infected oysters have also been found along coastlines of the more northern states, but historically, SSO morbidity and mortality has not been considered significant. Northeast regulatory agencies do not restrict shipments of oysters on the basis of positive SSO findings. In late spring 1998, oyster culturists in Katama Bay, Martha's Vineyard, Massachusetts, observed 20%–70% mortality of cultured stocks. The cause was identified as SSO, thus indicating that SSO can cause significant mortalities in some years in the northeast. Notably, in 1998 in both Connecticut and Massachusetts, SSO-like sporulating plasmodia were noted in sections of oysters in the late fall of the year. Although no other cases have since been identified in Massachusetts, SSO-like, fall sporulating organisms continue to be identified in Connecticut.

Haplosporidium nelsoni (MSX) also produces plasmodia in oyster tissues and is a cause of significant mortality in mid-Atlantic and northeast oyster populations. It is difficult to differentiate *H. nelsoni* and *H. costale* plasmodia using traditional diagnostic methods, and it is possible that SSO and SSO-like plasmodia have been misidentified as MSX over the last several years, resulting in the confusion between mortality associated with MSX and SSO.

In this study, species-specific DNA-based diagnostic PCR methods and in situ hybridization (ISH) were employed in conjunction with traditional histological examination to differentiate *H. nelsoni* and *H. costale* infections in cultured oyster populations in Massachusetts and Connecticut, with the main objective of determining to what extent mortality is attributable to SSO and/or MSX. Additional objectives were to identify the SSO-like organism and to determine if fall and winter infection characteristics (i.e., prevalence, tissue forms, and locations) of SSO are of value in predicting SSO-related disease severity in the following spring.

Results indicate that pathologists cannot reliably differentiate MSX, SSO, and SSO-like plasmodia in traditionally stained tissue sections. Using species-specific DNA primers and probes, PCR and ISH methods can differentiate between these infections. In addition, ISH can help quantify the relative proportion of SSO and MSX plasmodia within tissue sections. Some plasmodia identified in oyster tissues collected in the fall, which might have been identified as MSX, are positive for SSO and SSO-like organisms. The relationship of SSO-like organisms to SSO and MSX plasmodia continues to be studied. Combining the history of oyster mortality in a specific location with results from various diagnostic tests is the best way to predict morbidity and mortality associated with these oyster diseases in any population.

This work is sponsored by a NOAA/Sea Grant Oyster Disease Research Award.

RHODE ISLAND'S SHELLFISH RESTORATION PROGRAM IN RESPONSE TO THE *NORTH CAPE* OIL SPILL.

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On the evening of January 19, 1996, the tank barge *North Cape* struck ground off Point Judith, Rhode Island, and began leaking oil in the vicinity of two National Wildlife Refuges, several salt ponds, and public and private beaches. Wind and wave action dispersed the oil into the atmosphere, throughout the water column, and into the benthic sediment. Approximately 828,000 gallons of heating oil were released into the surrounding offshore and inshore environment, affecting large numbers of crustaceans, mollusks, birds, amphipods, and fish. It was determined that the spill was responsible for the loss of about 150 million surfclams, *Spisula solidissima*, with a total biomass of 379,000 kg, for a value of $1.5 million. The spill resulted in the formation of a natural resource trustee group, composed of Rhode Island Department of Environmental Management, the National Oceanic and Atmospheric Administration, and the United States Fish and Wildlife Service, to evaluate the injury to the natural resources and to plan the resulting restoration activities. Because the surfclam population should recover to natural baseline levels within 3–5 y, a compensatory shellfish restoration program will be launched in Narragansett Bay and in the coastal salt ponds. Beginning in 2002, the trustees will initiate a multifaceted and multispecies approach to shellfish restoration with programs for the eastern oyster, *Crassostrea virginica*; Northern quahog, *Mercenaria mercenaria*, and the bay scallop, *Argopecten irradians irradians*. The shellfish restoration strategy will utilize many techniques, which include a remote setting program for *C. virgincia* and spawning sanctuaries and spat collection for bay scallops, *A. irradians irradians*. The shellfish restoration initiatives in response to the *North Cape* oil spill disaster offer tremendous opportunities for Rhode Island's shellfish resources.

SPAT (SPECIAL PROGRAMS IN AQUACULTURE TRAINING) UPDATE, 2002. ESTABLISHING A MODEL FOR COMMUNITY-BASED SHELLFISH CULTURE AND RESTORATION. Kim Tetrault, R. Michael Patricio, and Mary Morgan.

Cornell Cooperative Extension of Suffolk County, Marine Environmental Learning Center, Southold, NY 11971.

Cornell Cooperative Extension of Suffolk County, New York, has expanded a component of their marine program division to include a series of educational and training initiatives collectively referred to as SPAT (Special Programs in Aquaculture Training). The SPAT campaign is now 1 y old and has made tremendous progress. The active membership of SPAT Master Shellfish Gardeners is currently 191 families strong, with attendance at the monthly workshop series (11, 2-h lectures) consistently exceeding 100 participants. More than 2,000 h have been logged into the community hands-on training sessions held weekly throughout the year. SPAT members assisted in culturing and planting millions of seed clams, oysters, and scallops in local waterways and selected test plots, as well as maintaining personal "garden" stocks. A new web site (www.cce.cornell.edu/Suffolk/MARprograms/Aquacultruemain.htm) now allows members to record data that have been collected on a monthly basis for growth and survival of cultured oysters (*Crassostrea virginica*). A community-built and operated hatchery on location is expected to be operational for the 2002 growing season, which will concentrate on the culture of bay scallops (*Argopecten irradians irradians*).

Various projects are planned for the 2002 season. These include restoration work on two community spawner sanctuaries funded by NOAA (oysters) and 5-Star (clams/scallop) grants. Two additional spawner sanctuaries, one for oysters and another for scallops, have been designated by the town of Southold. All sanctuaries will be stocked and monitored by SPAT trainees. The focus will be on early field planting and predator control techniques for select species of clams, oyster bed preparation and reef building, and intensification of scallop deployment at multiple stages within healthy eelgrass beds. The community hatchery is expected to significantly increase the production of bay scallops with a target goal of 5–10 million post-set for the 2002 season. A grant to examine the sociological components of the SPAT initiative has been awarded by an anonymous foundation. Two pilot educational programs, one at the third-grade level and one at the teenage level, are actively in progress. New internships in community-based shellfish restoration activities are available.

It is the intention of the Cornell Cooperative Extension SPAT initiative to establish a model for community-supported shellfish restoration efforts through intensive training and active participation. The founding members of SPAT are excited and prepared to meet the challenges in the upcoming year.

A NEW CLAM FOR CONNECTICUT. John Wadsworth,

Niantic Bay Shellfish, LLC, 15 First Street, Waterford, CT 06385; **Tessa Simlick** and **Nancy Balcom**, Connecticut Sea Grant, University of Connecticut, 1084 Shennecossett Road, Groton, CT 06340.

The razor clam *Ensis directus*, is one of the few untapped resources in shellfish aquaculture in the northeastern United States. The market supply of razor clams is low and inconsistent, particularly because of the difficulty in harvesting these fast-digging shellfish. If razor clam production were to become economically feasible and less labor intensive, there would be great opportunity to expand this underutilized species to niche markets. The Northeastern Regional Aquaculture Center (NRAC) has provided funding for the aquaculture industry to test various clam grow-out methods in four states: Connecticut, Massachusetts, New York, and Rhode Island. In Connecticut, a project was initiated to investigate razor clam grow-out in cages. Seed clams were obtained from the Aquaculture Research Corporation (ARC) in Dennis, Massachusetts, in September 2001. The clams were held initially in upwellers and then transferred to cages for field experiments. The project consisted of two experiments, the first of which was designed to compare razor clam growth rates in cages lined with felt set at two different heights. Replicate cages were lined with felt to the top of the cages or, the high position (H1). The remaining cages were lined up to the level of the sediment, or low position (H2). The second experiment was designed to compare razor clam growth rates between two different growing areas, the Niantic River (NR) and the Niantic Bay (NB) in Waterford, Connecticut. The clams were divided among replicate cages and stocked at a density of one clam/2.5 cm^2 (one clam/in.^{2}). Monthly inventories to determine clam density and growth rate (length and width to ±0.01 mm) were performed beginning in September 2001. As of early February, preliminary results showed that there was no significant difference in growth rates between felt heights (H1 vs. H2) or growing area (NR vs. NB) (ANOVA, $P < 0.05$). The clams grew in length from 18.84 ± 2.22 mm to 34.71 ± 4.46 mm. High densities were observed in the NR cages (>90% of original stocking densities), however, the clams were present in very low densities in the NB cages.

LIFE IN A TRAILER—DEVELOPMENT OF A NEW SHELLFISH HATCHERY AT THE MASSACHUSETTS MARITIME ACADEMY. **Bethany A. Walton,** Aquaculture Laboratory, Massachusetts Maritime Academy, 101 Academy Drive, Buzzards Bay, MA 02532.

The Aquaculture Lab at the Massachusetts Maritime Academy (MMA) in Buzzards Bay, Massachusetts, officially opened its doors in April 2001. Located at the southern entrance to the Cape Cod Canal on Taylor's Point, the hatchery is a cooperative venture between MMA and Barnstable County; the SouthEastern Massachusetts Aquaculture Center (SEMAC) also provides technical support. This facility is not a production-scale shellfish hatchery, but rather, a "teaching hatchery." This teaching hatchery serves as an integral component of an introductory aquaculture course taught at MMA to provide "hands-on" training for students in shellfish biology, hatchery techniques, and intermediate culture.

The building (a refurbished trailer that measures approximately 1100 ft^2) consists of two primary areas, one devoted to algae production and the other to larval production. The greenhouse area holds twelve 250-L Kalwall tubes and has space for several 3-L carboys and 1-L jugs. Microalgae production consists primarily of batch culture of more than 10 species. The larval production area has a capacity to produce approximately 30–50 million 1-mm larvae and holds four 937.5-L larval tanks; there is also a small area devoted to setting animals and broodstock holding tanks. A limited amount of upweller space is also on site. Two innovative features of the hatchery are its seawater system and protected interior surfaces. The seawater system is a multiple pump system that feeds into a common manifold consisting of four titanium submersible pumps with a capacity of 50 gal/min. In addition, the interior surfaces of the facility are protected by a commercial polyurethane formulation (typically used for spray-on truck bed liners) applied to the floor and walls to seal them from corrosion and water damage.

Not only will we continue to use the hatchery as a teaching tool, we will also be coordinating our 2002 spawning activities with the Eastham Aquaculture Technology and Training Center to maximize shellfish propagation and enhancement efforts in the Cape Cod area. We plan to produce razor clams (*Ensis directus*), bay scallops (*Argopecten irradians irradians*), and eastern oyster (*Crassostrea virginica*) larvae for remote sets in various Cape Cod towns. Currently, we are culturing a small number of razor clam larvae.

THE NEW OYSTER WARS: POLICY PERSPECTIVES ON THE INTRODUCTION OF *CRASSOSTREA ARIAKENSIS* IN THE CHESAPEAKE BAY. **Donald Webster,** University of Maryland, Wye Research & Education Center, P.O. Box 169, Queenstown, MD 21658.

Oyster harvests in the Chesapeake Bay have declined more than 90% in the past 50 y, largely due to the influence of the oyster diseases *Haplosporidium nelsoni* and *Perkinsus marinus*. Recent studies regarding the potential of the species *Crassostrea ariakensis* have shown that this animal may provide beneficial attributes desirable both for reconstruction of the oyster fishery as well as for environmental management of the Bay. However, because it is a nonindigenous species, there are factors to be considered prior to any large-scale introduction. Variations in the current status of the *Crassostrea virginica* resource, as well as historical differences of management techniques inherent in the two states, have led to challenges regarding open-water introductions, as well as plans to increase stocks of the nonnative oyster in the future. This paper focuses on the policy differences between Maryland and Virginia and the attitudes of various user groups that are interested parties in the potential introduction of *C. ariakensis* in the Chesapeake Bay.

DEVELOPMENTS IN SOFTSHELL CLAM HATCHERY AND NURSERY PRODUCTION ON MASSACHUSETTS' NORTH SHORE. **Scott Weston, Mark Fregeau,** and **Joe Buttner,** Northeastern Massachusetts Aquaculture Center and Department of Biology, Salem State College, Salem, MA 01970.

A major goal of the Northeastern Massachusetts Aquaculture Center (NEMAC) focuses on nurturing a sustainable aquaculture industry on Massachusetts North Shore amenable to existing social, economic, and environmental conditions. Several North Shore communities are exploring aquaculture as a means to diversify and supplement their capture fisheries while restoring and enhancing endemic populations. Historically, the softshell clam (*Mya arenaria*) supported significant commercial and recreational harvests. Efforts initiated in the1990s to augment natural populations now involve a half dozen towns. Culture protocols adapted to local conditions have demonstrated the efficacy of stocking 10- to 15-mm spat in spring. Availability of suitable seed is limited and has impeded stocking efforts. With the encouragement and participation of local communities, NEMAC has targeted reliable and ultimately large-scale production of spat as a priority objective.

NEMAC's second year of operation (2001) started early as

200,000 juvenile clams were collected by local shellfishers from the Rowley River in November 2000 and successfully overwintered in the Cat Cove Marine Laboratory. Clam survival approached 100%, though growth at 15–17°C averaged ~0.5 mm/mo between 3 January and 7 May. Clams were returned to the Rowley community and released in early May 2001 at approved sites. Starting in early July 2001 and continuing to the present, NEMAC personnel and North Shore shellfishers have monitored sites in the Rowley River to ascertain water quality, clam abundance, and optimal release sites.

NEMAC personnel induced four spawns of local clams between early May and mid July. Inadequate setups for settling larvae diminished survival of initial spawns and polyspermy adversely impacted larval development during the fourth spawn. NEMAC produced 75,000 post-set spat, supplemented by 500,000 clams imported from the Beals Island Regional Shellfish Hatchery (BIRSH) in Maine. All clams were cultured in the laboratory on *Tetraselmis chuii* and T-ISO. As clams reached ~3.5 mm in length, they were transferred to screen-bottom trays at 10,000 clams/tray (~3,600 cm^2). Approximately 30 periwinkles (*Littorina* sp.) were added to each tray to crop macroalgae and other fouling organisms. The first tray was stocked in mid July and stocking continued throughout the summer. All trays were covered with white plastic, floated in Smith Pool, and exposed to tidal flush. Initial growth averaged 2.5–5 mm/mo. At 8–9 mm, growth of clams diminished, which is indicative of overcrowding. Approximately 70% of the clams stocked in upwellers survived. Clams are being overwintered, 250,000 (8–15 mm) in spat bags suspended in Smith Pool and 150,000 (3–8 mm) are distributed in trays stacked in a trough with a continuous flow of filtered seawater. Laboratory-housed clams are fed 1–2 times daily with a *Tetraselmis chuii* and T-ISO mix. The objective is to release 200,000 spat of suitable size onto approved tidal flats in spring 2002. It is hoped and anticipated that on-going, collaborative restoration/enhancement efforts will evolve into commercial enterprises conceived and pursued by local shellfishers.

RAPID GROWTH OF BAY SCALLOPS, *ARGOPECTEN IRRADIANS IRRADIANS*, IN LONG ISLAND SOUND. **James C. Widman, Jr.** and **David J. Veilleux**, USDOC, NOAA, National Marine Fisheries Service, Northeast Fisheries Science Center, Milford Laboratory, Milford, CT 06460.

Bay scallops, *Argopecten irradians irradians*, were held in pearl nets to evaluate the suitability of western Long Island Sound as a culture site for scallops. Scallops were deployed at six locations off the coast of Darien, Connecticut. Pearl nets were anchored with a cement block and vertical lift was provided by an attached subsurface buoy. Nets were deployed singularly or in groups of three. Thirty scallops were measured to the nearest 0.1 mm, placed in each of nine pearl nets, and transported to the site in coolers filled with ambient seawater. Initial deployment was on July 25, 2001, and the experiment ended on February 6, 2002.

Survival was high, although there was some initial mortality. Survival for the entire experiment averaged 71% and ranged from 53% to 83%. After the initial mortality, survival after September 11 averaged 93% and ranged from 79% to 100%. The increase in survival after the September 11 sampling period indicates that there may have been some handling/transport problems.

Scallops with initial mean shell heights ranging from 6.8 to 7.7 mm on July 24, 2001, grew to mean shell heights ranging from 45.4 to 50.6 mm by February 6, 2001. Growth rates were high from July 24 until October 16, when water temperatures began to decline below 15°C. Less than 1 mm of growth occurred after December 4, when water temperatures were low. Rapid growth rates ranging from 0.36 to 0.49 mm/day were observed during the period from September 11 to October 16, 2001, a period of declining water temperatures.

For the experimental regime, Western Long Island Sound appears to be a suitable site for culturing bay scallops.

LIVESTOCK DOMESTICATION IN THE THIRD MILLENNIUM: ALL WET? **Gary H. Wikfors**, USDOC, NOAA, National Marine Fisheries Service, Northeast Fisheries Science Center, Milford Laboratory, Milford, CT 06460.

The dictionary defines "domestic" animals as those "kept by and for the use of man." The first domestication of land animals (sheep in what is now Turkey) is thought to have occurred between 13,000 and 9,000 y ago during the Neolithic-to-Paleolithic transition in human history. Since that time, the trajectory of human food acquisition has been away from the hunter-gatherer mode to increasingly sophisticated husbandry of both plants and animals. The one exception to this historical paradigm has been seafood. It appears, however, that mankind now is entering a transition period from harvest of wild populations in the sea to domestication for the same reasons that drove land-animal domestication—rising demand and limited supply of wildlife for harvest. We have the benefit of about 10,000 y of human experience to guide decisions about marine plant and animal domestication—both successes and failures. There has, however, been limited effort to transfer the broad principles of animal husbandry downstream; therefore, the first objective of this presentation will be to advance this transfer.

At this early stage in the transition to aquaculture, the first few global, industrial-scale aquaculture products—salmon and shrimp—have reached a point of development to impact world seafood markets, as well as generate controversy about the future of aquaculture expansion on several fronts, chiefly economic and environmental. Unquestionably, mistakes have been made with shrimp and salmon, as they have with agricultural livestock production methods. What lessons can be learned from modern agriculture and our limited experience with industrial aquaculture and how can these be related to broad principles identified from the history of animal husbandry? These questions will be discussed comparatively.

BLUE MUSSEL AQUACULTURE IN LONG ISLAND SOUND. **Lawrence Williams,** Jessie D., Inc., 68 Anchorage Drive, Milford, CT 06460; and **Tessa Simlick,** Connecticut Sea Grant, University of Connecticut, 1084 Shennecossett Road, Groton, CT 06340.

A pilot-scale investigation into the longline culture of blue mussels *Mytilus edulis* in Long Island Sound has been initiated. The project, in progress since the spring of 2001, includes collecting and grading seed for grow-out on dynamic longlines at three sites in Long Island Sound and comparing production rates. A horizontal head rope (100 ft in length) was submerged 6 ft below the surface (at MLW) and anchored with concrete blocks (150 lb total weight). Vertical seed collecting lines (11 ft in length) were attached to the head rope at 3-ft intervals. Seed collectors were placed over submerged mussels beds in Branford, Milford, Fairfield, and Stonington, Connecticut, in late April 2001. A large seed-set appeared in Stonington in early June 2001, however, mussels were not visible on the collectors in western LIS until mid-July 2001. In late September 2001, a second set of seed attached to the seed collectors.

ARACHIDONIC ACID REQUIREMENTS IN LARVAL SUMMER FLOUNDER, *PARALICHTHYS DENTATUS*. **Stephen Willey** and **David A. Bengtson,** Department of Fisheries, Animal and Veterinary Science, University of Rhode Island, Kingston, RI 02881; **Moti Harel,** University of Maryland Center of Marine Biotechnology, 701 East Pratt Street, Baltimore, MD 21202.

Highly unsaturated fatty acids (HUFAs) have been identified as essential fatty acids in marine fish and the dietary requirements for the n-3 (omega-3) HUFAs eicosapentaenoic acid (20:5n-3, EPA) and docosahexaenoic acid (22:6n-3, DHA) have been well documented. Only recently has attention been given to HUFAs of the n-6 series, in particular arachidonic acid (20:4n-6, AA). The impetus for much of this attention is the fact that AA is highly conserved during periods of starvation and also serves as a precursor in the biosynthesis of eicosanoids, physiologically active compounds that aid in stress response, among other things.

The AA requirements of larval summer flounder were determined for the rotifer- and *Artemia*-feeding stages. Experimental emulsions contained adequate n-3 HUFA ratios and emulsion levels of AA were set at 0%, 3%, 6%, 9%, and 12% (AA0, AA3, AA6, A9, and AA12). Examination of fatty acid levels in live feeds and larval tissues confirmed the physiological incorporation of fatty acids relative to dietary levels. In the first experiment, survival, growth, and salinity tolerance (2 h in 70‰) were measured at 18 days after hatch (dah) after feeding the larvae the various levels of AA. Larvae fed AA6-enriched rotifers were better able to survive the salinity tolerance test. AA enrichment up to 12% had no effect on growth and survival. In the second experiment, larvae fed AA0- or AA6-enriched rotifers until 23 dah, followed by unenriched 24- and 48-h *Artemia* nauplii until 32 dah. These larvae were then subdivided and fed AA-enriched *Artemia* from 33 to 45 dah. At the end of this experiment, larvae fed AA6-enriched rotifers had higher survival, increased growth, and survived better in the salinity tolerance test (2 h in 80‰) than did those fed AA0 (unenriched) rotifers. The AA enrichment of *Artemia* did not have any significant effect on these variables. Thus, the provision of AA6-enriched rotifers early in larval development may serve to enhance larval stress tolerance at the rotifer stage, while also increasing larval survival, growth, and stress tolerance later in the *Artemia* stage.

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ABSTRACTS OF TECHNICAL PAPERS

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CONTENTS

PERKINSUS

George R. Abbe, Brian W. Albright, Carol B. McCollough, Christopher F. Dungan and Stephen J. Jordan
Environmental effects on *Perkinsus marinus* infection rates, growth and survival among Dermo-disease-free juvenile oysters planted at three salinity regimes in the Patuxent River, Maryland 371
Bassem Allam and Susan E. Ford
Transport of particles across epithelia from oyster mantle cavity: a model for *Perkinsus marinus* invasion 371
Louis E. Burnett and Christopher S. Milardo
Effects of oyster emersion on the growth and the metabolism of *Perkinsus marinus* 371
David Bushek, John Scarpa and Susan E. Laramore
Susceptibility of the Caribbean oyster *Crassostrea rhizophorae* to *Perkinsus marinus* 371
Fu-Lin E. Chu, Eric Lund and Ellen Harvey
Does the unique fatty acid synthetic capability of *Perkinsus marinus* have implication for virulence? 372
Lewis E. Deaton and Percy J. Jordan
Effect of *Perkinsus marinus* on phenoloxidase activity in bivalve hemolymph 372
Christopher F. Dungan, Rosalee M. Hamilton, Carol B. McCollough, Kimberly S. Reece and Karen L. Hudson
Epizootic diseases in Chesapeake Bay clams 372
Vincent G. Encomio, Shawn Stickler and Fu-Lin Chu
Evaluation of physiological condition in Dermo resistant oysters 373
Tarquin Dorrington, Marta Gómez-Chiarri and Lenore Martin
Evaluation of antimicrobial peptides for recombinant feed-based delivery in shellfish aquaculture 373
Julie D. Gauthier, Jerome F. La Peyre and Jill A. Jenkins
Flow cytometric analysis of lectin binding to *in vitro* cultured *Perkinsus marinus* surface carbohydrates 373
Eileen E. Hofmann, John M. Klinck, Eric N. Powell, Susan E. Ford, Stephen Jordan and Eugene Burreson
Climate variability and Dermo disease in Chesapeake Bay 374
Stephen L. Kaattari, E. Alanna MacIntyre and Christopher G. Earnhart
Modulation of *Perkinsus marinus* functions by host-derived products 374
Jerome F. La Peyre, Amy D. Nickens, Sandra M. Casas and Antonio Villalba
Viability and growth of *Perkinsus marinus* and *Perkinsus atlanticus* at three temperatures 374
Eric D. Lund, Fu-Lin E. Chu and Philippe Soudant
Is the temperature and salinity-dependent virulence of *Perkinsus marinus* associated with increased lipid metabolism? 374
Carol B. McCollough, Christopher F. Dungan, Stephen J. Jordan, George R. Abbe and Brian W. Albright
Perkinsus marinus infection rates in specific-pathogen-free juvenile oysters planted at three salinity regimes in the Patuxent River, Maryland 375
Kennedy T. Paynter, Tim Koles and Don Meritt
Perkinsus prevalence in oysters produced in the hatchery and planted in the field 375
Sammy M. Ray, Thomas M. Soniat, Enrique V. Kortright and Lance Robinson
Recent trends in levels of infection of *Perkinsus marinus* in oysters from Galveston Bay, Texas: results of the Dermowatch Monitoring Program 375
Kimberly S. Reece
Utilization of molecular genetic data for detecting, identifying and describing *Perkinsus* species 376
Pilar Muñoz Ruiz and Marta Gómez-Chiarri
Protease activity in the eastern oyster *Crassostrea virginica* after experimental infection with the protozoan parasite *Perkinsus marinus* 376
S. M. Stickler, V. G. Encomio, S. K. Allen, Jr., F-L. E. Chu and J. F. La Peyre
"Natural Dermo resistance" in eastern oyster stocks: Chesapeake studies and defense-related activities 376

THE BIOLOGY AND CONSERVATION OF FRESHWATER MUSSELS

Alan D. Christian, David J. Berg and B. Crump
Spatial and temporal analysis of ecosystem processing of freshwater mussels in two headwater streams 377
Susan Rogers, Richard Biggins and Steven Ahlstedt
Conservation activities for freshwater mussels by the United States Fish and Wildlife Service 377
William F. Henley and Richard J. Neves
Diet evaluation for the freshwater mussel *Elliptio complanata* (Bivalvia: Unionidae) 377

Curt L. Elderkin, David J. Berg, Janice L. Metcalfe-Smith, Caryn C. Vaughn, Alan D. Christian and Sheldon I. Guttman
Hierarchical analysis of MtDNA variation in *Amblema plicata*, a widespread mussel species 378

Jeffrey T. Garner and Thomas M. Haggerty
Gametogenesis, spawning, brooding and glochidial discharge in *Megalonaias nervosa* (Bivlavia: Unionidae) from the Tennessee River in Alabama 378

Catherine M. Gatenby, Daniel A. Kreeger, Robyn Reinmiller and Richard J. Neves
Clearance rate and filtration efficiency of *Elliptio complanata* (Bivalvia: Unionidae) exposed to different laboratory conditions 378

Daniel A. Kreeger, Catherine M. Gatenby and Deborah Raksany
Variability in condition index and tissue biochemistry of *Elliptio complanata* held in the field and laboratory 378

Richard J. Neves, Jess W. Jones and William F. Henley
Propagation of endangered freshwater mussels in recirculating culture systems 379

Daniel E. Spooner and Caryn C. Vaughn
Small-scale ecological function of freshwater mussels (Family: Unionidae) in the Kiamichi River, Oklahoma 379

Caryn C. Vaughn, Daniel E. Spooner, Melissa Moore and Keith B. Gido
Comparative and experimental evidence for the functional role of freshwater mussels in streams 379

W.-H. Wong, Jeffrey S. Levinton and Benjamin Twining
Assimilation of microzooplankton by zebra mussels: the benthic zooplankton loop 380

GENETICS AND MOLECULAR BIOLOGY

Charles L. Brockhouse and Gillian Richard
Unscrambling the lobster genome: complex heterozygosity in the American lobster? 380

Bruno Ernande, Joel Haure, Lionel Degrémont, Edouard Bédier and Pierre Boudry
Genetical basis of the plasticity of the resource allocation in the Pacific oyster *Crassostrea gigas* 380

Ximing Guo, Jian Wang, Brenda J. Landau, Li Li, Gregory A. DeBrosse and Krista D. Buono
The successful production of tetraploid eastern oyster, *Crassostrea virginica* Gmelin 380

Matthew P. Hare and Stephen R. Palumbi
Genetic distinctiveness of inshore and offshore *Spisula* clams 381

Dennis Hedgecock, Sophie Hubert, Gang Li and Katharine Bucklin
A genetic linkage map of 100 microsatellite markers for the Pacific oyster *Crassostrea gigas* 381

Gregg G. Hoffman, Ami E. Wilbur, Martin H. Posey and Troy D. Alphin
A single-step multiplex PCR identification assay to distinguish megalopae of *Callinectes sapidus* from *Callinectes similis* in plankton samples 381

Brenda J. Landau, Arnaud Tanguy and Ximing Guo
Searching for differentially expressed genes in diploid and triploid eastern oyster, *Crassostrea virginica* Gmelin 382

Zinin Yu and Ximing Guo
A basic AFLP linkage map for the eastern oyster, *Crassostrea virginica* Gmelin 382

Mingfang Zhou and Standish K. Allen, Jr.
Reversion revisited in *Crassostrea ariakensis:* chromosome set instability in field trials across salinity regimes 382

EAST COAST BIVALVE INDUSTRY SESSION

Standish K. Allen, Jr.
An overview of the potential for *Crassostrea ariakensis* in the Chesapeake Bay 382

Gregory A. DeBrosse, David R. Jones and Eric N. Powell
Surf clam (*Spisula solidissima*) culture in southern New Jersey: hatchery culture protocols and preliminary field grow out results 383

M. Richard DeVoe and G. Ross Nelson
Coastal states permitting procedures for deploying a tidal powered upwelling nursery system 383

George E. Flimlin, Jr.
Relevant issues for the east coast shellfish aquaculture industry: finding commonality to foster unity 383

Aman Luthra and William Walton
Stakeholders' perceptions and challenges to participatory management: the case of the soft-shell clam, *Mya arenaria* L., industry in Maine 384
Sandra Macfarlane
Embracing aquaculture BMP's: a concept whose time has come as a good neighbor or just one more thing to deal with? 384
Dana L. Morse and John Riley
Optimization of tidal upweller design: project wrap-up 384
Robert B. Rheault and Jeffrey T. Gardner
Growth and economics of shellfish nursery culture in a commercially available floating upweller 385
Leslie N. Sturmer, John E. Supan and Charles M. Adams
Enhancing seed availability for the hard clam aquaculture industry through application of remote setting techniques ... 385
William C. Walton
Using shellfish seed as a public enhancement tool: a review of its use and success in the northeast US 385
Christopher G. Warfel, P.E.
Growth and economic advantages of distributed powered upwellers: creating a new aquaculture niche 385
Donald Webster
The new oyster wars: policy perspectives in the introduction of *Crassostrea ariakensis* in the Chesapeake Bay 386

BIVALVE DISEASE STATUS AND TRENDS

Bruce J. Barber and Katherine J. Boettcher
Recent expansion of juvenile oyster disease (JOD) in Maine 386
David Bushek, Dwayne Porter, Loren D. Coen, M. Yvonne Bobo and Donnia L. Richardson
Status and trends of Dermo and MSX in South Carolina 386
Lisa M. Ragone Calvo and Eugene M. Burreson
A decade of disease: a report of oyster disease monitoring efforts in the lower Chesapeake Bay 1990–2000 387
Ryan B. Carnegie, Gary R. Meyer, Janice Blackbourn, Susan M. Bower and Nathalie Cochennec-Laureau
Development and application of a PCR for *Mikrocytos mackini*, the causative agent of Denman Island disease in oysters 387
Susan E. Ford
Development of high disease resistance in a wild oyster population 387
Stephen J. Jordan and Jessica Vanisko
The long-term role of parasitic diseases in oyster population dynamics 387
Dale Leavitt, D. Murphy, W. Burt, W. Clark, M. Hickey, J. Moles and R. Smolowitz
Cape Cod bivalve diseases monitoring program, Year 1 388
Karen L. Mareiro, Marta Gómez-Chiarri, Katherine Kerr, Emily Carrington and Arthur Ganz
Prevalence and intensity of parasitic diseases in bivalves from Rhode Island waters 388
Spencer Russell, Salvatore Frasca, Jr., Richard A. French and Inke Sunila
Modification and field trials of a multiplex PCR for the detection of three protozoan pathogens of the eastern oyster, *Crassostrea virginica*, Gmelin 1871 388
Thomas M. Soniat, Enrique V. Kortright and Sammy M. Ray
Dermowatch: a web-based approach for monitoring the oyster parasite *Perkinsus marinus* *(Dermocystidium marinum)* 389
Mary F. Stephenson, Sharon E. McGladdery and Nancy A. Stokes
Detection of a previously undescribed Haplosporidian-like infection of a blue mussel *(Mytilus edulis)* in Atlantic Canada 389
Inke Sunila
Monitoring bivalve health in Long Island Sound 389

DISEASES OF CRUSTACEA

Donald C. Behringer, Jr., Mark J. Butler, IV and Jeffrey D. Shields
Ecological ramifications of disease in the Caribbean spiny lobster, *Panulirus argus* 390

Kathleen Castro and Thomas Angell
Field observations on the development and progress of a shell disease episode for American lobster in Rhode Island: 1995–2001 390
Terry Glover, Lauren Bergey and Judith S. Weis
Effects of parasites on behavior of grass shrimp, *Palaemonetes pugio* 390
Barbara Horney, Andrea Battison and Allan MacKenzie
Cytocentrifuge preparations: an alternate method to examine the hemocytes of the American lobster *Homarus americanus* 391
Gretchen A. Messick and Tom F. Nalepa
Parasites in *Diporeia* spp. amphipods from Lakes Michigan and Huron 391
Jeffrey D. Shields
Pathological alterations in the eyes of the American lobster, *Homarus americanus*, infected with *Paramoeba* sp. 391
Jeffrey D. Shields, Donald C. Behringer, Jr. and Mark J. Butler, IV
A pathogenic herpes-like virus from the spiny lobster, *Panulirus argus* 391

BLUE MUSSEL BIOLOGY AND CULTURE

John Brake, Jeffrey Davidson and Jonathan Davis
Observations on growth, gametogenesis, and sex ratio of triploid and diploid *Mytilus edulis* 392
Jeff Davidson, Frank Baothroyd, Neil McNair and Thomas Landry
The distribution and biology of an invasive tunicate in Prince Edward Island, Canada 392
Ellen L. R. Kenchington, Kenneth R. Freeman, Benedikte M. Vercaemer and Barry W. MacDonald
A field study of settlement depths of *Mytilus edulis* and *M. trossulus* in Nova Scotia, Canada, 1998–2000 392
Angeline R. LeBlanc, Gilles Miron and Thomas Landry
Nutrient uptake and release from fouling organisms associated with cultured mussels in Tracadie Bay, PEI 393
Afton McGowen, Matthew Gordon and Paul D. Rawson
Species-specific settlement patterns of blue mussels in Cobscook Bay, Maine 393
Marcelo Miranda, David Innes and Raymond Thompson
Genetic aspects of the blue mussel (*Mytilus edulis* and *Mytilus trossulus*) hybrid zone in Atlantic Canada 393
Valérie Moreau, Edwin Bourget and Réjean Tremblay
Small scale distribution of *Mytilus edulis* and *M. trossulus* in the baie des Chaleurs and the Gaspé Peninsula 393
Randy W. Penney, M. J. Hart and N. Templeman
Mussel culture in a mixed species (*M. edulis* and *M. trossulus*) zone—some commercial implications 394
Paul D. Rawson
Cladistic analysis of genetic differentiation between populations of the blue mussel, *Mytilus trossulus* 394
Judith Senechal and Jon Grant
Behavior and growth of juvenile mussels (*Mytilus* spp.) in suspended culture socks 394
Benoit Thomas, Valérie Moreau and Réjean Tremblay
Update on the distribution of two mussels species (*Mytilus edulis* and *Mytilus trossulus*) in the Quebec maritime regions 394
Réjean Tremblay, Valérie Moreau, Thomas Landry, Bruno Myrand and Cyr Couturier
Performance of *Mytilus edulis* and *Mytilus trossulus* in the Gulf of St. Lawrence: a laboratory experiment 395
J. Evan Ward, Sandra E. Shumway and Jeffrey S. Levinton
Blue mussels as model systems to investigate pallial cavity function in bivalves 395

HARMFUL ALGAL BLOOMS

Marie-Claude Archambault, Jon Grant, Monica Bricelj and Don Anderson
Effects of clay, used to control harmful algal blooms, on juvenile hard clams, *Mercenaria mercenaria* 395
Rita A. Horner
History, some recent HAB events, and their impacts on shellfish and finfish in Washington State 396
Jay R. Leverone and Norman J. Blake
Effects of the toxic dinoflagellate, *Karenia brevis*, on larval mortality and juvenile feeding behavior in the bay scallop, *Argopecten irradians* 396

Gary H. Wikfors, Jennifer H. Alix, Roxanna M. Smolowitz, Lacey Wallace and Hélène Hégaret
Detrimental effects of a recent *Prymnesium* isolate from Boothbay Harbor, Maine (USA) upon juvenile bay scallops *Argopecten irradians* 397

SHELLFISH BIOLOGY

Richard R. Alexander and Robert M. Baron
Burrowing-induced internal fractures and external abrasion in shells of the hard clam *Mercenaria mercenaria* from Raritan Bay, New Jersey 397
Eleanor A. Bochenek, Eric N. Powell, John M. Klinck and Eileen E. Hofmann
Influence of environment and food supply on survival of *Crassostrea gigas* larvae: a modeling study 397
Andrew T. Cogswell and Samia Sarkis
Growth characteristics of *Argopecten gibbus* juveniles reared in two suspended culture systems 398
W. R. Congleton, Jr., B. R. Pearce and M. Parker
Growth and dispersal studies of *Mya arenaria* using a numerical flow model 398
Zaúl García-Esquivel, Marco A. González-Gómez and Francisco Ley-Lou
Estimation of ingestion and biodeposition rates of the Pacific oyster, *Crassostrea gigas*, in a coastal lagoon of NW Mexico 398
Laura E. Gómez, Ma. Teresa Viana, Zaúl García-Esquivel, Armando Shimada and Louis R. D'Abramo
Effect of dietary protein/energy ratio on growth and metabolism of juvenile green abalone *(Haliotis fulgens)* 399
John Kraeuter, Eric N. Powell, Eileen E. Hofmann, John M. Klinck, Ray Grizzle, Monica Bricelj and Stuart Buckner
Modeling the growth of the hard clam, *Mercenaria mercenaria* 399
Eric N. Powell, Eleanor A. Bochenek, John M. Klinck and Eileen Hofmann
Influence of short term variations in food supply and critical periods on survival of *Crassostrea gigas* larvae 399
Melissa J. Southworth, Juliana M. Harding and Roger Mann
Population structure of the hard clam, *Mercenaria mercenaria*, in Hampton Roads, Virginia 400
Helen Woods, Ken Moore and Carl Hershner
An examination of potential conflict between hard clam aquaculture and SAV in the lower Chesapeake Bay 400

SCALLOP BIOLOGY AND CULTURE

V. Monica Bricelj, Anne Veniot, Céline Barré and Peter Beninger
Postlarval development of the gills and implications for feeding in the sea scallop, *Placopecten magellanicus* 400
Maureen Davidson
The effects of stocking density in pearl nets on survival, growth, and reproductive potential of the bay scallop, *Argopecten irradians irradians* 401
Scott Feindel and Daniel Schick
Developing a coastal Maine sea scallop enhancement program 401
Brad Harris and Kevin Stokesbury
Growth and movement of sea scallops in the southern part of the Great South Channel on Georges Bank: a tagging study 401
Daniel L. Jackson, Barry W. MacDonald, Shaka James, Benedikte Vercaemer, Ellen L. Kenchington and Andre Mallet
Investigations with triploid Atlantic sea scallops, *Placopecten magellanicus* 401
Lisa M. Milke, V. Monica Bricelj and Christopher C. Parrish
A comparison of microalgal diets for enhanced production of *Placopecten magellanicus* postlarvae 402
Shawn M. C. Robinson, Jim D. Martin and Eddy J. Kennedy
Impacts and control of the spionid polychaete, *Polydora websteri* on the sea scallop, *Placopecten magellanicus* in midwater culture 402
Gregg E. Rosenkranz and Douglas Pengilly
Development of a stock assessment program for weathervane scallops in Alaska 402
Fabrice Pernet, Réjean Tremblay and Edwin Bourget
Biochemical indicator of giant scallop *Placopecten magellanicus* quality: larval growth, competency and settlement ... 403
Kevin D. E. Stokesbury and Brad Harris
Examination of sea scallop, *Placopecten magellanicus*, aggregations using a video survey in closed areas of Georges Bank 403

PARASITE AND HOST DEFENSES

Steven M. Allen and Louis Burnett
The effect of pH on the killing activity of hemocytes in the Pacific oyster, *Crassostrea gigas* 403

Robert S. Anderson, Brenda S. Krans, Sharon McGladdery and Roxanna Smolowitz
Mucoid secretions protect QPX from antimicrobial agents 404

Gwénaëlle Choquet, Philippe Soudant, Christophe Lambert, Christine Paillard and Jean-Louis Nicolas
Measurement of *Vibrio tapetis* cytotoxic activity on *Ruditapes philippinarum* hemocytes by flow cytometry 404

Christie-Sue Decker and Robert S. Anderson
Chemotaxis of hemocytes of the hard clam, *Mercenaria mercenaria,* to Quahog parasite unknown (QPX) and other microorganisms 404

Maryse Delaporte, Jeanne Moal, Jean-françois Samain, Philippe Soudant, Gwénaëlle Choquet, Christophe Lambert and Christine Paillard
Effect of dietary fatty acid composition on lipid profiles of haemocyte membranes in oysters and clams and its impact on immune functions 404

William S. Fisher and Leah M. Oliver
Activation of oyster defenses by environmental contaminants 405

Christophe Lambert, Christine Paillard and Jean-Louis Nicolas
Improvement by probiotics of *Pecten maximus* larvae defense capacity, measured by chemiluminescence 405

Christophe Lambert, Philippe Soudant, Gwénaëlle Choqnet and Christine Paillard
Development of a flow cytometric measurement of oxidative metabolism product formation by *Crassostrea gigas* hemocytes and application to evaluate pathogenic *Vibrio* inhibiting capacity 405

Ann C. Mountz and Robert S. Anderson
Purification of a novel antimicrobial peptide from the eastern oyster (*Crassostrea virginica*) 405

Christine Paillard, Gwénaëlla Choqnet, Christophe Lambert, Philippe Soudant, Helen Reid and Harry Birbeck
Temperature effect on immunocompetence of clam *R. philippinarum* and on *V. tapetis* cytotoxicity 406

Christine Paillard and Susan E. Ford
Repeated hemolymph sampling of individual clams: what does it tell us about sampling procedures? 406

Eric J. Schott, José A. F. Robledo, Wolf T. Pecher, Florence A. Okafor and Gerardo R. Vasta
The antioxidant system of the protistan parasite, *Perkinsus marinus* 406

Philippe Soudant, Gwénaëlle Choquet, Christophe Lambert, Alain Marhic and Christine Paillard
Seasonal and culture site effects on the physiological, immunological and Brown Ring disease status of the manila clam *Ruditapes philippinarum* 406

P. Soudant, C. Lambert, G. Choquet, S. Ford, C. Paillard, L. Degremont, M. Delaporte, J. Moal, P. Boudry, P. Soletchnick, J.-P. Joly, M. Ropert, E. Bédier, A. Huvet and J.-F. Samain
Relationships between summer mortalities and defence mechanisms in families of *Crassostrea gigas* reared in different environmental conditions 407

Qing-Gang Xne, Jerome F. La Peyre, Aswani K. Volety and Fu-Lin E. Chu
Purification and characterization of lysozyme from plasma of eastern oysters (*Crassostrea virginica*) 407

SEA URCHIN BIOLOGY, PATHOLOGY AND CULTURE

Ralph Elston
Sea urchin mortality in Maine, initial case report and overview 407

Eddy J. Kennedy, Shawn M. C. Robinson, John Castell and G. Jay Parsons
Importance of dietary minerals and pigments for increasing somatic growth of juvenile green sea urchins (*Strongylocentrotus droebachiensis*) 408

Gregory MacCallum, Shawn Robinson, Sharan McGladdery and Mary Stephenson
Sea urchin disease concerns in Atlantic Canada 408

Michelle Moody and Charles W. Walker
Photoperiod, urchin "eyes" and gametogenesis 408

Esther C. Peters
Abiotic and biotic threats to sea urchin health: what price culture? 409

Michael P. Russell
Growth response and acclimation of green sea urchins to fluctuating salinity 409
Paul Waterstrat and Ted Creaser
Urchin health issues in Maine 409

LOBSTER BIOLOGY AND FISHERIES

Kathleen M. Castro, J. Stanley Cobb, Richard Wahle and John Catena
The effects of habitat addition and stock enhancement for American lobsters, *Homarus americanus* on artificial reefs in Rhode Island 410
Andrei Y. Chistoserdov, Feliza Mirasol and Roxanna Smolowitz
Characterization of microbial assemblagies involved in the development of shell disease in the American lobster, *Homarus americanus* 410
J. S. Cobb and M. Clancy
Changes in lobster populations in Narragansett Bay, Rhode Island, 1959–2000 410
Cem Giray and Deborah A. Bouchard
The role of *Vibrio fluvialis* and other bacterial species in lobster mortalities in Maine 410
Diane Kapareiko, Richard A. Robohm, John J. Ziskowski, George R. Sennefelder and Anthony Calabrese
Shell disease prevalence and severity in offshore American lobster populations 411
Wendy Norden and J. Stanley Cobb
Depth-related predation pressure on larval lobsters (*Homarus americanus*) prior to settlement 411
Susan A. Little, Win Watson and Bonnie Spinazzola
The effect of temperature on sexual maturity in the female lobster, *Homarus americanus* 411
James Manning, Bonnie Spinazzola, Patrice Farrey, David Casoni and Clare Grindal
Environmental monitors on lobster traps 412
Roxanna Smolowitz, Andrea Hsu, Erin Summers and Andrei Christoserdov
Lesions associated with recent epizootic shell disease in *Homarus americanus* on the northeast coast 412
Barbara A. Somers, Kathleen M. Castro, John Sorlien and Tom Angell
What can data from industry conducted tag-recapture programs tell us? A study of the American lobster (*Homarus americanus*) 412

OYSTER REEFS AND RESTORATION

Elizabeth M. Flynn and Kennedy T. Paynter, Jr.
A characterization of ideal habitat structure for the striped blenny *Chasmodes bosquianus* 413
Raymond Grizzle, Larry Ward, Jamie Adams, Semme Dijkstra and John Nelson
A comparison of acoustic techniques, videography, and quadrat sampling for characterizing subtidal oyster reefs........ 413
Kimberly A. Hamilton, D. LaDon Swann and William Burkhardt, III
Prevalence of enteric microorganisms in the eastern oyster (*Crassostrea virginica*) and their overlying waters at representative sites of an oyster gardening program in Mobile Bay, Alabama 413
J. C. Issacs, W. R. Keithly and A. Diagne
Use and value of oyster reefs among recreational fishermen in Louisiana 414
Earl J. Melancon, Jr., Dale Diaz and Badiollah Asrabadi
Relaying as a method to remove hooked mussels from oysters prior to reharvest for sale........ 414
Roger I. E. Newell, Evamaria W. Koch, Melissa K. Wood, Ray E. Grizzle, and Raleigh R. Hood
Modeling the influence of filtration by oyster stocks on turbidity and seagrass growth........ 414
Stewart Harris and Kennedy T. Paynter
The effect of stocking density on benthic community development and shell height in the eastern oyster, *Crassostrea virginica* 415
Rachel E. Sackett, Russ Peterson, Ami E Wilbur and Jim Swartzenberg
Evaluating the contribution of commercial oyster aquaculture to recruitment........ 415
Lisa Wall, Linda Walters, Kevin Johnson, Neysa Martinez and Ray Grizzle
Recruitment of the oyster *Crassostrea virginica* on intertidal reefs in areas with intense boating activity in the Indian River Lagoon, Florida........ 415

OFFSHORE FISHERIES

Todd Gedamke and William DuPaul
Integrating vessel tracking, catch data, and depletion models to estimate commercial scallop dredge efficiency 416

Kevin D. Gaff, William D. DuPaul and David B. Rudders
The use of a 4-inch (101 mm) sea scallop (*Placopecten magellanicus*) ring dredges in the context of an area management strategy 416

Deborah Hart and Paul Rago
Rebuilding sea scallop (*Placopecten magellanicus*) stocks using area closures and rotational fishing 416

Paul J. Rago and Deborah R. Hart
Fleet dynamics of the Atlantic sea scallop fishery 416

Farhad Rajabipour
Population structure of the Indian squid, *Loligo duvaucelii* Orbigny, 1848, in Iranian waters of the Oman Sea 417

Anne Richards, Michael Fogarty, David Mountain and Mirta Teichberg
Recruitment dynamics of northern shrimp (*Pandalus borealis*) in the Gulf of Maine 417

James R. Weinberg and Charles Keith
Deep sea red crabs off southern New England: has there been a fishery impact on the population? 417

DISEASES OF SHELLFISH: CONTRIBUTED SESSION

B. K. Diggles, P. M. Hine, V. L. Webb, E. W. Maas, J. Nichol, S. Wakefield, R. Roberts, C. S. Friedman, N. Cochennec-Laureau, K. S. Reece and N. A. Stokes
Mortalities of cultured abalone, *Haliotis iris,* infected by a novel haplosporidian 418

Ralph Elston, Dan Cheney, Brian MacDonald and Andrew Suhrbier
Low salinity tolerance in Manila clams *Venerupis philippinarum* 418

Sean E. Matson and Christopher Langdon
A specific pathogen free culture system for *C. gigas* larvae and spat 418

Gary R. Meyer and Susan M. Bower
Intramitochondrial crystals within the haemocytes of mussels (*Mytilus edulis*) experiencing unexplained mortalities 419

James D. Moore and Carolyn S. Friedman
Environmental detection of the rickettsiales-like prokaryote causing withering syndrome in abalone 419

J.-L. Nicolas, M. Garnier, M. Gay and F. Leroux
Vibrio associated with juvenile oyster during summer mortality in France 419

Wolf T. Pecher, José A. F. Robledo, Cathleen A. Coss and Gerardo R. Vasta
Further molecular characterization of *Perkinsus andrewsi* and related isolates 420

José A. F. Robledo, Gerardo R. Vasta, Patrícia A. Nunes and M. Leonor Cancela
In vitro culture of *Perkinsus atlanticus* from clams *Tapes decussatus:* characterization of the rRNA gene 420

José A. F. Robledo, Eric. J. Schott, Adam G. Marsh and Gerardo R. Vasta
Gene discovery in *Perkinsus marinus* using expression sequence tags (EST) 420

José A. F. Robledo and Gerardo R. Vasta
The Nramp gene and competition for available iron between *Crassostrea virginica* and *Perkinsus marinus* 420

Arnaud Tanguy, Susan E. Ford and Ximing Guo
Characterisation of gene expression in response to *Perkinsus marinus* and *Haplosporidium nelsoni* infections in the eastern and Pacific oysters 421

BIVALVE HABITAT SUITABILITY AND THE ROLE OF BIVALVES IN ECOSYSTEMS

Loren D. Coen, Richard K. Wallace and Nancy Hadley
How community-based oyster restoration can enhance research efforts: examples from South Carolina and Alabama 421

William S. Fisher
Oyster habitat suitability as a component of resource management 421

Dana M. Frank and J. Evan Ward
Continuous monitoring of pumping pressure and valve gape in the oyster *Crassostrea virginica* in response to changes in environmental parameters 422

Raymond Grizzle, Jennifer Greene and Mark Luckenbach
A simplified seston uptake model for bivalves: preliminary field tests 422
Mark Luckenbach, Janet Nestlerode, Paige Ross, Jr. and Alan Birch
The influence of reef architecture and scale on success of oyster reef restoration 422
Jennifer Mugg Pietros and Michael A. Rice
Effects of *Crassostrea virginica* populations on sedimentation, phytoplankton species composition and ammonia cycling in experimental mesocosms 423
Thomas M. Soniat
A modified habitat suitability index for the eastern oyster, *Crassostrea virginica* 423
S. Gregory Tolley, Aswani K. Volety, Emily C. Lindland and James T. Winstead
Use of oyster habitat by reef-resident fishes and decapod crustaceans in the Caloosahatchee Estuary, Florida 423
Aswani K. Volety, S. Gregory Tolley and James T. Winstead
Effects of freshwater releases and season on oysters (*Crassostrea virginica*) in Caloosahatchee Estuary, Florida 424
James T. Winstead, Aswani K. Volety and S. Greg Talley
Parasitic and symbiotic fauna inhabiting oysters (*Crassostrea virginica*) and mud crabs (*Panopeus herbstii*) sampled from Caloosahatchee Estuary, Florida 424

POSTERS

Patrick Baker and Amy Benson
Habitat and ecology of green mussels, *Perna viridis*, in Florida 424
Shirley Baker, Ed Phlips, David Heuberger, Clay Montague and Leslie Sturmer
Introducing the CLAMMRS project: clam lease assessment, management and modeling using remote sensing 425
Andrea Battison, Barbara Horney, Richard Cawthorn and Allan MacKenzie
Hemocytes of *Homarus americanus* stained with a modified Wright-Giemsa stain: description and comparison to current classification schemes 425
Karine Bouilly, Alexandra Leitão, Helen McCombie and Sylvie Lapègue
Impact of atrazine on aneuploidy in the Pacific oyster, *Crassostrea gigas* 425
François Bourque, Bruno Myrand and Marcel Roussy
Status of the *Mytilus edulis* stock which supplies high-quality spat to mussel growers in Iles-de-la-Madeleine (Gulf of St. Lawrence) 425
Jason P. Breves and Andrew E. Tate
Predator induced phenotypic response by the common periwinkle (*Littorina littorea*) 426
Sandra M. Casas, Antanio Villalba, Jerome F. La Peyre, Kimberly S. Reeee and Carlos Azevedo
Continuous *in vitro* culture of *Perkinsus atlanticus*, parasite of the carpet shell clam *Tapes decussatus* 426
Lise Chevarie, Bruno Myrand, François Bourque, Michel Giguère, Lizon Proveneher, Philippe Archambault and Réjean Tremblay
A R&D program to develop *Mya arenaria* culture in Iles-de-la-Madeleine (Gulf of St. Lawrence) 426
Vania R. Coelho and Jeffrey D. Shields
Loss of eye pigmentation in two gammaridean amphipods from the biosphere 427
Tricia L. Cranmer, Daniela Zima and Richard R. Alexander
Shell repair rates in surgically damaged valves of the blue mussel (*Mytilus edulis*) and the ribbed mussel (*Geukensia demissa*) from New Jersey 427
Alan J. Erskine and Standish K. Allen, Jr.
Over-wintering of *Crassostrea ariakensis* in land-based systems in Virginia 427
Chwan-Hong Foo and Jerome F. La Peyre
Irradiation of oyster primary cell cultures with ultraviolet-light to eliminate bacterial and protozoal contaminants 428
B. Gagnaire, H. Thomas-Guyon and T. Renault
In vitro effects of heavy metals and atrazine on Pacific oyster, *Crassostrea gigas*, haemocytes 428
Holly A. Gefroh, Matthew J. Jenny, Ryan B. Carnegie, Kevin L. Schey and Robert W. Chapman
Inducible antibacterial activity in oyster (*Crassostrea virginica*) hemolymph 428

Jennifer Greene, Raymond Grizzle, David Burdick, Larry Ward and Ann Reid
Constructing shellfish reefs in a polluted, urban estuary: scientists join with the community to work for a common goal ... 429
Rashel V. Grindberg, Erin C. Rasnake, Michael Savarese and Aswani K. Volety
The eastern oyster as an indicator species to establish restoration targets in southwest Florida estuaries ... 429
Raymond Grizzle, Jamie Adams and Linda Walters
Long-term changes in intertidal oyster reefs in a Florida lagoon potentially caused by boating activities: an analysis of aerial photographs from 1943–2000 ... 430
Raymond Grizzle, Jennifer Greene, Stephen Jones, Mark Luckenbach and Roger Mann
An oyster (*Crassostrea virginica*) reef restoration experiment in New Hampshire involving CROSBreed stock and native transplants ... 430
William J. Hargis, Jr., Helen E. Woods, Rebecca Arenson, Sharon Dewing, Arman Kaltayev, Elizabeth Mountz, Marcia R. Berman and Dexter S. Haven
What certain 19th and early 20th century navigational and species purpose survey charts reveal about changes in the oyster reef morphology of the lower Chesapeake Bay ... 430
William J. Hargis, Jr., Helen E. Woods, Rebecca Arenson, Elizabeth Mountz, Marcia R. Berman and Sharon Dewing
Morphology of a Chesapeake Bay oyster reef system in 1871–1873 ... 431
Cindi A. Hoover and Patrick M. Gaffney
Geographic variation in nuclear genes of the eastern oyster *Crassostrea virginica* ... 431
Nicola J. Kernaghan, Eileen Monck, Carla Wieser and Timothy S. Gross
Characterization and manipulation of sex steroids and vitellogenin in freshwater mussels ... 431
Edouard Kraffe, Philippe Soudant, Yanic Marty, Nelly Kervarec and Pierre Guenot
A specific association of docosahexaenoic fatty acid with cardiolipins of some marine bivalves ... 431
Brenda S. Kraus, Amy E. Beaven and Robert S. Anderson
Hemocyte-mediated defense responses of the lobster *Homarus americanus* ... 432
Megan K. G. La Peyre, Amy D. Nickens and Jerome F. La Peyre
Potential elimination of the protozoan pathogen *Perkinsus marinus* from eastern oysters by freshet events ... 432
Jean Lavallée and Donald J. Rainnie
A field investigation of the effects of V-notching on the health and susceptibility to infection of ovigerous female American lobsters ... 432
Li Li and Ximing Guo
A preliminary linkage map for the Pacific oyster *Crassostrea gigas*, constructed with RAPD and AFLP markers ... 433
Karin B. Lohrmann, Elisabeth von Brand and Cristian Gallarda
Gonadal maturation of triploid scallops *Argopecten purpuratus* Lamarck, 1819 ... 433
Bortolini R. José Luis, Torres G. M. Pilar and Montoya R. Leobardo
Detection of white spot syndrome virus (WSSV) in carcinologic faun associated to shrimp culture of Sinaloa, Mexico, using Polymerase Chain Reaction (PCR) and *in situ* hybridization ... 433
Gregory MacCallum, Jeffrey Davidson, Garth Arsenault, Sharon McGladdery, Michelle Maillet and Neil MacNair
Factors, risks and significance of emergent neoplasia diseases in cultured and wild soft-shell clams (*Mya arenaria*) in Atlantic Canada ... 434
Clyde L. MacKenzie, Jr.
Identity of United States mollusk production declines in the 1900s ... 434
Aaron P. Maloy, Katherine J. Boettcher and Bruce J. Barber
Development of a PCR-based assay for detection of the JOD-associated *Roseobacter* ... 434
Nassrin Mashaii
Preliminary study about feeding ecology of the rock lobster, *Panulirus homarus*, Linnaeus, 1785, at Iranian seashores of Oman Sea ... 434
Sean McDermott, David Burdick, Raymond Grizzle and Jennifer Greene
Evaluation of the structure and function of a created blue mussel (*Mytilus edulis*) reef ... 435
Coren A. Milbury and Patrick M. Gaffney
Genetic monitoring of oyster stock enhancement in the Choptank River, Chesapeake Bay ... 435

Daniel P. Molloy, Denise A. Mayer, Michael J. Gaylo, Kathleen T. Presti, Alexander Y. Karatayev and Lyubov E. Burlakova
Progress in the biological control of zebra mussels with microbial toxin 435
Thomas E. Mullen, Jr. and Salvatore Frasca, Jr.
Characterization of a parasitic amoeba in the American lobster by molecular systematics 436
Kim-Lien T. Nguyen, Jerome F. La Peyre and Terrence R. Tiersch
Seasonal changes in cell proliferation of oyster tissues 436
Amy D. Nickens, Jerome F. La Peyre and Sandra M. Casas
Development of a medium to induce hypnospore formation and zoosporulation of *Perkinsus marinus* 436
Rodney Peters and David Raftos
The effects of *Marteilia sydneyi* on the host defense responses of the Sydney rock oyster, *Saccostrea glomerata* 437
Alan J. Power, Mary Sweeney-Reeves, Todd C. Recicar, Dodie M. Thompson and Randal L. Walker
Population biology of melongenid whelks in the intertidal zone in Wassaw Sound, Georgia 437
Deanna L. Prince, Robert Bayer, Christina Cangleton, Shannon Calby, Danielle LaVine, Danielle Volmuth, Katrina Brooks, Margaret Berry, William Congleton and John Vetelino
A rapid method for assessing stress in the American lobster using a hand held glucometer 437
Michael Sheppard, Florian Rambow, Marc E. Frischer and Richard F. Lee
Routes of *Hematodinium* sp. transmission into blue crabs 438
Shelly M. L. Tallack
The relationship between black spot disease and limb-loss in *Cancer pagurus* from the Shetland Islands, Scotland 438
Anne Thessen, Q. Dortch, T. M. Soniat and G. J. Doucette
Oyster grazing on toxic and non-toxic *Pseudo-nitzschia* and *Thalassisiora weisfloggii*, and *Ditylum brightwellii* 438
Jessica M. Vanisko, Mary Christman, Kennedy T. Paynter and Stephen J. Jordan
Historic eastern oyster (*Crassostrea virginica*) dredge and patent tong surveys in the Maryland portion of the Chesapeake Bay 439
Anna Walker, Michael Sheppard, Richard F. Lee and Marc Frischer
Hematodinium infection in blue crabs, spider crabs and stone crabs 439
Linda Walters, Kevin Johnson, Lisa M. Wall, Neysa Martinez and Ray Grizzle
Shell movement and juvenile survival of the oyster *Crassostrea virginica* on intertidal reefs adjacent to waters with intense boating activity in the Indian River Lagoon, Florida 439
Win Watson, Steve Jury, Jennifer Wishinski, Dan O'Grady, Walter Golet, Darren Scapel, Heidi Pye and Chris Rillahan
Development and testing of a simple field system for monitoring mussel shell gape size 439

PERKINSUS

ENVIRONMENTAL EFFECTS ON *PERKINSUS MARINUS* INFECTION RATES, GROWTH AND SURVIVAL AMONG DERMO-DISEASE-FREE JUVENILE OYSTERS PLANTED AT THREE SALINITY REGIMES IN THE PATUXENT RIVER, MARYLAND.

George R. Abbe* and **Brian W. Albright,** Academy of Natural Sciences Estuarine Research Center, St. Leonard, MD 20685; **Carol B. McCollough, Christopher F. Dungan,** and **Stephen J. Jordan,** Sarbanes Cooperative Oxford Laboratory, Oxford, MD 21654.

Specific pathogen-free (SPF) oysters were set on oyster shell and transplanted to three sites in the Patuxent River, Maryland along a salinity gradient to investigate environmental effects of *Perkinsus marinus* on infection rates, growth and survival. Oysters were held in trays on PVC structures 0.1 m off bottom on natural oyster bars. Several thousand spat were deployed at each site, and 100 oysters in a separate tray were followed for growth and mortality. From September 2000 to September 2001 salinities at Holland Point (HP, upper river), Gatton (GAT, mid) and Town Creek (TC, lower) averaged 11.1, 13.0 and 14.4, respectively. Oysters were examined monthly for growth and mortality and 30 were collected from each site for assay of *P. marinus* infections by whole body burden technique. This allowed determination of time to initial infection and subsequent progression of disease. An additional 30 from the natural population at each site were also examined monthly by rectal tissue assay. Oysters (initially 25 mm) at HP, GAT and TC grew 23, 34 and 27 mm, respectively, and survival was 95, 98 and 94% during the first 12 months. Mean intensity of dermo disease among feral populations (on a scale of 0–7) at HP, GAT and TC ranged from 1.1 to 4.2, 0.7 to 4.6 and 0.7 to 4.7, respectively, and averaged 2.51, 2.72 and 2.79. It appears that salinity had little effect on growth, survival and infection intensity during the first year; however, it is generally later that damage occurs. Preliminary data suggest that a mid-river site might be the best area to locate oysters, but additional data during subsequent years may dictate otherwise.

TRANSPORT OF PARTICLES ACROSS EPITHELIA FROM OYSTER MANTLE CAVITY: A MODEL FOR *PERKINSUS MARINUS* INVASION.

Bassem Allam and **Susan E. Ford,*** Haskin Shellfish Research Laboratory, Rutgers University, Port Norris, NJ 08349.

The digestive tract has long been considered the major portal of entry for the oyster parasite, *Perkinsus marinus*, but recent studies suggest that the mantle and gill may also be import invasion sites. We used 5-μm latex beads, placed in the mantle cavity, to determine whether *P. marinus* could be actively transported across mantle and gill epithelia. At intervals after incubation, oysters were processed for histological and cytological analysis. After 2 h, beads were observed in hemocytes at the epithelial surface and within the epithelium of the gill and mantle. After 6 to 16 h, beads were found within hemocytes in the underlying tissues. Sixteen hours following contact, only about 1% of hemocytes withdrawn from the adductor muscle contained beads, although most of those that did contained multiple beads. Over the next 96 h, the fraction of hemocytes with beads gradually declined. Meanwhile, tissue sections showed that beads were progressively transferred toward the digestive tract. These results emphasize the role of mantle and gill epithelia as portals of entry for *P. marinus* and other microorganisms, and suggest a role for hemocytes in their transport.

EFFECTS OF OYSTER EMERSION ON THE GROWTH AND THE METABOLISM OF *PERKINSUS MARINUS*.

Louis E. Burnett* and **Christopher S. Milardo,** Grice Marine Laboratory, University of Charleston, SC, 205 Fort Johnson, Charleston, SC 29412

We examined the effects of pH, oxygen, carbon dioxide, and temperature on the metabolism of the oyster parasite *Perkinsus marinus* simulating conditions that occur within the tissues of the Eastern oyster *Crassostrea virginica* during air exposure in the summer. *P. marinus* was cultured in a modified JL-ODRP medium in ambient air at high humidity. The culture medium was gassed with appropriate CO_2, N_2 and O_2 mixtures, and pH adjusted in order to simulate the conditions present in oyster hemolymph. Oxygen uptake of the parasite decreased proportionately with decreasing ambient oxygen, and decreasing pH increased *P. marinus* respiration under low CO_2 conditions ($p = 0.0006$); under 15 torr CO_2, there was no relationship between pH and oxygen uptake. Increased CO_2 levels (simulating conditions during oyster air exposure) significantly increased parasite O_2 uptake ($p < 0.0001$), as did increased temperature ($p < 0.0001$). Cycling temperature (at pH 6.3, 7.1 and 7.6) in 6-hour intervals from 25°C–35°C did not affect culture growth; however, when cycled from 25°C–40°C, cells showed a significant decrease in growth ($p < 0.0001$). Similarly, cells grown at 35°C and 15 torr ambient CO_2 showed decreased growth. These results suggest that summer environmental conditions in the intertidal zone and especially in the tissues of oysters play a role in keeping *P. marinus* infections of *C. virginica* at sub-lethal levels (SC Sea Grant R/ER-14).

SUSCEPTIBILITY OF THE CARIBBEAN OYSTER *CRASSOSTREA RHIZOPHORAE* TO *PERKINSUS MARINUS*.

David Bushek,* Baruch Marine Field Laboratory, PO Box 1630, University of South Carolina, Georgetown, SC 29442; **John Scarpa** and **Susan E. Laramore,** Harbor Branch Oceanographic Institution, Ft. Pierce, FL 34946.

The oyster pathogen *Perkinsus marinus* is a formidable problem for the American oyster, *Crassostrea virginica*. The Caribbean oyster *C. rhizophorae* is a closely related species of *C. virginica*.

but little is known of its resistance to *P. marinus* (Dermo). Therefore, Dermo resistance was compared between the Caribbean and American oyster.

Two Dermo-free families were produced and reared separately, under quarantine, for each species. Oysters from each family were challenged once via shell-cavity inoculations with either saline (control) or one of two genetically distinct isolates of *P. marinus:* ATCC 50762 from Fort Pierce, FL or ATCC 50783 from Cotuit Bay, MA. Parasite dosages were adjusted to 1×10^5 *P. marinus* per gram of wet tissue weight for each oyster. Oysters were fed phytoplankton twice a day and water was exchanged weekly for 16 wk. A natural infection study was also performed by placing oysters from each family in concrete tanks that received 7–10 L/min unfiltered seawater from the Indian River Lagoon and examining the oysters for infection over the course of 214 days. Infections in each experiment were determined using the whole-body burden method.

Fifteen America oysters and one Caribbean oyster died during the challenge experiment. Log10 transformed *P. marinus* burdens in surviving oysters were significantly heavier for Caribbean oysters (1.25 ± 0.08) compared to American oysters (0.72 ± 0.06). Control oysters did not develop infections (0.09 ± 0.01). In the natural infection study, parasites were detected in all groups after 103 days, but intensities were negligible and there was no difference in burdens between the two species. At the end of the study (day 214), most oysters had died (survival: 0 to 27%). *Perkinsus* prevalence was 100% in all surviving *C. virginica*, but 10% of the *C. rhizophorae* had escaped infection. There was no significant difference between species, however, in body burdens of survivors at the end of the natural exposure experiment. Results from the two studies indicate that Caribbean oysters are as susceptible to North American isolates of *Perkinsus*, but may be somewhat more tolerant of heavier parasite loads.

DOES THE UNIQUE FATTY ACID SYNTHETIC CAPABILITY OF *PERKINSUS MARINUS* HAVE IMPLICATION FOR VIRULENCE?

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The fatty acid synthetic capability in all the studied parasitic protozoans are limited. *Perkinsus marinus* distinguishes itself from all of the other studied parasitic protozoans by its ability to synthesize a wide range of saturated and unsaturated fatty acids. Most interestingly, *P. marinus* utilizes a two carbon substrate (^{13}C-acetate) to synthesize the essential fatty acid, arachidonic acid (AA). The ability of *P. marinus* to synthesize AA is novel. No parasitic protozoan has been reported to be capable of synthesizing AA *de novo*. There is building evidence in the mammalian system that excess AA-derived eicosanoids (powerful intercellular signaling molecules) may be harmful to host defenses. Synthesis of AA increased dramatically from day 1 to day 3 in cultured *P. marinus* meronts. It has been suggested that AA metabolites may potentially affect the cellular immune function and the inflammatory response to infection. This research was funded by Metabolic Biochemistry Program, National Science Foundation (MCB9728284).

EFFECT OF *PERKINSUS MARINUS* ON PHENOLOXIDASE ACTIVITY IN BIVALVE HEMOLYMPH.

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Phenoloxidase has been implicated as a component of host defense in a variety of invertebrates, including molluscs. The enzyme is found in the hemolymph and hemocytes of bivalves. *Perkinsus marinus* is a protist that causes disease in oysters, but does not infect *G. demissa.* We collected hemolymph from the mussel *Geukensia demissa* and the oyster *Crassostrea virginica* and measured the phenoloxidase activity in the blood with a colorimetric assay. *Perkinsus marinus* was added to aliquots of hemolymph and the phenoloxidase activity compared to that in hemolymph not exposed to *P. marinus* at 1, 2, 4, and 6 hrs after addition of the protist. After 2 hours of incubation, the phenoloxidase activity in both *G. demissa* and *C. virginica* hemolymph mixed with *P. marinus* was significantly lowered. These results suggest that *P. marinus* may suppress some components of the host defense mechanisms of bivalves.

EPIZOOTIC DISEASES IN CHESAPEAKE BAY CLAMS.

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Chesapeake Bay commercial clam landings have consistently declined to less than 2% of peak harvests during the past ten years, prompting Maryland DNR resource assessment surveys that included disease diagnostics. During 2000, ten sampled commercial clam populations (8 *Mya arenaria* softshell clams and 2 *Tagelus plebeius* razor clams) all showed *Perkinsus* sp. infections at prevalences of 30–100%. Seven of eight sampled *M. arenaria* populations also showed disseminated neoplasia (DN) prevalences of 3–37%, but neither sympatric *T. plebeius* sample was affected by DN disease. Depleted and diseased softshell clam populations, especially Eastern Bay stocks affected by both diseases, were projected to suffer heavy disease mortalities. Although both mesohaline commercial clam species were reported as *Perkinsus marinus* hosts in 1954, *Perkinsus* sp. infections in Maryland *M. arenaria* populations were not detected histologically in routine survey samples until 1990, the same year in which the current decline in landings began. Axenic in vitro *Perkinsus* sp. isolates were readily acquired from both clam hosts sampled during 2000, and from three additional clam species (*Macoma balthica, Mulinia lateralis, Rangia cuneata*) sampled during 2001. In vitro cell cycle and genetic characteristics of *Perkinsus* sp. isolates from Chesapeake Bay clams differ from those of *P. marinus* isolates from sympatric oyster hosts.

EVALUATION OF PHYSIOLOGICAL CONDITION IN DERMO RESISTANT OYSTERS.

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Distinct oyster stocks (F1 progeny) were grown in the field to compare resistance to Dermo. After two years, significant differences in mortality and growth were observed. To compare the effects of parasitic stress (*Perkinsus marinus*) on physiological condition and energy reserves between oyster stocks, energy reserves (glycogen, lipids, and total protein) were quantified and evaluated for their contribution to overall physiological condition. Biochemical indices were correlated with changes in shell height, condition index, and *P. marinus* infection. Total glycogen and protein contents increased with shell height. Results also indicate seasonal variation in condition index, which was highest in winter and decreased during the summer when infection levels were the highest. Glycogen also exhibited a similar trend. These trends were consistent between sites and over all oyster stocks, but variation between stocks was not significant, despite differences in infection intensities and mortalities. Although condition indices decreased as infections increased, data were not strongly correlated. This project was supported by ODRP, NOAA (Virginia Sea Grant # VA-OD-99-3).

EVALUATION OF ANTIMICROBIAL PEPTIDES FOR RECOMBINANT FEED-BASED DELIVERY IN SHELLFISH AQUACULTURE.

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The long-term goal of this research is to develop recombinant strains of microalgae expressing antimicrobial peptides (AMPs) that could be fed to bivalves for treatment of infectious diseases. We screened for candidate AMPs that are toxic against marine pathogens without inhibiting the growth of candidate hosts for eukaryotic expression. Pleurocidin (from the winter flounder *Pleuronectes americanus*) and tachyplesin (from the horseshoe crab *Tachypleus tridentatus*) were active against *Vibrio* spp. in the range of salinity, pH and protease concentrations found in the digestive system of oysters (Minimum Inhibitory Concentration MIC_{50} at 24 hours 6.25–25 μM). Tachyplesin and pleurocidin had no effect on the growth of *Perkinsus marinus*. The yeast strains *Pichia pastoris* and *Saccharomyces cerevisiae* were resistant to tachyplesin and pleurocidin activity ($MIC_{50} > 200\ \mu M$), whereas three algal strains (*Skeletonema costatum, Chlamydomonas reinhardtii* and *C. pulsatilla*) were sensitive ($MIC_{50} < 50\ \mu M$). While proteases from *P. marinus* significantly reduced the antimicrobial activity of pleurocidin, they did not affect the activity of tachyplesin. These results are consistent with the finding that the substrate specificity of *Perkinsus* proteases is similar to that of elastase. Oyster clearance rates of *C. reinhardtii, C. pulsatilla,* and *P. pastoris* were similar to those of *Tetraselmis lucia*, a commercially available alga used in aquaculture.

FLOW CYTOMETRIC ANALYSIS OF LECTIN BINDING TO *IN VITRO* CULTURED *PERKINSUS MARINUS* SURFACE CARBOHYDRATES.

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Parasite surface glycoconjugates are frequently involved in cellular recognition and colonization of host. The present study identifies surface carbohydrates by flow cytometric analysis of fluorescein isothiocyanate-conjugated lectin binding. Lectin binding specificity was confirmed by sugar inhibition and Kolmogorov-Smirnov statistics. Clear, measurable separation between fluorescence peaks and no parasite autofluorescence were observed. Parasites (GTLA-5 & P-1 strains) harvested at log phase growth from a protein-free medium reacted strongly with wheat germ agglutinin (WGA) and concanavalin A (Con A), reflecting presence of N-acetyl-D-glucosamine and glucose/mannose moieties, respectively. Both strains also bound, although with lower intensity, *Maclura pomifera* agglutinin (MPA) and *Bauhinia purpurea* agglutinin (BPA) (N-acetyl-D-galactosamine specific lectins), peanut agglutinin (PNA) (terminal galactose specific), and *Griffonia simplicifolia* II (GSII) (N-acetyl-D-glucosamine specific). Background fluorescence levels were detected for *Ulex europaeus* agglutinin I (L-fucose specific) and *Limulus polyphemus* agglutinin (sialic acid specific). The order of lectin binding intensity differed between strains: GTLA-5 reacted with MPA>GSII>PNA>BPA, whereas P-1 reacted with PNA>MPA>BPA>GSII, which may result from differential expression of glycoconjugates throughout log phase growth. Fluorescence microscopy revealed that PNA bound with high intensity primarily to schizonts (dividing cells), whereas the other lectins bound with approximately equal intensity among parasite growth stages. Future efforts will determine if *P. marinus* rapid recognition and internalization involves a specific lectin-carbohydrate interaction.

CLIMATE VARIABILITY AND DERMO DISEASE IN CHESAPEAKE BAY.

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A mathematical model that includes biological and environmental processes that contribute to the observed annual cycles of intensity and prevalence of the disease, Dermo, has been used to determine the effect of predicted climate variations on Eastern oyster (*Crassostrea virginica*) populations in Chesapeake Bay. Simulations that use the salinity change predicted for Chesapeake Bay by the Hadley Center Climate model for conditions of doubled CO_2 suggest that the present pattern of Dermo disease in the Bay will be altered to the benefit of the oysters. The predicted salinity change in Chesapeake Bay for conditions of doubled CO_2 produces an overall decrease in Bay salinity, which results in a decrease in the prevalence and intensity of Dermo disease. However, for reduced salinity conditions, there is a trade-off between reduced disease levels and reduced reproductive capacity of the oysters. Also, conditions of increased food can offset the effects of either increased or decreased salinity on Dermo disease levels. The simulated total oyster biomass changes suggest that for conditions of doubled CO_2, northern Chesapeake Bay oyster populations may not be viable over the long term without external inputs of juveniles. However, oyster populations in the Rappahannock and York Rivers increase or have stable biomass over time for most climate change conditions. One implication of these results is that oyster populations in southern Chesapeake Bay may sustain the Bay-wide oyster fishery. These simulations provide a basis for suggesting management plans for diseased oyster populations under conditions of climate change.

MODULATION OF *PERKINSUS MARINUS* FUNCTIONS BY HOST-DERIVED PRODUCTS.

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In an attempt to induce physiological changes *in vitro* that would be comparable to those elicited by natural infection, we have co-incubated *P. marinus* cells with extracts of *Crassostrea* spp tissues and plasma. Co-incubation with these host-derived materials gave rise to a variety of complex effects including altered differentiation, protease expression, growth rates, infectivity, and *in vitro* parasite mortality. Preliminary evidence is suggestive that a suite of such functions could be employed as a prognostic tool for the selection of resistant oyster stocks. However, variations in the relative activity of these materials suggest that preparative procedures and/or conditions of storage could confound such analyses. Thus, while indicative of prognostic value, the precise mechanisms of these biological effects must be resolved.

VIABILITY AND GROWTH OF *PERKINSUS MARINUS* AND *PERKINSUS ATLANTICUS* AT THREE TEMPERATURES.

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The ability to culture several protozoan parasites of the genus *Perkinsus* allows comparison of their growth and tolerance given different environmental conditions. This information is important to understand the distribution of these molluscan parasites and can be used to predict zones for high risk of mortalities of host populations. The effects of temperature on *P. marinus* and *P. atlanticus* were determined because temperature is a major factor controlling the prevalence and intensity of infection in their respective host, the eastern oyster, *Crassostrea virginica,* and the carpet shell clam, *Tapes decussatus.* Three isolates of each species were added to 96-well plates at a seeding density of 10^5 cells/ml and incubated at 4°C, 15°C and 28°C for 15 days. The parasite density, viability, size and metabolic activity were measured every other day starting on day 2. Parasite density was measured with a hemacytometer. Parasite viability was determined by neutral red uptake. Parasite metabolic activity was assessed by measuring the cellular bioreduction of the fluorescent dye Alamar Blue. The most significant result was that all three isolates of *P. atlanticus* multiplied at 15°C whereas no increase in parasite number of any *P. marinus* isolates was detected at 15°C. The growth rate of *P. marinus* and *P. atlanticus* was comparable at 28°C and neither species grew at 4°C. While perkinsosis is generally considered a warm water disease, this study suggests there will be differences in the temperature at which different *Perkinsus* species kill their respective hosts.

IS THE TEMPERATURE AND SALINITY-DEPENDENT VIRULENCE OF *PERKINSUS MARINUS* ASSOCIATED WITH INCREASED LIPID METABOLISM?

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Temperature and salinity significantly affect the rate of proliferation and development of *Perkinsus marinus* and the progression of the disease in its host, the eastern oyster. To better understand how these environmental parameters affect the nutritional metabolism of this parasite, the effects of temperature and salinity on the lipid metabolism of *P. marinus* were investigated. Using axenic cultures grown at 3 temperatures and 2 salinities, the uptake, incorporation and metabolism of two fluorescent lipid analogues in meronts and the lipolytic activities of meronts and extracellular proteins (ECPs) were determined. Uptake and bioconversion of the lipid analogues were positively correlated to temperature. Salinity did not affect the uptake, but bioconversion of these two components was lower at low salinity. Triacylglycerol lipase activity of cell homogenates and ECPs were positively correlated to temperature. Phospholipase activity was not detectable in ECP. This research was supported by NSF (MCB9728284).

PERKINSUS MARINUS INFECTION RATES IN SPECIFIC-PATHOGEN-FREE JUVENILE OYSTERS PLANTED AT THREE SALINITY REGIMES IN THE PATUXENT RIVER, MARYLAND.

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Specific-pathogen-free (SPF) seed oysters were set and reared in filtered and sterilized Patuxent River water. They were transferred to three natural oyster bars in the Patuxent River along a salinity gradient. Deployment sites were located at Town Creek (TC, down river), Gatton (GA, mid-river), and Holland Point (HP, upriver). Samples of 30 oysters were assayed at 2, 4, and 8 weeks post-deployment for infection by *Perkinsus marinus*, using an enhanced RFTM whole body burden technique. Assays continued at 4-week intervals until three consecutive samples from each area tested positive for presence of *P. marinus*. Three discrete spat sets and deployments were made: September 2000, June 2001, and August 2001. Animals at sites TC and GA September 2000 deployments acquired *P. marinus* infections within 2 weeks, with prevalences of 10% and 3% respectively. Positive results at low prevalences and intensities continued for 8 weeks. Sites TC and GA June 2001 deployment acquired infections within 2 weeks, with 10% and 13% prevalences respectively, and positive results continued for 8 weeks with increasing prevalences. Site HP acquired one infection (3%) between 2 and 4 weeks. Positive results continued through two additional 4-week sampling intervals, with increasing prevalences. All August 2001 deployments acquired infections within 2 weeks, with prevalences of 7% at TC, 87% at GA, and 3% at HP, and positive results continued for 8 weeks. Infection intensities among these samples ranged from 1–23 hypnospores per host animal. The GA deployment was 100% infected by 8 weeks post-deployment. These results show that juvenile oysters acquire *P. marinus* infections as early as 2 weeks after placement in dermo disease endemic areas, and that these infections persist in the planted populations over time.

PERKINSUS PREVALENCE IN OYSTERS PRODUCED IN THE HATCHERY AND PLANTED IN THE FIELD.

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Since 1995, millions of oyster spat have been produced by the Horn Point oyster hatchery. Most of those oysters have been planted on oyster bars in various locations in the Maryland portion of Chesapeake Bay in association with oyster restoration efforts. Oysters planted in most of these areas were tested for *P. marinus* prior to being planted and then regularly tested, in some cases for as many as five years, after planting. Salinity, temperature and dissolved oxygen data were also collected at most sites. Data were analyzed from all monitored sites planted with *Perkinsus*-free spat over the past 5 years. Analysis showed that very few oysters were infected with *P. marinus* during their first year. In fact, very few oysters were infected during their second and even third years. Of the nearly 2,000 oysters collected in 2001 that were planted on restoration sites in 1998, 1999 or 2000, only 44 were infected. In addition, infections appear to be more related to local infection foci than salinity. Infection rates in hatchery-produced oysters are lower than expected compared to the naturally set oysters transplanted by the State as part of the repletion program.

RECENT TRENDS IN LEVELS OF INFECTION OF *PERKINSUS MARINUS* IN OYSTERS FROM GALVESTON BAY, TEXAS: RESULTS OF THE DERMOWATCH MONITORING PROGRAM.

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Since December 1998 oysters in Galveston Bay have been monitored for levels of the parasite *Perkinsus marinus* as part of the Dermo Watch Program. Monthly sampling of six reefs (Redfish, Hannah's, Frenchy's, Fisher's, Confederate and April Fool) and three leases provides good spatial coverage and adequate temporal resolution for management purposes. Each month oysters are collected, their length (L) measured, and checked for weighted incidence (WI) of infection; water temperature and salinity are also determined. Initial WI, T, S, and L are used by a model embedded in the DermoWatch web site (www.blueblee.com/dermo) to calculate a time to critical level of disease (t-crit). The t-crit is the number of days that it would take to reach a critical WI of 1.5 (assuming no change in T and S). Values of t-crit are low when T and S are high. The effects of drought conditions during 1999 and 2000 were reflected in higher WI and lower t-crit values throughout the Bay. Oyster growers and managers can use estimates of t-crit to manage disease. For example, heavily infected populations of oysters with t-crit values equal to or approaching zero can be moved to lower salinity sites or harvested before they die of disease.

UTILIZATION OF MOLECULAR GENETIC DATA FOR DETECTING, IDENTIFYING AND DESCRIBING *PERKINSUS* SPECIES. **Kimberly S. Reece,*** Virginia Institute of Marine Science, The College of William and Mary, Gloucester Point, VA 23062.

Morphological characters are unreliable for distinguishing among *Perkinsus* species. In addition, traditional detection methods for *Perkinsus* species such as histological analyses or the Ray's fluid thioglycollate assay do not differentiate among the species. Therefore, *Perkinsus* species designations are based largely on differences in hosts and/or geographic ranges. In recent years several species-specific molecular detection assays have been developed targeting DNA sequence differences among described species. DNA sequence data is also being used to support and validate descriptions of new *Perkinsus* species. Results from several molecular genetic studies indicate that caution must be employed, however, to ensure that adequate molecular data is available to appropriately develop molecular diagnostics and to discriminate among species. Intra-, as well as inter-specific sequence variation should be examined to confidently target a particular nucleic acid sequence in molecular diagnostic assays or to use DNA sequence data from a locus in phylogenetic analyses or for taxonomic discrimination. Data will be presented suggesting that genetic variation that was previously thought to represent sequence differences between two *Perkinsus* species is observed within the genome of a single *Perkinsus* species isolated from two different hosts. In another study, a comprehensive examination of sequence variation among *Perkinsus* species within the internal transcribed spacer region of the ribosomal RNA gene complex supports designation of a new *Perkinsus* species that is closely related to *P. atlanticus* and *P. olseni*. Currently, *P. atlanticus* and *P. olseni* cannot be distinguished based on available molecular sequence data suggesting that synonymization of these two species may be warranted pending results of more extensive and complete analyses.

PROTEASE ACTIVITY IN THE EASTERN OYSTER *CRASSOSTREA VIRGINICA* AFTER EXPERIMENTAL INFECTION WITH THE PROTOZOAN PARASITE *PERKINSUS MARINUS*. **Pilar Muñoz Ruiz** and **Marta Gómez-Chiarri,*** Department of Fisheries, Animal and Veterinary Sciences, University of Rhode Island, Kingston, RI 02881.

Perkinsus marinus, an endoparasitic protozoan parasite, has long been recognized as a serious oyster pathogen that is often blamed for widespread mortality of the Eastern oyster *Crassotrea virginica*. In order to investigate the molecular interactions between *P. marinus* and oyster hemocytes during early infection, *Perkinsus*-free Eastern oysters (Taylor Shellfish, Washington, USA), were inoculated either in the mantle cavity or the aductor muscle with cultured cells of *P. marinus*. Protease and antimicrobial activity were measured in filtered supernatants of hemolymph collected 4 hours, 24 hours, 4 days and 2 months after experimental infection. No antimicrobial activity was detected in hemolymph supernatants of parasitized or control oysters. Protease activity in hemolymph supernatants collected 4 h after experimental infection was significantly higher in parasitized oysters than in control oysters. There were no differences in secreted protease activity between control and parasitized oysters sampled 24 hours, 4 days and 2 months after experimental infection. Gelatin-gel electrophoresis (zymography) analysis showed the presence of 5 high molecular weight bands (60–220 KDa) with protease activity in both control and parasitized oysters. Large variations in both the amount of protease activity and the relative abundance of each protease were observed between individual oysters. The presence of low molecular weight proteases (35–50 KDa), corresponding to *P. marinus* serine proteases, was observed in hemolymph supernatants of a small proportion of experimentally infected oysters.

"NATURAL DERMO RESISTANCE" IN EASTERN OYSTER STOCKS: CHESAPEAKE STUDIES AND DEFENSE-RELATED ACTIVITIES. **S. M. Stickler,* V. G. Encomio, S. K. Allen, Jr.** and **F-L. E. Chu,** Virginia Institute of Marine Science, College of William and Mary, Gloucester Point, VA 23062; **J. F. La Peyre,** Louisiana State University, Baton Rouge, LA 70803.

The restoration of Eastern Oyster populations can be accelerated, in part, by using stocks of oysters resistant to the parasitic disease Dermo, caused by *Perkinsus marinus*. Similarly, identifying correlated defense activities will expedite the breeding process for disease resistance. We used putatively Dermo-resistant wild stocks from both the Gulf of Mexico and the Chesapeake Bay and a disease-resistant hatchery strain, and compared the resistance of their progeny to Dermo disease in a two-year common garden experiment at two Dermo-enzootic Chesapeake Bay sites. Using a modified body burden assay, we determined *Perkinsus* infection levels and Dermo-related mortality were considerably higher in Chesapeake stocks than in Louisiana stocks. Chesapeake stocks experienced wide variation in both disease infection levels and mortality. These results indicate a genetic basis for disease resistance, even within a single region. Monthly hemocyte counts and hemocyte and serum potential for killing *P. marinus*, and plasma protein, lysozyme, and protease inhibition levels were obtained for correlation with *Perkinsus* infection levels. This project was supported by ODRP, NOAA (Virginia Sea Grant # VA-OD-99-3).

THE BIOLOGY AND CONSERVATION OF FRESHWATER MUSSELS

SPATIAL AND TEMPORAL ANALYSIS OF ECOSYSTEM PROCESSING OF FRESHWATER MUSSELS IN TWO HEADWATER STREAMS. Alan D. Christian, Department of Zoology, Miami University, Oxford, OH 45056; **David J. Berg**, Department of Zoology, Miami University, 1601 Peck Blvd., Hamilton, OH 45011; **B. Crump**, Caddo Ranger District, Ouachita National Forest, 912 Smokey Bear Lane, Glenwood, AR 71943.

The objectives were to determine food resources, food resource nutrient composition, tissue nutrient composition and N and P excretion rates of two species of mussels from two Little Darby Creek, OH and two Ouachita River, AR headwater sites. Seston stable isotope analysis revealed no differences in $\delta^{15}N$ values between sites within a streams or seasons, however, summer $\delta^{13}C$ values were significantly different than spring and fall $\delta^{13}C$ and $\delta^{15}N$ values. No differences in mussel $\delta^{15}N$ values were observed between sites within a stream, but were significantly different seasonally and different regionally. Seston nutrient concentrations were similar between sites within a stream and among seasons. No significant differences in nutrient tissue concentrations were observed between species within a stream, sites, or seasons. Excretion rates of N and P were lowest in spring and highest in fall for all species. Average N:P ratios were below 16:1 for some species and above 16:1 for other species. Nutrient limitation experiments may have indicated N and P limitation in the fall. Isotope values were regionally different, locally similar, temporally variable and suggest mussels utilize a subset of the seston, possibly bacteria. Mussels may be providing an important ecosystem role by providing limiting nutrients to primary producers.

CONSERVATION ACTIVITIES FOR FRESHWATER MUSSELS BY THE UNITED STATES FISH AND WILDLIFE SERVICE. Susan Rogers,* US Fish and Wildlife Service, Conway, AK 72032; **Richard Biggins**, US Fish and Wildlife Service, Asheville, NC 28801; **Steven Ahlstedt**, US Geological Survey, Knoxville, TN 37828.

The Fish and Wildlife Service (FWS) is active in freshwater mussel conservation through several approaches: habitat restoration, propagation and reintroduction of juveniles, collaboration with partners, and conservation research. The FWS is involved in conserving both federally listed and unlisted species. Before any recovery program can be successful, however, habitat must be restored and protected. A program such as that enacted in partnership with The Nature Conservancy on the Clinch River can be particularly successful in reaching this goal. Partnerships are integral to the FWS's freshwater mussel recovery programs. For example, the Tennessee Valley Authority and the Army Corps of Engineers also have worked with the FWS on mussel conservation in relation to their water resource projects. In addition to habitat restoration, propagation efforts have been ongoing for approximately 10–15 y, and endangered mussel populations throughout the country are being augmented with propagated juveniles. Finally research is being conducted to determine the causes of the bottleneck in survival that juvenile mussels experience around 60 d of age. Further information needs that would aid the FWS's conservation efforts include quantification of the impacts of sedimentation from dredging and navigation projects on mussel populations, and the impacts of pesticides on glochidial transformation success.

DIET EVALUATION FOR THE FRESHWATER MUSSEL *ELLIPTIO COMPLANATA* (BIVALVIA: UNIONIDAE). William F. Henley* and **Richard J. Neves**, Virginia Cooperative Fish and Wildlife Research Unit, Department of Fisheries and Wildlife Sciences, Virginia Tech, Blacksburg, VA 24061.

To determine the effects of holding conditions and different algal diets on freshwater mussels, the tissues of adult eastern elliptio freshwater mussels, *Elliptio complanata* (56–106 mm) were tested for physiological and gametogenic condition over 8 sampling events. Treatments included a reference group from the source population, the Nottoway River, Virginia (NR), and 3 captive treatments. Captive treatments were mussels fed *Scenedesmus quadricauda* (S), *Neochloris oleoabundans* (N), and a no feed treatment (NF). Patterns in levels of soluble protein, glycogen, and percentages of moisture differed over the course of the experiment ($p < 0.01$). Production of ripe and developing gametes did not differ in the treatments ($p = 0.22$), but the NF mussels ceased gametogenesis in spring. Carbohydrate levels of the N and S animals did not differ from those of the NF mussels ($p = 0.99$). Soluble protein levels of the N and S mussels remained relatively high throughout the experiment, those of the NF animals declined, and those of the NR varied. Percent moisture in tissues of the treatments differed over time ($p < 0.001$). Levels of total lipids in the mantle tissues of the treatment groups, as well as muscle fiber diameters in the foot, provided useful complimentary data for assessing body condition.

HIERARCHICAL ANALYSIS OF MTDNA VARIATION IN *AMBLEMA PLICATA*, A WIDESPREAD MUSSEL SPECIES.

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Knowledge of genetic structure of target species is essential for the development of effective conservation plans. *Amblema plicata* is a common, widespread freshwater mussel species. Six or seven individuals from six populations of *Amblema plicata* within the Lake Erie and Ohio River drainages were analyzed by sequencing restriction fragments from a 652-base portion of the mitochondrial cytochrome oxidase I (COI) gene and a 530-base portion of the nuclear internally transcribed spacer (ITS-1) gene. Percent sequence divergence was determined and the results were analyzed by calculating Tamura-Nei distances between individuals and then constructing neighbor-joining trees from the distance matrix. Two haplotypes were identified for the COI gene and all populations shared these haplotypes. Greater diversity was found for the ITS-1 gene, with a total of six haplotypes, although each population had only one or two haplotypes. Cluster analysis revealed no real geographic structuring for either gene. These preliminary results are in striking contrast to those we have found using allozymes. The latter group of loci showed significant differentiation between the Ohio River and Lake Erie basins. Further investigation is required in order to explain this apparent contradiction.

GAMETOGENESIS, SPAWNING, BROODING AND GLOCHIDIAL DISCHARGE IN *MEGALONAIAS NERVOSA* (BIVALVIA: UNIONIDAE) FROM THE TENNESSEE RIVER IN ALABAMA.

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The annual gametogenic cycle of *Megalonaias nervosa* was monitored over a two-year period by observing temporal changes in histological preparations of gonadal tissues. In male specimens, temporal changes in cell numbers and relative abundance were evaluated by counting different germ cell types along transects through gonadal acini. Temporal changes in female specimens were quantified by measuring diameters of oocytes and counting the numbers of oocytes per acinus. The cycles observed in both male and female *M. nervosa* differed from those observed in other members of the Ambleminae. Little activity was observed through most of the year, culminating in a flurry of activity in late summer and early autumn. In male specimens, acini filled with spermatozoa in July and August and all were released in September. In female specimens, oocytes developed and grew quickly during the period in which spermatogenesis occurred in males and all oocytes were released to the marsupia over a short period, showing a high degree of spawning synchronicity with the males. Glochidia quickly matured and were present in both inner and outer gill demibranchs until December.

CLEARANCE RATE AND FILTRATION EFFICIENCY OF *ELLIPTIO COMPLANATA* (BIVALVIA: UNIONIDAE) EXPOSED TO DIFFERENT LABORATORY CONDITIONS.

Catherine M. Gatenby,* Daniel A. Kreeger and **Robyn Reinmiller,** Patrick Center for Environmental Research, Academy of Natural Sciences, Philadelphia, PA 19103; **Richard J. Neves,** Virginal Cooperative Fish And Wildlife Research Unit, Department of Fisheries and Wildlife Sciences, Virginia Tech, Blacksburg, VA 24061-0321.

As part of an ongoing research program improve laboratory culture protocols for native freshwater mussels, we quantified and compared feeding rates and particle size preferences of mussels, *Elliptio complanata*, that were exposed to different holding conditions in the laboratory. Two culture parameters were examined: the importance of a burrowing substrate (sand) and the effect of food quality (natural seston vs. lab-cultured microalgae). A Multi-Sizer was used to measure and compare clearance rates for the whole diet (2–63 μm diameter), specific size fractions of the diet (e.g., 2–3 μm), and for various sized carboxylate microspheres. By comparing clearance rates for different sized particles, we calculated their relative filtration efficiency, which tended to be greatest for moderately large sized particles averaging 7–10 μm in diameter. Both clearance rate and filtration efficiency varied considerably between sediment/no sediment conditions, and among dietary treatments of different quality. These parameters were also monitored for periods of up to 60 d to determine whether *E. complanata* adjust either feeding rates or particle size preferences as an adaptive response to differences in physical (e.g. sediment) or nutritional conditions.

VARIABILITY IN CONDITION INDEX AND TISSUE BIOCHEMISTRY OF *ELLIPTIO COMPLANATA* HELD IN THE FIELD AND LABORATORY.

Daniel A. Kreeger,* Catherine M. Gatenby and **Deborah Raksany,** Patrick Center for Environmental Research, Academy of Natural Sciences, Philadelphia, PA 19103.

Like their marine counterparts, native freshwater mussels can be sufficiently abundant to affect key ecological functions (e.g., energy, biogeochemical cycles) and perform important ecosystem services (e.g., removal of suspended particulate matter, use as bioindicators). The effectiveness of freshwater mussels depends in part on their physiological rate functions, which may vary with their physiological condition. Our goal was to determine the level of variability in physiological condition of *Elliptio complanata*, a common unionid in the Atlantic drainage. Adults were subjected to a variety of laboratory and field conditions. In the lab mussels were held in continuous flow chambers with or without sediment and fed isocaloric rations of either natural seston or lab-cultured microalgae. Field populations were caged and transplanted to waters of varying quality. Condition index and proximate tissue biochemistry (protein, lipid, carbohydrate, ash) were monitored for 6 weeks in the lab and 1 year in the field. Both condition and tissue biochemistry of wild mussels varied seasonally, as expected for adults undergoing reproductive cycling. However, irrespective of these normal seasonal patterns, condition and tissue biochemistry also varied among all experimental comparisons; food quality (seston > algal diets), sediment conditions (sediment > no sediment) and environmental conditions (field site differences). Although *E. complanata* is considered to be one of the more hardy unionids, these results indicate that its physiological condition is sensitive to nutritional and environmental cues. Therefore, physiological fitness measures for *E. complanata* should prove useful as bioindicators, and studies of ecological processing by these animals should quantify physiological rate functions under a variety of conditions.

PROPAGATION OF ENDANGERED FRESHWATER MUSSELS IN RECIRCULATING CULTURE SYSTEMS. **Richard J. Neves,* Jess W. Jones** and **William F. Henley**, Virginia Cooperative Fish and Wildlife Research Unit, Department of Fisheries and Wildlife Sciences, Virginia Tech, Blacksburg, VA 24061.

A propagation facility for the production, culture, and release of endangered juvenile mussels has been established at Virginia Tech, to address the conservation needs of 70 federally listed mussel species in the United States. After years of research on species' life histories, substratum and food requirements, and efficacy of culture systems, the facility has been successful in culturing juvenile mussels for release to natal rivers. Nine species have been cultured thus far, and over 250,000 juveniles have been released to 3 rivers in Tennessee and 2 streams in Virginia. These culture systems are seemingly suitable for holding and conditioning adult mussels for spawning, and graduate student experiments are ongoing to test the range of applications to mussel conservation. A recent grant from the National Fish and Wildlife Foundation has allowed construction of a new facility to test the recirculating culture systems on a larger production scale, to begin in 2002. The new facility, coupled with an adjacent pond, will provide additional research opportunities and conditions for long-term rearing of juvenile and adult mussels.

SMALL-SCALE ECOLOGICAL FUNCTION OF FRESHWATER MUSSELS (FAMILY: UNIONIDAE) IN THE KIAMICHI RIVER, OKLAHOMA. **Daniel E. Spooner*** and **Caryn C. Vaughn**, Oklahoma Biological Survey and Department of Zoology, University of Oklahoma, Norman, OK 73019.

Freshwater mussel (Family Unionidae) biomass and species diversity is declining as a result of recent and historic anthropogenic impacts. Despite this marked trend, very little is known concerning the ecology of unionids with respect to stream ecosystem function and local macroinvertebrate assemblages. We performed a 12-month field enclosure experiment to examine the role of unionids in streams and document their associated sediment and epilithic communities. Treatments were two mussel species, *Actinonaias ligamentina* and *Amblema plicata*, shell treatment, and a non-mussel sediment treatment. We observed colonization of algae, ash-free dry mass and invertebrates in the sediment and on the shells over three time periods: 1 month, 3 months, and 12 months. Chlorophyll a, AFDM and invertebrate abundance were higher in treatments containing live mussels. This trend was significant for months where stream flows were low, but non-significant during periods of high flow. However, there were no significant differences between species despite observed trends. This study indicates that freshwater mussels have the capacity to impact local algae, invertebrate and organic matter dynamics. However this relationship appears to be context dependent and may be overridden by advective forces such as flow.

COMPARATIVE AND EXPERIMENTAL EVIDENCE FOR THE FUNCTIONAL ROLE OF FRESHWATER MUSSELS IN STREAMS. **Caryn C. Vaughn,* Daniel E. Spooner, Melissa Moore** and **Keith B. Gido**. Oklahoma Biological Survey and Department of Zoology, University of Oklahoma, Norman, OK 73019.

Both the overall abundance and species richness of freshwater mussels are declining at an alarming rate. If mussels play critical roles in the functioning of river ecosystems, this significant loss of benthic biomass may result in alterations of ecosystem processes and functions, and also may impact other stream organisms that have co-evolved with mussel assemblages. We used a comparative field study and laboratory experiments to investigate the functional role of unionids in streams. From 1999–2001, we sampled benthic macroinvertebrates, meiofauna and fishes living in and around mussel beds in nine rivers of the Quachita Uplands of Oklahoma and Arkansas. Data analyzed to date indicate that densities of some benthic groups, notably oligochaetes and filtering caddisflies, are significantly related to unionid density. Laboratory experiments compared community respiration rates, water column nutrient concentrations, algal clearance rates, excretion rates, and biodeposition rates of *Actinonaias ligamentina* and *Amblema plicata*. Both species exhibited a strong, linear relationship between biomass and most ecosystem processes at small spatial scales. However, there were only subtle differences between species in their effects. Our results indicate that unionids can have strong effects on ecosystem processes when biomass is high, but that these effects are context dependent and can be overridden by physical forces.

ASSIMILATION OF MICROZOOPLANKTON BY ZEBRA MUSSELS: THE BENTHIC ZOOPLANKTON LOOP. W.-H. Wong* and Jeffrey S. Levinton, Department of Ecology and Evolution, State University of New York, Stony Brook, NY 11794-5245; Benjamin Twining, Marine Sciences Research Center, State University of New York, Stony Brook, NY 11794-5000.

Bivalves living at high densities exert strong effects on the water column. Following their invasion of North American rivers and lakes, zebra mussels (*Dreissena polymorpha*) caused major declines in both phytoplankton and microzooplankton. We tested the hypothesis that the microzooplankton are a potential food source for zebra mussels, and by extension, other bivalves. We labeled phytoplankton with ^{14}C and fed them to two species of rotifers found abundantly in the Hudson River, which had declined following the zebra mussel invasion. The labeled rotifers were fed to zebra mussels and we estimated clearance rates and assimilation of carbon. For both species of rotifers the assimilation efficiency was 40–50%. Before zebra mussels dominated the Hudson River in 1992, the assimilation from rotifers was about 2 to 3 times higher than its routine metabolic rate and also contributed about 0.349 (J/h) to 0.662 (J/h) to mussels growth and reproduction, conferring a positive scope for growth. Since the zebra mussels became abundant, the assimilation is still sufficient to explain about 16.4% to 23.1% of the mussels routine metabolic rate. Therefore, rotifers play a conceivably large role in the zebra mussels energy budget whether at high rotifer concentrations (before the invasion) or at low rotifer concentrations (after the invasion).

GENETICS AND MOLECULAR BIOLOGY

UNSCRAMBLING THE LOBSTER GENOME: COMPLEX HETEROZYGOSITY IN THE AMERICAN LOBSTER? Charles L. Brockhouse* and Gillian Richard, Marine Aquatic Genetics, Biological Sciences, U. South Alabama, Mobile, AL 36688.

The American Lobster, *Homarus americanus* Edwards, is both a highly important economic species and a model for invertebrate physiology. In spite of this, remarkably little is known about any aspect of the genetics of this species. In view of the long breeding cycle, genomics approaches are clearly preferable to classical breeding genetics. As a prelude to a genomics program, we have undertaken genome size and meiotic studies. Using Feulgen staining densitometry, we determined the *H. americanus* genome size to be approximately 2.7×10^9 base pairs. Confocal microscopy was used to study meiosis in male lobsters. The chromosome number was found to be $n = 108$, in approximate agreement with previous studies. Large numbers of univalents with chiasma to multiple other univalents were observed at metaphase I. Most or all of the chromosomes were observed in these conformations. This is strongly indicative of heterozygous interchanges among the chromosomes, a condition which has the genetic effect of linking many chromosomes into a single linkage group.

GENETICAL BASIS OF THE PLASTICITY OF RESOURCE ALLOCATION IN THE PACIFIC OYSTER *CRASSOSTREA GIGAS*. Bruno Ernande, CNRS-CREMA, 17137 L'Houmeau, France; Joel Haure, IFREMER-LCPL 85230 Bouin, France; Lionel Degrémont, Edouard Bédier and Pierre Boudry* IFREMER-LGP, 17390 La Tremblade, France.

Phenotypic and genetic correlations between fitness-related life-history traits—such as survival, growth and reproductive allocation—have to be considered to better understand selective processes, both in the wild and in breeding programs. Quantitative genetic experiments, based on nested half-sib mating designs, were performed in the Pacific oyster *C. gigas* (1) to estimate genetic variance and the response to selection and (2) to document phenotypic and genetic trade-offs between life history traits. Physiological trade-offs, *i.e.* plastcity of resource allocation, were shown to vary among genotypes. Genetic polymorphism was primarily observed for the plasticity of reproductive effort. In addition, the degree of plasticity in reproductive effort appeared to be genetically correlated with mean survival. The signs of some significant genetic correlations were found to reverse accross environments. These results could explain the maintenance of genetic polymorphism for the studied traits. These may help us to better understand the causes of summer mortalities in *C. gigas* juveniles, on which a national program recently started in France.

THE SUCCESSFUL PRODUCTION OF TETRAPLOID EASTERN OYSTER, *CRASSOSTREA VIRGINICA* GMELIN. Ximing Guo,* Jian Wang, Brenda J. Landau, Li Li, Gregory A. DeBrosse and Krista D. Buono, Haskin Shellfish Research Laboratory, Rutgers University, 6959 Miller Avenue, Port Norris, NJ 08349.

Triploid eastern oyster grow 30–40% faster than normal diploids. Fast growth reduces not only culture duration and cost, but also losses from diseases inflicted mortality, which represents a serious problem in the eastern oyster. The ideal way to produce triploids is through diploid x tetraploid mating. Triploid production from tetraploid is as simple as producing normal diploids, 100% effective and free from genetic defects caused by polar body inhibition. Tetraploid Pacific oysters have been successfully produced and commercialized. A small number of tetraploid eastern oysters were previously produced but lost. Here we reported the production of a large number of tetraploid eastern oysters. The tetraploids were produced using the Guo-Allen method—inhibiting polar body I in eggs from triploids fertilized with normal sperm. Triploid females were individually confirmed by flow cytometry before use. Fertilized eggs were treated with 0.5 mg/L cytochalasin B from about 10 to 30 min post-fertilization (PF). Thirteen replicates were made, and eight produced significant numbers of spats (>100). Ploidy analysis at two months PF found tetraploid spat in all replicates, ranging from 10 to 100%. Over 4000 tetraploid spats were produced from the eight surviving replicates. Tetraploids were larger than triploids and diploids within groups. At five months PF, about 10% of the tetraploids changed to primarily triploid/tetraploid mosaics. The tetraploids were made from a Rutgers disease-resistant stock, which demonstrated strong resistance to MSX, a parasitic disease. The combination of superior growth and disease-resistance may make triploid eastern oysters extremely valuable for aquaculture production.

GENETIC DISTINCTIVENESS OF INSHORE AND OFFSHORE *SPISULA* CLAMS.

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Spisula solidissima is restricted to the western North Atlantic where it is commercially harvested by dredge from shelf waters between Georges Bank and Cape Hatteras. A closely related taxon, *S. solidissima similis*, inhabits the Gulf of Mexico and shallow coastal waters along the Eastern Seaboard. Although studies of the population biology and demography of *Spisula* clams have been productive because of the ability to age cohorts from annular growth rings, few data have been published on range distributions and patterns of genetic variation. The mitochondrial and nuclear DNA sequences presented here represent a preliminary effort to describe the phylogeny and biogeography of *Spisula* clams and relatives in the North Atlantic. Mitochondrial cytochrome oxidase I and nuclear calmodulin intron sequence data show the southern/inshore subspecies is genetically very distinct from *Spisula solidissima* populations from the Gulf of St. Lawrence (Canada), Georges Bank and offshore from Ocean City, Maryland. Despite the restricted inshore habitat of *S.s. similis*, genetic variation within this taxon is exceptionally high, suggesting a large effective population size or population substructure among coastal populations.

A GENETIC LINKAGE MAP OF 100 MICROSATELLITE MARKERS FOR THE PACIFIC OYSTER *CRASSOSTREA GIGAS.*

Dennis Hedgecock,* Sophie Hubert, Gang Li, and **Katharine Bucklin,** University of California, Davis, Bodega Marine Laboratory, Bodega Bay, CA 94923-0247.

A genetic linkage map will be an important tool for improving the Pacific oyster *Crassostrea gigas*, the most farmed aquatic species in the world. Combining 91 newly cloned and 23 previously published microsatellite DNA markers, we obtained 114 microsatellite DNA markers for testing on three outbred families, using 11-day-old larvae, to reduce segregation distortion caused by recessive deleterious mutations (Launey & Hedgecock 2001 Genetics 159:255). Of the 102 markers that are informative in at least one family, 98 are placed on a consensus map comprising 10 linkage groups and 880 centimorgans (cM; *cf.* karyotype of 10 chromosome pairs with an estimated length of 600–1000 cM based on chiasma frequency). Map distances and, occasionally, marker order differ between parents and among families. Of the 79 new microsatellites tested on four other *Crassostrea* species, 76 may be amplified from *C. angulata*, 65, from *C. sikamea*, 30, from *C. ariakensis*, and 8, from *C. virginica*. Decline in ability to amplify these markers across congeneric species, together with a high frequency of null alleles within *C. gigas* (40% of the loci are segregating for a null allele in at least one family), suggests that primer-target DNA sequences are rapidly evolving in oysters.

A SINGLE-STEP MULTIPLEX PCR IDENTIFICATION ASSAY TO DISTINGUISH MEGALOPAE OF *CALLINECTES SAPIDUS* FROM *CALLINECTES SIMILIS* IN PLANKTON SAMPLES.

Gregg G. Hoffman,* Ami E. Wilbur, Martin H. Posey, and **Troy D. Alphin,** Department of Biological Sciences and the Center for Marine Science, 5600 Marvin K. Moss Lane, University of North Carolina at Wilmington, Wilmington, NC 28409.

Understanding recruitment variation in economically important species such as the blue crab, *Callinectes sapidus*, requires the collection, processing, and identification of large numbers of larval animals. The identification process typically involves sorting of specimens based on morphological characters under a microscope, and subsequent enumeration of the species of interest. For some species combinations, morphological identification is complicated by a paucity of clear diagnostic features that reliably survive the collection process. In southeastern estuaries, the study of the recruitment dynamics of *Callinectes sapidus* is complicated by the co-occurrence of its congener, *C. similis*. We have developed a multiplexed PCR technique that efficiently and accurately distinguish *C. sapidus* and *C. similis* larvae. This assay relies on the use of species specific primers to amplify distinctly sized PCR (polymerase chain reaction) products and targets a portion of the mitochondrial cytochrome oxidase I gene. We have applied this technique to larval samples (megalopae) collected on settlement traps deployed during the 2000 and 2001 season in the Cape Fear estuary (North Carolina) to discern temporal and spatial patterns in larval abundance.

SEARCHING FOR DIFFERENTIALLY EXPRESSED GENES IN DIPLOID AND TRIPLOID EASTERN OYSTER, *CRASSOSTREA VIRGINICA* GMELIN.

Brenda J. Landau,* Arnaud Tanguy, and **Ximing Guo,** Haskin Shellfish Research Laboratory, Institute of Coastal and Marine Sciences, Rutgers University, 6959 Miller Avenue, Port Norris, NJ 08349.

Triploid shellfish are useful for aquaculture because of their sterility, superior growth and improved meat quality. Triploids also provide a unique model system for analysis of certain biological processes. Triploids grow faster than diploids in most bivalves studied so far including oysters. This phenomenon has been referred to as triploid gigantism. Although several hypotheses have been proposed to explain triploid gigantism, we know little about changes in gene expression in triploid oysters. The identification of unique expression profiles in triploids may enhance our understanding of basic mechanisms of growth regulation. Because triploids are sterile, comparison of expression profiles during gametogenesis between diploid and triploid oysters may lead us to genes involved in sexual maturation and meiosis. We have begun a study searching for differentially expressed genes in triploid eastern oyster in the aim to better understand the physiological processes involved in growth and reproductive differences between diploids and triploids. Subtractive libraries were made to identify differentially expressed genes in both diploid and triploid oysters. Selected clones from these libraries are being sequenced. Results from the sequence analysis will be presented at the meeting.

A BASIC AFLP LINKAGE MAP FOR THE EASTERN OYSTER, *CRASSOSTREA VIRGINICA* GMELIN.

Ziniu Yu* and **Ximing Guo,** Haskin Shellfish Research Laboratory, Institute of Coastal and Marine Sciences, Rutgers University, 6959 Miller Avenue, Port Norris, NJ 08349.

Genetic markers and maps are needed to map and improve economically important traits in oysters. Amplified fragment length polymorphisms (AFLPs) are popular markers for linkage mapping in many agricultural species because of the relative ease of developing a large number of reliable markers at low cost. We tested AFLP markers for linkage mapping in the eastern oyster, *Crassostrea virginica* Gmelin. Selective amplification primers were labeled with fluorescent dyes, and amplified fragments were detected with the ABI 310 genetic analyzer and analyzed with the GenScan software. Forty primer combinations were screened, and ten of them were selected for linkage analysis of 81 progenies from a reference family derived from a Rutgers disease-resistant stock. The 10 primer pairs produced 212 polymorphic markers in the reference family; and 150 of them were informative or segregating in one of the parents. Chi-square analysis indicated that 136 (91%) markers segregated in Mendelian ratio, while the other 14 (9%) showed significant segregation distortion ($p < 0.05$). A basic linkage map was constructed with 84 segregating markers from the male parent. The markers formed 10 linkage groups, corresponding to the haploid number of 10 chromosomes in this species. The map covered a total of 439 cM, with average interval of 5.2 cM per marker. Additional markers including microsatellites are being added to this first linkage map of the eastern oyster.

REVERSION REVISITED IN *CRASSOSTREA ARIAKENSIS:* CHROMOSOME SET INSTABILITY IN FIELD TRIALS ACROSS SALINITY REGIMES.

Mingfang Zhou* and **Standish K. Allen, Jr.,** Aquaculture Genetics and Breeding Technology Center, Virginia Institute of Marine Science, Gloucester Point, VA 23062.

VIMS has been systematically examining the potential of several non-native species for their potential in alleviating the serious decline of oyster stocks in the Virginia portion of Chesapeake Bay. One that has emerged as quite promising is the Suminoe oyster, *Crassostrea ariakensis*. We were interested in determining the stability of triploids from the period of seed to harvest (about 18 months). About 150 individually marked triploid *C. ariakensis* and 100 triploid *C. virginica* were deployed at each of seven sites in Virginia and North Carolina. Every three months, all triploid *C. ariakensis* were biopsied for hemolymph to determine the occurrence and extent of reversion (development of diploid cells within a triploid) by flow cytometry. As in other studies, *C. ariakensis* outgrew *C. virginica* at all sites. Overall, 26 mosaics were found among 919 individuals tested. The highest incidence of reversion was uncovered in medium salinity (average: ~5%) with high and low salinities 2% or less. Frequency of diploid cells within individual mosaics was generally less than 10% although we observed several "streakers" that obtained 65%, 46% and 28% diploid cells by study's end. These results demonstrate that the "risk of reversion" in commercial aquaculture of triploid *C. ariakensis* will likely be very low, but emphasizes that reversion is an inherent quality of autopolyploid oysters in the *Crassostrea* genus and persists through time. Therefore, the principal risk for recovery of reproductive potential among triploids seems to reside in unharvested and "lost" oysters that remain in the Bay for long periods of time.

EAST COAST BIVALVE INDUSTRY SESSION

AN OVERVIEW OF THE POTENTIAL FOR *CRASSOSTREA ARIAKENSIS* IN THE CHESAPEAKE BAY.

Standish K. Allen, Jr., Aquaculture Genetics and Breeding Technology Center, Virginia Institute of Marine Science, Gloucester Point, VA 23062.

VIMS has been systematically examining the potential of several non-native species for their potential in alleviating the serious decline of oyster stocks in the Virginia portion of Chesapeake Bay.

One that has emerged as quite promising is the Suminoe oyster, *Crassostrea arkiakensis*. Field trials with sterile triploids have shown a general resistance to disease, rapid growth rate, and high survival. Formal and informal test marketing of the product has been similarly successful. As with any non-native species, serious concerns exist over the long-term implications of introduction. Some of these issues are associated with simultaneous introduction of pests or pathogens and some with the ecological effects of sustained population growth in the Bay. By culturing this species in the hatchery, pest and pathogen issues are largely, but not wholly, addressed. Aquaculture of triploid-only individuals mitigates, but does not eliminate, most of the ecological concerns. Aquaculture of hatchery raised sterile triploid seed represents an intermediate solution to assisting the industry between abandoning non-native research and wholesale introduction of diploids. The industry potential is enormous, but there are lingering questions about how precisely this "revolution" will proceed.

SURF CLAM (*SPISULA SOLIDISSIMA*) CULTURE IN SOUTHERN NEW JERSEY: HATCHERY CULTURE PROTOCOLS AND PRELIMINARY FIELD GROW OUT RESULTS. Gregory A. DeBrosse,* David R. Jones, and **Eric N. Powell,** Haskin Shellfish Research Laboratory, Institute of Marine and Coastal Sciences, Rutgers University, 6959 Miller Avenue, Port Norris, NJ 08349.

The state of New Jersey harvests over 90% of the worlds surf clams, *Spisula solidissima* (Dillwyn, 1817), with an annual value exceeding $30 million. The commercial fishing industry targets large clams, typically those larger than 80 mm shell length. These clams are used primarily for clam strips and chowders. Clams smaller than 70 mm cannot be fished economically, even though their per bushel value may be high. The raw and steamed clam trade uses primarily soft-shell clams (*Mya arenaria*) and hard clams (*Mercenaria mercenaria*). In the case of *Mercenaria*, the majority of clams utilized are produced by aquaculture, and this industry in New Jersey alone produced approximately $6 million worth of clams in 1998. The surf clam grows well in New Jersey, at about twice the rate of hard clams. Cultured yearling surf clams compare favorably with hard and soft-shell clams in a variety of product forms; steamed, fried, and on the half-shell, and therefore represent another potential species for these markets. A key to *Spisula* aquaculture success is to grow clams to marketable size (~50–55mm) in one year. To do this requires spawning and rearing animals out of season (e.g., during fall, winter, and early spring). This requires conditioning of brood stock in the hatchery, and defining the parameters of successful larval and juvenile culture. The specific parameters of brood stock conditioning, larval and juvenile culture, and preliminary field grow out data will be described in the presentation.

COASTAL STATES PERMITTING PROCEDURES FOR DEPLOYING A TIDAL POWERED UPWELLING NURSERY SYSTEM. M. Richard DeVoe,* South Carolina Sea Grant Consortium, 287 Meeting Street, Charleston, SC 29401; **G. Ross Nelson,** General Engineering Laboratories, 2040 Savage Road, Charleston, SC 29407.

Shellfish aquaculture is a growing segment of the U.S. aquaculture industry. Several nursery techniques have been developed for nursery growout of oysters from seed produced by hatcheries, including land-based runway systems and upwellers, and both bottom and off-bottom nurseries. The Tidal-Powered Upwelling Nursery System (TPU) was developed as a low cost alternative to these traditional nursery methods. The TPU has the advantage of being a floating structure using tidal power to upwell water into bins that house oyster seed.

A questionnaire was sent to coastal state permitting contacts during the year 2000 to determine if the TPU is a viable nursery option for oyster growout for culturists in their states, to determine the permits that would be necessary to deploy the TPU, and to estimate the costs associated with these permits. From this initial survey, four states were selected for a more complete analysis of the permits necessary to operate the TPU—Massachusetts, South Carolina, Washington, and Alaska. The permitting processes in these states were analyzed to compare and contrast the regulatory structures among these four states and with the results of the initial survey.

RELEVANT ISSUES FOR THE EAST COAST SHELLFISH AQUACULTURE INDUSTRY: FINDING COMMONALITY TO FOSTER UNITY. George E. Flimlin, Jr.,* Rutgers Cooperative Extension, 1623 Whitesville Rd., Toms River, NJ 08755.

Hatchery supported shellfish aquaculture on the East Coast of the United States has been growing since the 1970s. Once practically obscure, growers are now in every state bordering the Atlantic with over 60 hatcheries or seed nurseries scattered among the coastal states. Some of the newer states involved in hard clam, *Mercenaria mercenaria*, or American oyster, *Crassostrea virginica*, culture have shown very quick industry growth while places where shellfish aquaculture took hold over 25 years ago have practically slowed to a stand-still. With growth however comes scrutiny by governmental agencies, both state and federal, increased regulations, competition for space, environmental issues, disease concerns, and other challenges. Presently individual states have associations which can address concerns as they arise but there is no cohesive organization which can represent the common problems, as well as, promote pro-active activities for the growers throughout the region. This discussion will review the industry priorities respective to the social, political and scientific or technological problems that face the shellfish growers. A survey of growers, associations, hatchery/nursery operators, and extension personnel provides the input. A compilation of these issues will serve as a basis for the formation of a shellfish aquaculture industry group on the East Coast.

STAKEHOLDERS' PERCEPTIONS AND CHALLENGES TO PARTICIPATORY MANAGEMENT: THE CASE OF THE SOFT-SHELL CLAM, *MYA ARENARIA* L., INDUSTRY IN MAINE. **Aman Luthra*** and **William Walton,** University of Maine at Machias, Machias, ME 04654.

Local management of the soft-shell clam (*Mya arenaria*, L.) fishery in Maine involves the interaction of several different communities including commercial harvesters, shellfish committee personnel, scientists and the state, who share a common interest of increasing the productivity of their mudflats. Efficient management is hindered due to a lack of communication between these different communities. In 2000–2001, we surveyed 26 municipal shellfish committee chairs (7 in eastern, 8 in mid-coast and 11 in southern region) and interviewed 41 commercial harvesters (11 in eastern, 14 in mid-coast and 16 in southern region) to identify the knowledge gaps that exist between these 2 groups and across the 3 regions. Responding towns differed significantly in the number of commercial licenses issued and the price of recreational and commercial licenses across the 3 regions. Interviewed harvesters differed regionally in the number of active clamming months per year and in the number of commercial licenses held by each harvester. Both harvesters and chairs identified pollution, predation and overfishing as the factors that most severely limited the number of harvestable clams on the flats. Both groups identified a diverse array of predators with seagulls (*Larus* spp.) and green crabs (*Carcinus maenas*) topping their list. Harvesters identified raccoons (*Procyon lotor*) as the third most damaging predator while only one chair mentioned it. Stock enhancement is being pursued at different intensities and with different foci among the responding towns in the three regions. While most towns in mid-coast (57%) and eastern region (71%) are transplanting wild seed as a stock enhancement strategy, only responding towns from mid-coast (50%) and southern Maine (33%) are planting hatchery-reared seed. Both harvesters and chairs were opposed to reducing the two-inch minimum as well as leasing of intertidal flats. The survey and interviews identified areas of agreement and disagreement among these communities involved in the management of soft-shell clams, and these results can be used to guide both research and improved management.

EMBRACING AQUACULTURE BMP'S: A CONCEPT WHOSE TIME HAS COME AS A GOOD NEIGHBOR OR JUST ONE MORE THING TO DEAL WITH? **Sandra Macfarlane,*** Coastal Resources Specialists, Orleans, MA 02653.

In a labor intensive, high risk and competitive industry frought with uncertainty and reliance on natural conditions for growing food, initial efforts to foster an industry-wide or area-wide agreement on the most environmentally sound and "good neighbor" approach to the business have received a luke-warm reception at best from many growers. However, misinformation is rampant outside the industry from stakeholders who also have a vested interest in the manner in which the aquaculture is conducted in their back yard. In order for aquaculture to be sustainable, issues such as water quality, disease prevention and control, and care and maintenance of gear as well as animals is essential. Other stakeholders must be convinced that the aquaculture operation is conducting itself in the most prudent of manner on these issues and adopting BMP's or a Code of Practice is an effective mechanism to project a positive image. However, bringing all the voices to the table and bridging gaps in understanding among stakeholders is a crucial element in developing effective BMP's.

OPTIMIZATION OF TIDAL UPWELLER DESIGN: PROJECT WRAP-UP. **Dana L. Morse*** and **John Riley,** Maine Sea Grant Program/UMaine Cooperative Extension, Darling Marine Center, Walpole, ME 04573.

A fifth-scale model of a commonly-used tidal upweller design was built, and tested in the tow tank at the University of Maine, in Orono. The goal was to better understand the effects of design components on overall flow through the system, thus allowing future construction to be maximally efficient, and therefore profitable.

Three engineering considerations were tested at different speeds in the laboratory: scoop size, silo outlet opening size, and reduction of mooring line forces. Site visits were also made to local shellfish hatcheries, to examine the effects of shellfish seed size, bed depth, and shellfish species, on frictional flow loss in an upweller silo.

Results indicate that it is critical that the upweller platform maintains a level profile, as changes in horizontal aspect have adverse effects on flow and growth. Silo outlets should be made as non-restrictive as possible, by increasing the diameter of the outlet holes, submergence of the outlets, or by removing the upper portion of the silo wall entirely, and protecting against shellfish loss with plastic netting. Enlarging the scoop area over the initial design size has a limited effect on increasing flow through the upweller. Smaller shellfish seed tended to create stronger frictional head losses than the larger seed. Lastly, placement of an escape vent in the rear of the upweller can help to maintain a level profile for the platform.

In general, the original Mook design was well thought out. Though some modifications have resulted in performance improvements, the approach is sound and to the extent possible, makes use of the potentials afforded by tidal action.

GROWTH AND ECONOMICS OF SHELLFISH NURSERY CULTURE IN A COMMERCIALLY AVAILABLE FLOATING UPWELLER.

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A small, commercially-available, floating upweller that serves as a dual-use dock and work platform has been deployed in several sites on the east coast. Five seasons of testing and development have led to production of a unit that is durable, functional, economical to operate and easy to maintain. Several producers claim that the device has resulted in substantial improvements in their production economics by accelerating growth, reducing variation in growth rates and cutting labor costs.

Growth and economic data from several Coastal Aquacultural Supply floating upwellers are compared with other conventional nursery culture methods such as a "Taylor float" and rack and bag systems. Capitol costs, labor costs, maintenance and operational expenses are described as well as siting and permitting considerations.

ENHANCING SEED AVAILABILITY FOR THE HARD CLAM AQUACULTURE INDUSTRY THROUGH APPLICATION OF REMOTE SETTING TECHNIQUES.

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Hard clam, *Mercenaria mercenaria,* aquaculture has developed rapidly in the southeastern United States. Adequate seed availability is a major industry concern and has recently faced critical shortages. Technical procedures were developed and demonstrated to determine the economic feasibility of transferring remote setting technology from the Pacific Northwest molluscan shellfish industry to the hard clam culture industry. Competent pediveliger larvae obtained from commercial hatcheries were refrigerated, stored overnight and delivered chilled to remote set locations for evaluation of technique, site and season. Participating land-based nurseries were modified to incorporate mechanical filtration of water supply, remote setting tanks and downwellers. Management regimes evaluated over 4 trials included: 1) supplemental feeding with a commercial algal paste versus none, and 2) duration of shipping. Biological features documented included survival and time to reach a 1 mm seed, the minimum size presently obtained by nurseries. Results will provide for operational procedures and guidelines for remote setting of hard clam seed. The economic characteristics associated with the remote setting system will be described. Costs associated with producing the seed, including the original larvae cost, will be compared with the current market price for 1 mm seed. Remote setting of hard clam seed would allow nursery operators to become less dependent upon traditional seed sources and ensure a reliable supply of seed to growers.

USING SHELLFISH SEED AS A PUBLIC ENHANCEMENT TOOL: A REVIEW OF ITS USE AND SUCCESS IN THE NORTHEAST US.

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In response to dwindling stocks of shellfish (hard clams, *Mercenaria mercenaria,* oysters, *Crassostrea virginica,* soft shell clams, *Mya arenaria,* etc.) and increased fishing pressure, fisheries managers are implementing a variety of restrictive and proactive measures. One proactive 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 shellfish that survive and grow to a legally harvestable size, and indirectly by increasing larval supply and subsequent year classes. This technique has been widely adopted in southern New England for hard clams and in northern New England for soft shell clams. Despite such implementation, quantitative assessments are rare. Here I review 1) local managers' perceptions of the success of such programs based on interviews and surveys, 2) quantitative and experimental studies of the survival of shellfish seed, and 3) known limiting factors. Although perceived and actual survival rates span the spectrum, this variation can largely be attributed to predation, which is a function of seed size at least in hard clams. Unlike private aquaculture, however, the scale of public seeding generally prohibits the use of nets to protect the seed. To illustrate the choices faced by public managers, I will present a case study of potential public seeding strategies of hard clams in Wellfleet, Massachusetts, highlighting the costs, risks and benefits.

GROWTH AND ECONOMIC ADVANTAGES OF DISTRIBUTED POWERED UPWELLERS: CREATING A NEW AQUACULTURE NICHE.

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Distributed powered upwellers, (also known as grid-isolated, or renewable energy based upwellers) allow upweller technology to be used in areas where traditional sources of electrical/mechanical energy are not possible or desirable. The author presents the growth and cost performance findings from two years of research in the development of distributed generation upwellers utilizing solar energy. The design and prototypes went through several changes during the two years to facilitate its operability in a harsh environment. The present configuration has proved to have high reliability, provided for good growth, low mortality, and good ergonomics. Growth of *Crassostrea virginica* averaged 320% over base case for a relatively short four month growing seasons. The major benefits of this technology is the use of upwelling technology in areas that are not conducive to traditional utility powered upwellers, allowing for the benefits of upwellers to be realized in locations traditionally thought not to be ammenable to such technology.

THE NEW OYSTER WARS: POLICY PERSPECTIVES ON THE INTRODUCTION OF *CRASSOSTREA ARIAKENSIS* IN THE CHESAPEAKE BAY.

Donald Webster, University of Maryland, Wye Research & Education Center, P.O. Box 169, Queenstown, MD 21658.

Oyster harvests in the Chesapeake Bay have declined over ninety percent in the past fifty years due largely to the influence of *Haplosporidium nelsoni* and *Perkinsus marinus*. Recent studies regarding the potential of the species *Crassostrea ariakensis* have shown that this animal may provide many features desirable in the reconstruction of the oyster fishery as well as environmental management of the Bay. However, since it is a non-indigenous species, there are many factors to be considered in its introduction. Variations in the current status of the *C. virginia* resource, as well as historical differences of management techniques inherent in the two states have led to challenges regarding open-water introductions as well as plans to increase stocks of the non-native oyster in the future. This paper focuses on the policy differences between Maryland and Virginia and the attitudes of various user groups that are interested parties in the potential introduction of *C. ariakensis* in the Chesapeake Bay.

BIVALVE DISEASE STATUS AND TRENDS

RECENT EXPANSION OF JUVENILE OYSTER DISEASE (JOD) IN MAINE.

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Juvenile oyster syndrome has caused mortalities of cultured Eastern oysters, *Crassostrea virginica*, in Maine since 1988. We have identified a likely etiological agent, a novel taxa in the *Roseobacter* clade of the marine alpha-proteobacteria. In oysters exhibiting signs of the disease (reduced growth and uneven valve growth), this bacterium comprises 40–95% of the total bacteria colonizing gill and mantle surfaces. Until recently, all documented outbreaks in Maine have occurred in the Damariscotta River. One strategy employed by oyster growers to avoid JOD mortality has been to use nursery sites outside the Damariscotta River. In the summer of 2000, however, mortalities of 70% in the New Meadows River and 20% in Maquoit Bay were reported by growers. In 2001, mortalities of 40% and 50% occurred in the New Meadows River and Sheepscot River, respectively. Thus in the last two years, JOD mortalities occurred in three additional locations, and in all cases, animals were heavily colonized by the same species of *Roseobacter*. The potential for utilizing genetic signatures of this bacterium for epidemiology will be discussed.

STATUS AND TRENDS OF DERMO AND MSX IN SOUTH CAROLINA.

David Bushek* and **Dwayne Porter,** Baruch Institute, University of South Carolina, PO Box 1630, Georgetown, SC 29442; **Loren D. Coen, M. Yvonne Bobo,** and **Donnia L. Richardson,** Marine Resources Research Institute, SCDNR, 217 Fort Johnson Rd., Charleston, SC 29412.

South Carolina has a relatively abundant and extensive population of oysters throughout the state. Dermo (*Perkinsus marinus*) and MSX (*Haplosporidium nelsoni*) are both present, but they do not cause widespread oyster mortality as reported in other regions. Surprisingly, little attention has been paid to understand why this difference exists. In South Carolina, virtually all oysters inhabit high salinity (20–35 ppt) estuaries where they form extensive fringing and mounding reefs in the 1.5 to 2 m intertidal zone. Their intertidal existence exposes them to daily temperature fluctuations that often exceed 30°C, with winter extremes below freezing and summer extremes that can exceed 50°C.

Since 1972, SCDNR's Marine Resources Research Institute (MRRI) has been documenting the occurrence of Dermo throughout the state. MSX has been documented since at least 1986 and monitored since 1994. In addition, the NOAA-funded Urbanization of Southeastern Estuarine Systems (USES) project has monitored these diseases in pristine and developed areas to identify potential relationships with different land-use patterns and landscapes. The data indicate that *P. marinus* is ubiquitous and abundant throughout the state. Prevalence and intensity peak from late summer to early winter, but unlike populations in the northeast, the parasite remains prevalent throughout the year. By comparison, *H. nelsoni* is relatively rare or altogether absent with mean prevalence generally below 20%. *Haplosporidium nelsoni* is known to be very sensitive to low salinities (10ppt or less). Low salinity is also often invoked as a major control that reduces prevalence and intensity of *P. marinus*. In South Carolina, most sites where oysters thrive rarely encounter such low salinities for more than a few hours. Furthermore, lowest salinities occur during low tide when the oysters are exposed. Instead, elevated temperatures may exceed the thermal tolerances of the parasites just long enough to shift the host-parasite dynamic in favor of the host. In this talk, we summarize recent findings and discuss implications for intertidal oysters along the southeast Atlantic coast of the United States.

A DECADE OF DISEASE: A REPORT OF OYSTER DISEASE MONITORING EFFORTS IN THE LOWER CHESAPEAKE BAY 1990–2000. **Lisa M. Ragone Calvo*** and **Eugene M. Burreson,** Virginia Institute of Marine Science, School of Marine Science, College of William and Mary, Gloucester Point, VA 23062.

In the late 1980s oyster populations in the Virginia portion of the Chesapeake Bay were significantly impacted by epizootics of the oyster parasites *Perkinsus marinus* and *Haplosporidium nelsoni* as a consequence of dry and warm climatic conditions. The distributions of both parasites were widespread and the range of *P. marinus* was extended into upper tributary areas that had historically been disease free. Since that time VIMS has been extensively monitoring lower bay oyster populations for the presence of the pathogens via annual fall surveys of 30–40 lower bay oyster populations, monthly surveys of four oyster reefs in upper James River, and monthly summer surveys of naïve sentinel oyster that are transplanted to a site in the lower York River in May of each year. *Perkinus marinus* continues to persist in upper tributary areas, despite the occurrence of several years that were relatively wet and cold. Record high prevalences and intensities of *P. marinus* were observed in 1999 and 2000, and the parasite was found in all areas where significant oyster stocks remain. *Haplosporidium nesloni* prevalences were generally low in 1990, 1993, and 1998; however, record high levels were observed in 1999. In 1999, epizootics of the parasite were extensive and caused severe oyster losses in many areas. Clearly, both pathogens continue to be a significant threat to lower Cheaspeake Bay oysters. The correlation of disease patterns and environmental conditions will be discussed.

DEVELOPMENT AND APPLICATION OF A PCR FOR *MIKROCYTOS MACKINI*, THE CAUSATIVE AGENT OF DENMAN ISLAND DISEASE IN OYSTERS. **Ryan B. Carnegie,* Gary R. Meyer, Janice Blackbourn,** and **Susan M. Bower,** Fisheries and Oceans, Science Branch, Pacific Biological Station, Nanaimo, British Columbia, Canada V9R 5K6; **Nathalie Cochennec-Laureau,** IFREMER, Laboratoraire de Génétique, Aquaculture et Pathologie, BP 133, Ronce les Bains, F-17390 La Tremblade, France.

Mikrocytos mackini has caused Denman Island disease of *Crassostrea gigas* and *Ostrea edulis* in British Columbia, Canada since at least 1960. A small protistan parasite, *M. mackini* is commonly observed with standard histopathological techniques around the green pustules characteristic of Denman Island disease. In oysters with subclinical infections, however, *M. mackini* is not easily detected, and it is rarely observed before March and after June. Its mode of transmission, life cycle, and taxonomic affinities are unknown.

Our objectives were to design and validate molecular assays for *M. mackini* and to use these tools to re-examine the seasonal prevalence of *M. mackini* infections. We sequenced 1457 base pairs of *M. mackini* SSU rDNA and developed a PCR specific for this gene. We used this PCR to screen palp DNA samples from cultured *C. gigas* from Henry Bay, Denman Island, BC monthly (N = 100/month) from January to December 2001. PCR detected *M. mackini* more sensitively than did standard histopathology. PCR-based *M. mackini* prevalence estimates met or exceeded histopathology-based estimates in every month, and only two oysters *M. mackini*-positive by histopathology were misdiagnosed as negative by PCR. PCR confirmed a trend in seasonal *M. mackini* prevalence identified earlier by histopathology. Prevalence peaked in April and particularly May, when 22% of lower intertidal *C. gigas* were *M. mackini*-positive by PCR (12% by histopathology). However, PCR also detected *M. mackini* in the palps of apparently healthy *C. gigas* in every month of the year (through September 2001), a first indication that *M. mackini* may persist subclinically in oyster populations year round.

DEVELOPMENT OF HIGH DISEASE RESISTANCE IN A WILD OYSTER POPULATION. **Susan E. Ford,** Haskin Shellfish Research Laboratory, Rutgers University, 6959 Miller Avenue, Port Norris, NJ 08349.

In 1957–1959, the introduced parasite, *Haplosporidium nelsoni*, killed 90–95% of the oysters (*Crassostrea virginica*) in lower Delaware Bay and about half of those in the upper Bay. Shortly thereafter, *H. nelsoni*-caused mortalities in the native population declined, equalling that of first-generation selectively bred oysters. For two decades, no further change in the wild population occurred, although steady improvement was achieved by continued selective breeding. Survival of the wild population is though to have plateaued because most oysters inhabited the upper bay and were protected from *H. nelsoni* by low salinity. From 1957 through 1989, *H. nelsoni* prevalence was cyclic, but overall high (annual maxima of 50 to 90%). After 1989, however, prevalence in wild oysters rarely exceeded 30% even though unselected oysters continued to become heavily infected, and molecular detection indicated that infective stages were present throughout the Bay. This apparent "second step" in the development of resistance in the native oysters occurred after the incursion of *H. nelsoni* into the upper bay in the mid 1980s, with widespread and heavy oyster mortalities occurring for the first time since the 1957–59 epizootic.

THE LONG-TERM ROLE OF PARASITIC DISEASES IN OYSTER POPULATION DYNAMICS. **Stephen J. Jordan** and **Jessica Vanisko.** Sarbanes Cooperative Oxford Laboratory, 904 S. Morris St., Oxford, MD 21654.

Populations of the Eastern oyster, *Crassostrea virginica*, suffer high rates of mortality from Dermo, MSX, or both diseases throughout most of the range of the species. The principal effect of high mortality rates on population structure is truncation of the size distribution, skewing the populations toward smaller oysters. Secondary effects include reductions in population biomass, harvests, and spawning potential, although there does not appear to be a strong effect on recruitment of juveniles. In Chesapeake Bay, the diseases, in combination, have had their most negative effects since the late 1980s, when Dermo spread throughout the population. Over the past decade, disease intensity and oyster mortality rates have been strongly dependent on salinity, intensifying in drought years, moderating during wet periods, and affecting most severely sub-populations in higher salinity regions. Time series of oyster population data from Maryland have been used to construct a model that simulates trends in the population at various rates of disease mortality, fishing mortality and recruitment. Current rates of total mortality appear not to be sustainable without significant management intervention.

CAPE COD BIVALVE DISEASE MONITORING PROGRAM, YEAR I.

Dale Leavitt,* **D. Murphy**, **W. Burt**, and **W. Clark**, Cape Cod Cooperative Extension, Barnstable, MA 02630; **M. Hickey** and **J. Moles**, Department of Marine Fisheries, Pocassett, MA 02559; **R. Smolowitz**, Marine Biological Laboratory, Woods Hole, MA 02543.

The effect of disease on bivalve populations is an important factor in determining management methods for the culture, harvest and movement of bivalves. In order to provide the information needed by extension and regulatory agencies and the aquaculturists, to effectively manage these populations on Cape Cod, MA, a monitoring program has been established as a cooperative project between Cape Cod Cooperative Extension Agency and the MA Department of Marine Fisheries. Hard clams, Eastern oysters and soft shell clams will be monitored over the course of each year. It is anticipated that the monitoring program will become a routine method for disease information gathering in MA.

Oysters and hard clams were collected in the fall of 2001. Twenty-five oysters from each site underwent testing for *Perkinsus marinus* using Thioglyocollate culture methods. Samples were also evaluated histologically for MSX and SSO. Sixty hard clams from each selected site were examined grossly for QPX. Twenty-five of these were examined histologically. In 2002, twenty five soft shell clams from several sites will monitored. Results of the first year of monitoring will be presented at this session.

PREVALENCE AND INTENSITY OF PARASITIC DISEASES IN BIVALVES FROM RHODE ISLAND WATERS.

Karen L. Mareiro,* and **Marta Gómez-Chiarri**, Fisheries, Animal, and Veterinary Science, University of Rhode Island, Kingston, RI 02881; **Katherine Kerr** and **Emily Carrington**, Department of Biological Sciences, University of Rhode Island; **Arthur Ganz**, Division of Fish and Wildlife, Department of Environmental Management, Wakefield, RI 02879.

The goal of this Shellfish Disease Survey is to assess disease prevalence and intensity in Rhode Island bivalves. Eastern oysters (*Crassostrea virginica*) were collected from several wild populations and local farms in August and November 1998–2001. Hard clams (*Mercenaria mercenaria*) were collected from selected locations in 1998, 2000 and 2001. Dermo disease (caused by *Perkinsus marinus*) is widespread in Rhode Island oysters, while prevalence of Multinucleated Sphere X (caused by *Haplosporidium nelsoni*) and Seaside Organism (caused by *Haplosporidium costale*) is low and restricted to a few locations. Prevalence of Dermo disease ranged from 0–100%, remaining low in oysters from aquaculture leases and a few wild locations. Intensity of Dermo disease has remained unchanged or increased since 1998. Several factors could be responsible for differences in prevalence of Dermo disease, including genetic factors, lack of infective particles and differences in environmental conditions. Trematode infections were common in Rhode Island oysters, but high prevalences of heavy infections were found only in wild oysters from Point Judith Pond and Block Island. Wild oysters from Pawcatuck River showed a high prevalence of the apicomplexan parasite *Nematopsis ostrearum.* No Quahog Parasite Unknown (QPX) was detected in Rhode Island hard clams.

MODIFICATION AND FIELD TRIALS OF A MULTIPLEX PCR FOR THE DETECTION OF THREE PROTOZOAN PATHOGENS OF THE EASTERN OYSTER, *CRASSOSTREA VIRGINICA*, GMELIN 1871.

Spencer Russell,* **Salvatore Frasca, Jr.**, and **Richard A. French**, Department of Pathobiology and Veterinary Science, University of Connecticut, Storrs, CT 06269; **Inke Sunila**, Bureau of Aquaculture, Department of Agriculture, Department of Agriculture, State of CT, Milford, CT 06460.

Populations of eastern oysters (*Crassostrea virginica*) along the eastern coast of North America have repeatedly experienced outbreaks of epizootic disease and mass mortality due to infections by protozoal parasites. As polymerase chain reaction (PCR) methodologies become routine laboratory procedures and diagnostic tools of choice, it is imperative to incorporate PCR quality controls to avoid false positive and negative results. In this study we describe the modification of a previously developed multiplex PCR (MPCR) for the detection of the eastern oyster parasites *Haplosporidium nelsoni, Haplosporidium costale* and *Perkinsus marinus* by incorporation of a quality control extraction and amplification PCR product from primers directed against an 805 base-pair (bp) sequence of the 28S SSU rRNA sequence of *Crassostrea virginica.* The modified MPCR simultaneously amplified 805bp, 564bp, 301bp and 151bp fragments of the SSU rRNA of *C. virginica, H. nelsoni, P. marinus* and *H. costale*, respectively. The *C. virginica* gene product tests the quality of extracted DNA used to support amplification in an optimized and appropriately performed PCR. In addition, we examined the species specificity and sensitivity of the newly modified MPCR for the detection of *H. nelsoni, H. costale* and *P. marinus* and compared its performance to that of the conventional diagnostic techniques of histopathological tissue examination and the Ray/Mackin fluid thioglycollate medium assay. Five hundred thirty oysters were sampled from 12 sites along the East Coast of the United States from the Gulf of Mexico to New England throughout the entire range of these parasites. Our results indicate that the newly modified MPCR is specific for the detection of *H. nelsoni, H. costale* and *P. marinus*. Through spike/recovery experiments, the sensitivity of the MPCR for *H. nelsoni, H. costale* and *P. marinus* was 100 fg, 10 fg and 100 fg, respectively, from 25 mg of oyster tissue. The modified MPCR detected 118 of 530 (22%) oysters with *H. nelsoni*, 12 (2%) oysters with *H. costale* and 212 (40%) oysters with *P. marinus* infections. The RMFT assay detected *P. marinus* infection in 163 (31%) oysters. Histopathological examination detected *H. nelsoni* and *H. costale* infections in 30 (6%) and 4 (0.8%) oysters, respectively. The MPCR is a more sensitive diagnostic assay for the detection of *H. nelsoni, H. costale* and *P. marinus*, and the inclusion of an oyster quality control product in the multiplex limits the number of false negative results from extraction or PCR failures.

DERMOWATCH: A WEB-BASED APPROACH FOR MONITORING THE OYSTER PARASITE *PERKINSUS MARINUS (DERMOCYSTIDIUM MARINUM)*.

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A web site called DermoWatch (www.blueblee.com/dermo) is being used to monitor *Perkinsus marinus* (= *Dermocystidium marinum*) in Galveston Bay, Texas. The main page provides the most recent data from nine locations. Data include measured water temperature (T) and salinity (S), calculated percent infection and weighted incidence (WI) of parasitism, and estimated time to a critical level of disease (t-crit). (The t-crit is calculated as the time in days to reach a critical WI if 1.5, assuming no change in T and S.) By expressing the solution in days, oyster growers can make informed decisions about when to move or harvest their oysters. Furthermore, they can relate observed mortalities to estimates of t-crit, and thus *P. marinus* ceases to be an "unseen" killer. With a utility called the DermoCalculator the web site is useful, not just in Galveston Bay, but wherever *P. marinus* is found. It allows anyone with information on T and S, oyster length and initial WI to determine a t-crit. A limitation of the approach in Galveston Bay is that t-crit is calculated from a single (monthly) measurement of T and S. A monitoring station is being constructed in Louisiana with which real-time measures of T and S will be used to constantly update calculations of t-crit. More frequent inputs of T and S should increase the reliability of the t-crit estimates.

DETECTION OF A PREVIOUSLY UNDESCRIBED HAPLOSPORIDIAN-LIKE INFECTION OF A BLUE MUSSEL (*MYTILUS EDULIS*) IN ATLANTIC CANADA.

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Microscopic examination of a blue mussel, *Mytilus edulis* Linnaeus, with an unusual macroscopic appearance revealed the presence of a haplosporidian-like infection throughout the soft tissues. This is the first documented occurrence of this group of parasites in Atlantic Canada. Large numbers of blue mussels and American oysters, *Crassostrea virginica* Gmelin, are sampled on an ongoing basis to monitor their disease profiles within Atlantic Canada. Twelve years of histological sampling has established a disease profile including many protozoans and diseases of local concern but no infectious agents resembling those listed by the Office International des Epizooties (OIE). Samples were sent immediately to the Reference Laboratory for Haplosporidiosis at the Virginia Institute for Marine Science, where they were screened for OIE-notifiable disease agents by in situ hybridization using *Haplosporidium nelsoni* and *H. costale*-specific DNA probes. These did not hybridize with the parasite in the mussel. The spores of the parasite are operculate and measure 3–5 microns by 6–8 microns. They show a diversity of forms from roughly spherical to pyriform with or without filamentous extensions. These resemble the haplosporidians from *Mytilus californianus* and *Mytilus edulis* from California (*Haplosporidium tumefacientis*), as well as a *Haplosporidium* sp. described from mussels in Maine. As with these previous descriptions, the current infection, although massive, was not associated with any haemocyte defense response indicative of an acute pathogenic infection. No other infections have been found in Atlantic Canada, and the significance for the lucrative Atlantic Canadian mussel industry is unknown.

MONITORING BIVALVE HEALTH IN LONG ISLAND SOUND.

Inke Sunila, State of Connecticut, Department of Agriculture, Bureau of Aquaculture, Milford, CT 06460.

Long Island Sound (LIS) has a viable, economically important culture of eastern oysters (*Crassostrea virginica*) and quahogs (*Mercenaria mercenaria*). Connecticut has 65,000 underwater acres leased for bivalve culture, New York 3,500 in LIS. Bivalve health is constantly monitored in the field and hatchery operations, and diagnostic service is available in the case of mortalities. Oysters and clams are diagnosed for inflammatory responses (acute or chronic), degenerations (ceroidosis, atrophy), cell and tissue death (necrosis vs. apoptosis), hemodynamic derangements (hemorrhage, edema), growth derangements (hyperplasia, metaplasia) and tumors (benign or malignant). Possible pathological irritants, such as infective agents or environmental factors are identified. An epizootic caused by *Haplosporidium nelsoni* (MSX) in 1997–1998 caused a 76% reduction in oyster production. At the present time *Haplosporidium costale* (SSO) and possibly other haplosporidian species occur as coinfections with MSX contributing to an annual loss of 13% in oyster stocks. Impact of the 1997–98 MSX epizootic still affects the oyster industry since those year classes would be on the market now. Dermo disease (*Perkinsus marinus*) occurs with a high prevalence (80%) and a low intensity (1 on Mackin Scale). It has not been associated with significant field mortalities. On the contrary, hard clam harvest has seen a six-fold increase during the last five years. Hard clams in LIS are healthy, and the ecosystem does not appear to provide optimal conditions for the clam parasite QPX to proliferate. We have recently adapted molecular methods to monitor bivalve diseases.

DISEASES OF CRUSTACEA

ECOLOGICAL RAMIFICATIONS OF DISEASE IN THE CARIBBEAN SPINY LOBSTER, *PANULIRUS ARGUS*.

Donald C. Behringer, Jr.* and **Mark J. Butler, IV**, Old Dominion University, Norfolk, VA 23529; **Jeffrey D. Shields**, Virginia Institute of Marine Science, Gloucester Point, VA 23062.

Spiny lobsters have few reported diseases. The identification and prevalence of pathogenic diseases in wild populations of spiny lobsters are poorly known. We recently discovered the first pathogenic viral disease (HLV-PA) known from a lobster, in this case the Caribbean spiny lobster, *Panulirus argus*. Our findings suggest that the disease alters the behavior and ecology of this species in fundamental ways, in part via remarkable changes in the social behavior of healthy individuals in response to diseased conspecifics. Both field and laboratory data show significant avoidance of infected lobsters by healthy conspecifics. Since 1999, we have identified infected juvenile lobsters at 75% to 100% of the 14 nursery habitat sites that we have surveyed twice a year (summer and winter) in the middle and lower Florida Keys, USA. The disease is highly prevalent with prevalences of up to 40% in juveniles (mean = 8%). Infected animals are often moribund, exhibit lethargy, and have milky or chalky hemolymph. Prevalences in initial challenge trials using infected hemolymph from infected donors resulted in a prevalence of 90% (n = 20, control n = 10). The virus appears to be highly pathogenic and moderately lethal with deaths occurring after 60–90 days in inoculation trials. An intriguing epidemiological twist is that commercial and recreational fishing activities for this economically valuable species may potentially contribute to the spread of the pathogen.

FIELD OBSERVATIONS ON THE DEVELOPMENT AND PROGRESS OF A SHELL DISEASE EPISODE FOR AMERICAN LOBSTER IN RHODE ISLAND: 1995–2001.

Kathleen Castro, University of Rhode Island Fisheries Center, Kingston, RI; **Thomas Angell**, RI Department of Environmental Management, Division of Fish and Wildlife, Wakefield, RI 02880.

From 1995 to 2001, shell disease in the American lobster was monitored in Rhode Island waters including Narragansett Bay, Rhode Island and Block Island Sound and the offshore areas of Block and Hudson Canyons. A tag-recapture study conducted by RI lobstermen also included a shell disease category. In the inshore population, a significant increase in frequency and severity of the disease was documented beginning in 1996, reaching an 31% proportion infected overall in 2001. Spatial information is available and describes a rapid increase in the Upper East, Lower East and West Passages from 1997–1998. Proportions in the Upper East Passage and West Passage in Narragansett Bay continue to rise, while the proportion infected in the Lower East Passage has declined. Proportion observed with the disease is correlated with the molting period, with rapid increases in September and October after the major molt. In 2000 and 2001, there appears to be a shift to higher infection rates in small animals and greater number of males and immature females than previously observed. Tag-recapture data is providing information on the progress of the disease on individual lobsters and re-infection percentages after a molt. Field data such as these may provide valuable information concerning the causes and consequences of the disease on the lobster populations.

EFFECTS OF PARASITES ON BEHAVIOR OF GRASS SHRIMP, *PALAEMONETES PUGIO*.

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The effect of parasites on grass shrimp *Palaemonetes pugio* was studied using shrimp from three sites which had varying levels of contamination. There was an inverse relationship between level of contamination and level of parasites. Parasitized shrimp had either *Microphallus sp.*, *Microphallus* hyperparasitized by *Urosporidium sp.*, or *Probopyrus pandalicola* parasites. *Microphallus* and its hyperparasite are endoparasites within muscle, while *P. pandalicola*, which lives in the gill chamber, is considered an ectoparasite. Parasitized shrimp were compared to unparasitized controls on latency to swimming in a novel environment, activity, freezing to a startle stimulus, and latency to finding food. Shrimp from the least contaminated site had the longest latencies and lowest activity levels. Shrimp with *P. pandalicola* had the longest freezing times. Shrimp with *Urosporidium* tended to have longer swimming latencies than controls. *Microphallus* did not systematically affect behavior, even in shrimp in which they were numerous. Although site differences have a greater effect on behavior than parasite load, there is a complex relationship between contamination, parasite levels and behavior.

CYTOCENTRIFUGE PREPARATIONS: AN ALTERNATE METHOD TO EXAMINE THE HEMOCYTES OF THE AMERICAN LOBSTER *HOMARUS AMERICANUS*.

Barbara Horney and **Andrea Battison,*** Department of Pathology and Microbiology; **Allan MacKenzie**, The AVC Lobster Science Centre, Atlantic Veterinary College, University of Prince Edward Island, Charlottetown, PE, Canada, C1A 4P3.

Current techniques used for examination of crustacean hemocytes can be labour intensive, costly, and have a slow turnaround time as is the case with transmission electron microscopy. Phase contrast microscopy, although inexpensive and rapidly performed, does not provide a permanent record of the results.

Cytocentrifugation of anticoagulated hemolymph samples, followed by staining with a modified Wright's-Giemsa stain, provided an excellent means to evaluate the hemocytes of *Homarus americanus*. The technique is simple to perform, requires relatively inexpensive laboratory equipment, and provides a permanent record of results within one hour of sample collection. Optimal results were obtained when slides were prepared within 6 to 8 h of sample collection.

Preliminary findings using hemolymph samples from the rock crab, *Cancer irroratus* suggest that this technique could be compatible for the examination of hemocytes of other crustacean species.

PARASITES IN *DIPOREIA* SPP. AMPHIPODS FROM LAKES MICHIGAN AND HURON.

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Diporeia populations began to decline in Lake Michigan in 1992, just three years after the nonindigenous zebra mussel *Dreissena polymorpha* was first reported. *Diporeia* are detritivores, feeding upon organic material freshly settled from the water column. In turn, they are fed upon by most fish species found in Lake Michigan and are a major food-web link between pelagic production and upper trophic levels. Although the decline in *Diporeia* populations was thought to be due to zebra mussels intercepting food material (i.e., algae) before it settles to the bottom, sampling efforts indicate sufficient food is still available to the amphipods, and *Diporeia* lipid content remain high, indicating the population is not deprived of food. An alternative explanation for the amphipod population decline may be pathogens.

Diporeia amphipods were sampled to assay the prevalence of disease and see whether prevalence of disease varied by time or location. Surveys revealed numerous parasites in amphipod tissues including virus, rickettsia-like microorganisms, fungus, a haplosporidan, microsporidans, external ciliates, gregarines, and worms. Prevalence of nodules and parasites varied among surveys, dates sampled, and locations sampled. No one etiologic agent has been identified as causing the amphipod population decline but several parasites identified during this investigation including microsporidans, rickettsia-like microorganisms, haplosporidan, and fungus likely result in amphipod mortalities.

PATHOLOGICAL ALTERATIONS IN THE EYES OF THE AMERICAN LOBSTER, *HOMARUS AMERICANUS*, INFECTED WITH *PARAMOEBA* SP.

Jeffrey D. Shields,* Virginia Institute of Marine Science, Gloucester Point, VA 23062.

In September, 2001, 31 lobsters, *Homarus americanus*, from Western Long Island Sound were examined for pathologies associated with infection by *Paramoeba* sp. Only 1 animal (3.2%) presented with gross morbidity and pathology indicative of a presumptive infection by *Paramoeba* sp. Histologically, light to moderate infections were observed in 35.5% of the lobsters. Several altered tissues were observed in the lamina ganglionaris, optic nerve complex and ommatidia of the eyes. Eye pathologies ranged from minor shifts or losses of optic pigments associated with the optic nerves, to necrosis of the optic nerve with a complete shift of pigments into the ommatidia. In more severe cases, the optic nerves were obliterated, with a partial to complete disruption of the basement membrane proximal to the ommatidia. Necrosis of the retinular cells varied with the severity of the damage to the optic nerves. There was marked infiltration of hemocytes into the space formerly occupied by the optic nerves, and infiltration of hemocytes into the ommatidial complex. In addition, infected animals showed relatively high intensities of non-specific granulomas in many tissues. The high prevalence of non-specific granulomas indicates that the lobsters in WLIS continue to be subjected to a variety of disease-inducing stressors such as parasitic or microbial infection, or exposure to metals or other toxicants. Interestingly, the severity of the pathology in the eyes was not well correlated with the intensity of infection of the amoebae in the optic nerves. However, more lobsters should be examined to fully assess severity in relation to disease.

A PATHOGENIC HERPES-LIKE VIRUS FROM THE SPINY LOBSTER, *PANULIRUS ARGUS*.

Jeffrey D. Shields,* Virginia Institute of Marine Science, Gloucester Point, VA 23062; **Donald C. Behringer, Jr.**, Old Dominion University, Norfolk, VA 23529; **Mark J. Butler, IV**, Old Dominion University, Norfolk, VA 23529.

A pathogenic herpes-like virus was diagnosed from juvenile Caribbean spiny lobsters from the Florida Keys. Moribund lobsters had characteristically milky hemolymph that lacked the ability to clot. With light microscopy, the virus infected hyalinocytes and semigranulocytes, but not granulocytes. Infected hemocytes had emarginated, condensed chromatin, hypertrophied nuclei and faint nuclear bodies resembling Cowdry-type-A inclusions. With electron microscopy, the large (187 nm ± 15 nm sd.) icosahedral, capsid-enclosed nonoccluded virions were diffusely spread around the border of the condensed chromatin with virogenic stroma present in the cytoplasm and free in the hemolymph. In some cases, virions were found in connective tissue cells. Virion and capsid assembly occurred within the nucleus of infected cells, but envelopes were not apparent. The virus ranged in prevalence over time from 6% to 8% with certain foci reaching up to 40%. The virus was transmissible to uninfected lobsters using inoculations of raw hemolymph from infected animals and through feeding trials. Inoculated animals showed morbidity and began dying from the virus after 60–80 d. Adult lobsters have not been observed with the infection. Additional infection trials, impacts on the social behavior of juveniles, and a TEM study to identify the agent are currently underway.

BLUE MUSSEL BIOLOGY AND CULTURE

OBSERVATIONS ON GROWTH, GAMETOGENESIS, AND SEX RATIO OF TRIPLOID AND DIPLOID *MYTILUS EDULIS*.

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Concerns in the Prince Edward Island mussel aquaculture industry over product quality during, and immediately after the spawning period prompted research on developing triploid *Mytilus edulis*. Triploid shellfish are sterile and retain quality during and after the spawning period.

Field evaluations of diploid and triploid mussels demonstrated that triploids had a greater growth rate than diploids. The growth difference was evident in just nine months after deployment in highly productive waters. This difference was not detectable in less productive waters until the second year of growth, suggesting possible differential growth of triploids versus diploids, related to environment. Mussels in the less productive waters were notably less sexually mature in the first year, therefore the differential performance of triploids may have been related to spawning. Triploids had a mean shell length 1.05% larger than diploids in the less productive waters versus 8.09% larger in the highly productive waters. Triploids examined after a spawning event showed no histological evidence of spawning, while 71% of diploids showed some evidence of spawning. Shell length, relative soft tissue weight, and condition index were all higher in triploids. This resulted in an increase in dry tissue weight of 62.82% and a mean shell length increase of 10.95% when triploids were compared to diploids at one site after the local spawning event. As well, a highly skewed sex ratio confirmed a previous study on *Mytilus galloprovincialis* showing a male dominant sex ratio in triploid mussels.

THE DISTRIBUTION AND BIOLOGY OF AN INVASIVE TUNICATE IN PRINCE EDWARD ISLAND, CANADA.

Jeff Davidson* and **Frank Boothroyd,** Atlantic Veterinary College, University of Prince Edward Island, 550 University Avenue, Charlottetown, Prince Edward Island, Canada, C1A 4P3; **Neil McNair,** PEI Department of Fisheries, Aquaculture and Environment, P.O. Box 2000, Charlottetown, Prince Edward Island, Canada, C1A 7N8; **Thomas Landry,** Department of Fisheries and Oceans, Science Branch, Maritimes Region, Gulf Fisheries Center, P.O. Box 5030, Moncton, New Brunswick, Canada, E1C 9B6.

The presence of the club tunicate *Styela clava* was recently noted in Eastern Prince Edward Island (PEI), Canada. This tunicate presents a significant fouling problem for the blue mussel (*Mytilus edulis*) farms located in affected areas. The spread of this new tunicate species in the waters of PEI is presently limited to a few rivers and seems to be mainly from anthropological mode as opposed to natural mode. Investigation on its reproductive biology is being conducted through the monitoring of gonad development and seasonal recruitment. Preliminary results are suggesting that spawning may occur throughout the summer months, while recruitment is limited to a relatively short period. The impact of this new fouling organism is also being investigated by evaluating its competition for food in relation to the blue mussels. Methods to control this fouling problem are presently being developed and tested. The eradication of this invasive tunicate from PEI waters is considered impractical and therefore the development of farm management strategies is considered as the only economically viable solution.

A FIELD STUDY OF SETTLEMENT DEPTHS OF *MYTILUS EDULIS* AND *M. TROSSULUS* IN NOVA SCOTIA, CANADA, 1998–2000.

Ellen L. R. Kenchington, Kenneth R. Freeman,* Benedikte M. Vercaemer, and **Barry W. MacDonald,** Department of Fisheries & Oceans, Bedford Institute of Oceanography, PO Box 1006, Dartmouth, NS, Canada, B2Y 4A2.

Larvae of *Mytilus edulis* and *M. trossulus* were shown previously to have different settlement preferences in the laboratory. However, it was not known whether these results could be extrapolated to field conditions with the much greater degree of environmental variability. Consequently, both temporal and spatial variability in larval settlement between these species, with depth were examined in the field. The relative proportion of *M. edulis* spat was determined using genetic markers at each of 3 depths (1, 3, 5 m) at intervals over the spawning season (minimum 3 collections) for 3 consecutive years at one site in Parrang Cove, St. Margaret's Bay, Nova Scotia. In the last year a second site in Ship Harbour, Nova Scotia was studied to add a spatial dimension to the project. There was no significant interaction between sites, years and timing with respect to depth of settlement. *M. edulis* consistently settled deeper than *M. trossulus* although there was both temporal and spatial variability in the relative percentages of *M. edulis* collected. In addition to opening up certain ecological questions, these results show promise for direct application to husbandry practices at mussel farms having both species and where aquaculturists wish to preferentially collect the more commercially desirable *M. edulis*.

NUTRIENT UPTAKE AND RELEASE FROM FOULING ORGANISMS ASSOCIATED WITH CULTURED MUSSELS IN TRACADIE BAY, PEI. **Angeline R. LeBlanc*** and **Gilles Miron,** Université de Moncton, Moncton, NB E1A 9B6; **Thomas Landry,** Department of Fisheries and Oceans, Science Branch, Maritimes Region, Gulf Fisheries Center, P.O. Box 5030, Moncton, New Brunswick, Canada, E1C 9B6.

Fouling organisms are causing concerns among mussel growers in PEI, Canada. Most of these foulers are sedentary filter feeders, and are therefore a potential competitor with mussels for resources. This could translate into a reduction in meat yield in mussels. We carried an experiment to determine the relative impact of fouling organisms on the uptake and release of nutrients. Chlorophyll a, ammonium, suspended particulate matter and oxygen were investigated. This preliminary study was undertaken in December 2000, before the ice cover, and showed that foulers had only a small effect on nutrient use. Foulers accounted for about one tenth of chlorophyll *a* consumption by mussels and foulers together. They also contributed about one tenth of the ammonium released by mussels and foulers. There was no significant use of suspended particulate matter by the mussels or the foulers. The use of oxygen was not significantly different between mussels and mussels with foulers. A temporal and spatial investigation has been initiated in 2001 and preliminary results will be discussed.

SPECIES-SPECIFIC SETTLEMENT PATTERNS OF BLUE MUSSELS IN COBSCOOK BAY, MAINE. **Afton McGowen, Matthew Gordon,** and **Paul D. Rawson,*** School of Marine Sciences, University of Maine, Orono, ME 04468-5751.

The blue mussels *Mytilus edulis* and *M. trossulus* are sympatric throughout much of the Canadian Maritime Provinces, as well as in easternmost Maine. We have observed marked variation in the frequency of adult *M. trossulus* mussels among sites within Cobscook Bay, Maine. Little is known regrading the degree to which local variation in species-specific larval supply versus post-settlement mortality determine the relative frequency of this species in Gulf of Maine mussel populations. To examine the importance of larval supply, we deployed and sampled mussel spat collectors (ropes) on a monthly basis at three separate sites within Cobscook Bay during the summer and fall of 2000. There was significant spatial and temporal variation in the number of settlers observed on these collectors. Using molecular techniques, we have also estimated the frequency of *M. trossulus* and *M. edulis* spat in each sample. Appreciable frequencies of *M. edulis* spat were observed at all locations throughout much of the experiment wherease sizeable frequencies of *M. trossulus* were more temporally restricted. Our results suggest that species-specific differences in post-settlement mortality must be invoked to explain populations containing exclusively *M. trossulus* adults within Cobscook Bay.

GENETIC ASPECTS OF THE BLUE MUSSEL (*MYTILUS EDULIS* AND *MYTILUS TROSSULUS*) HYBRID ZONE IN ATLANTIC CANADA. **Marcelo Miranda*** and **David Innes,** Dept. Biology, Memorial University or Newfoundland, St. Johns, NF, A1C 3X9; **Raymond Thompson,** Ocean Science Center, Memorial University of Newfoundland, St. Johns, NF, A1C 3X9.

The blue mussel (*Mytilus edulis* and *M. trossulus*) hybrid zone in Atlantic Canada provides an opportunity to study the process of speciation, adaptation and species interaction. Species composition in different size classes was determined at several aquaculture sites and natural populations in Newfoundland using nuclear DNA markers. *M. trossulus* and hybrids occur at a higher frequency in the smaller size classes and *M. edulis* dominates in larger size classes. This pattern suggests that *M. trossulus* in these areas may have a higher mortality rate or a shorter life span than *M. edulis*. In addition, offspring from inter and intra-specific crosses were reared in the laboratory to study the dynamics of hybridization and species differences. Fertilization rate, larvae abnormality and sperm competition were used to estimate barriers to hybridization at the gamete stage. Growth rate and survival of the different families were compared during the larval stage, spat (6 months) and until maturity (18 months). These data were used to assess hybrid fitness and also to compare the performance of both species, which recently had been a major concern to the mussel industry.

SMALL SCALE DISTRIBUTION OF *MYTILUS EDULIS* AND *M. TROSSULUS* IN THE BAIE DES CHALEURS AND THE GASPÉ PENINSULA. **Valérie Moreau*** and **Edwin Bourget,** GIROQ, Département de biologie, Université Laval, Ste-Foy, Québec, Canada, G1K 7P4; **Réjean Tremblay,** Centre Aquicole Marin-Université du Québec à Rimouski, Grande-Rivière, Québec, Canada, G0C 1V0.

Mytilus edulis and *M. trossulus* are the two species found in the Baie des Chaleurs and the Gaspé Peninsula. To date, few studies bring out the effects of environmental factors (temperature, salinity, tidal height, wave action) on the local or regional distribution of these two species. Mussels were sampled on rocky shores according to a factorial design including six locations, three degrees of wave exposure (exposed, semi-exposed, sheltered) and two intertidal level (mid and low). PCR amplification using a diagnostic DNA marker (*Glu-5*) was used to distinguish species. Species relative frequencies show no clear patterns of distribution with wave exposure or tidal height. Although, there appears to be a pattern of distribution at the regional scale, this pattern could not be related to salinity or temperature gradients observed.

MUSSEL CULTURE IN A MIXED SPECIES (*M. EDULIS* AND *M. TROSSULUS*) ZONE—SOME COMMERCIAL IMPLICATIONS. **Randy W. Penney,* M. J. Hart,** and **N. Templeman,** Department of Fisheries and Oceans, Science, Oceans and Environment Branch, P. O. Box 5667, St. John's, Nfld, Canada, A1C 5X1.

The island of Newfoundland, Canada, is a zone where two blue mussel species, *M. edulis* and *M. trossulus,* overlap in distribution. Typically, indigenous populations at most sites contain mixtures of both species and hybrids. Sites in close proximity (eg. <10 kilometers) often vary as much in their relative species proportions as sites hundreds of kilometers apart. Intra-site growth variability is significantly affected by the mixed-species nature of these stocks. In a 14 month commercial rearing trial of rope-cultured mussels from sleeving to harvest at three farm sites, the *M. edulis* stock component had higher growth rates in shell and total weight than the sympatric *M. trossulus* at all three farms, while inter-specific shell length growth rates were different at only one farm. Growth rates of hybrids were typically intermediate between the two. Mortality rates were similar between sympatric *M. edulis* and *M. trossulus* at all three sites. We conclude that naturally-occurring stock genetic variability may have a significant impact on commercial production indices at farm sites within zones of species overlap.

CLADISTIC ANALYSIS OF GENETIC DIFFERENTIATION BETWEEN POPULATIONS OF THE BLUE MUSSEL, *MYTILUS TROSSULUS.* **Paul D. Rawson,** School of Marine Sciences, University of Maine, Orono, ME 04468-5751.

Two species of blue mussel, *Mytilus edulis* and *M. trossulus,* have overlapping distributions on the Atlantic coast of North America. Populations containing a mixture of these species and their hybrids are commonly observed throughout the Canadian Maritime Provinces. Recent work, in my lab, has shown that the range of *M. trossulus* extends well into the Gulf of Maine, much further south than previously observed. In this study, I have employed a DNA sequence-based cladistic analysis to examine whether the presence of *M. trossulus* in the Gulf of Maine is due to a recent range expansion. DNA sequences for a portion of the female mitochondrial lineage D-loop region (~670 base pairs) were obtained from 140 *M. trossulus* by PCR amplification and direct sequencing. Analysis of these sequences indicates there is significant genetic divergence between Atlantic and Pacific *M. trossulus* populations. In contrast, there was no evidence of genetic differentiation or a reduction in genetic diversity among the Atlantic coast populations. These results are contrary to what would be expected if Gulf of Maine *M. trossulus* populations have only recently been established.

BEHAVIOR AND GROWTH OF JUVENILE MUSSELS (*MYTILUS* SPP.) IN SUSPENDED CULTURE SOCKS. **Judith Senechal*** and **Jon Grant,** Oceanography Dpt, Dalhousie University, Halifax, NS B3H 4J1.

Suspended mussel culture is based on loading high densities of juvenile mussels into mesh socks, and hanging them from floats. This leads to severe intraspecific crowding, and potentially reduces growth and mussel yield. The highly mobile juveniles position themselves according to size, shell gape, ambient current and food, among other factors. Despite this critical stage in culture, there are little data on behavioral mechanisms that lead to adult density and growth rate. We conducted a series of field and laboratory experiments with culture socks to examine the effects of stocking density, species (*Mytilus edulis* and *Mytilus trossulus*) and environment on early development of the culture population. *In situ* photography was used to capture a time series of mussel images in experimental socks at a commercial farm in Ship Harbor, Nova Scotia. Moored CTD-current meters and water sampling were used to characterize the sites. Image analysis on mussel size, gape, and position was used to quantify temporal changes over several months in the socks. In the laboratory, socks were hung in a tall flow-through tank with variable temperature, current speed and direction, allowing controlled experiments on mussel emergence and growth. Initial results are discussed in the context of optimizing husbandry practices.

UPDATE ON THE DISTRIBUTION OF TWO MUSSELS SPECIES (*MYTILUS EDULIS* AND *MYTILUS TROSSULUS*) IN THE QUEBEC MARITIME REGIONS. **Benoit Thomas,*** Centre aquacole marin, MAPAQ, Grande-Rivière, Québec, G0C 1V0; **Valérie Moreau** and **Réjean Tremblay,** Centre aquacole marin-Université du Québec à Rimouski, Grande-Rivière, Québec, G0C 1V0.

This study attempted to determine the distribution of the two species of mussels and hybrids along the main Quebec maritime regions of the Gulf of St. Lawrence, as well as evaluate the biannual pattern of distribution among different sites. Following 1996 near shore mussel sampling, we started a study based on mussel spat fixed on artificial collectors immersed at 8 sites along the 460 km long Gaspe peninsula and at 5 sites along some 1000 km of the North Shore of the Gulf of St. Lawrence. Biannual sampling was conducted at these sites following initial results obtained in 1997. At all sites, collectors and thermographs were immersed at 2 m in mid-June (sea bottom depth: 18 m) and sampled in mid-October of the same year. Sampling the next June was also conducted along sites located along the North Shore, where spat growth was found to be low the first season and site accessibility was limited. In 1996 and 1997, analyses were done by electrophoresis on MPI and by diagnostic DNA marker (GLU-5) for other years. According to the initial two year results, a predominance of *M. trossulus* occurs on the northern side of the Gaspe peninsula as well as in the extreme east of the North Shore. The best sites for presence of *M. edulis* was in the mid-region of the Gaspe peninsula as well as the western side of the North Shore. Results show that some sites are variable, with yearly variations. This annual change or steadiness in the proportion of the two species will permit mussel growers to orient towards better *M. edulis* spat provision sites; accepting, for now, the hypothesis that the commercial value of *M. trossulus* is lower.

PERFORMANCE OF *MYTILUS EDULIS* AND *MYTILUS TROSSULUS* IN THE GULF OF ST. LAWRENCE: A LABORATORY EXPERIMENT. Réjean Tremblay* and **Valérie Moreau,** Centre aquacole marin-Université du Québec à Rimouski, Grande-Rivière, Québec, G0C 1V0; **Thomas Landry,** Gulf Fisheries Center, DFO, Moncton, New Brunswick, E1C 9B6; **Bruno Myrand,** Station technologique Maricole, MAPAQ, Cap-aux-Meules, Québec, G0B 1B0; **Cyr Couturier,** Marine Institute, Memorial University of Newfoundland, St John's, Newfoundland, A1B 3X5.

During the past 15 years, reciprocal transfer experiments with mussels, *Mytilus edulis* and *M. trossulus* in Gulf of St. Lawrence have shown that their performance, expressed in term of growth and survival, is different between stocks. Moreover, the relative performance level of a stock may vary between sites. These results suggest that the performance of mussels is probably genetically based. The genetic diversity of mussels in the Gulf of St. Lawrence could be very important in relation to their ability to adapt to the highly variable environmental conditions. The goal of this study, is to characterise the performance of different mussel stocks and to determine the relation between physiological parameters and genetic traits, for both species of mussels. These analysis were conducted on mussels spat sampled from artificial collectors in Québec, Prince Edward Island, New-Brunswick and Newfoundland. Bi monthy growth and survival data was collected from twelve different mussel stocks maintained in laboratory with non-treated seawater, over a one year period. Temperature, seston and chlorophyll-a were monitored. Physiological measurements, including scope for growth and basal metabolism, as well as genetic analysis, were conducted on each stock. Genetic analysis included species determination by PCR technique and genetics variability determined by electrophoresis technique. Results will be discussed in terms of mussel culture strategy for spat supply.

BLUE MUSSELS AS MODEL SYSTEMS TO INVESTIGATE PALLIAL CAVITY FUNCTION IN BIVALVES. J. Evan Ward,* and **Sandra E. Shumway,** Department of Marine Sciences, University of Connecticut, Groton, CT 06340; **Jeffrey S. Levinton,** Department of Ecology & Evolution, S.U.N.Y., Stony Brook, NY 11794.

For over fifty years, mussels in the genus *Mytilus* have been used as model systems to study aspects of bivalve behavior, physiology, and genetics. Because of their relatively simple, nonplicate, homorhabdic ctenidium, mussels are also ideal animals in which to investigate general mechanisms of particle handling. In this talk we will discuss our studies on particle capture, transport, and selection in mussels and how they have furthered our understanding of pallial cavity function and its connection to ecosystem processes. In particular, we will examine where gaps in our knowledge exist and compare and contrast feeding processes in mussels with those of other bivalves.

For our studies, mussels were delivered polystyrene tracer particles alone or in combination with defined diets consisting of either ground, aged *Spartina* sp. (3–10μm), similar sized phytoplankton (*Rhodomonas* sp.), or a 50/50 mixture of both at three concentrations (10^3, 10^4, 10^5 particles ml^{-1}). Particle capture, transport, and handling by the pallial organs were studied *in vivo* by means of video-endoscopy and discrete samples of particulate material were collected from various ciliated tracts. Image analysis was used to track particle movement on the ctenidia and labial palps, and flow cytometry was used to analyze samples for evidence of particle selection. Particle depletion and handling time experiments were also performed to measure residence times on the ctenidia and labial palps. Results indicate the following: 1. ctenidal filaments are directly involved in particle capture, 2. diet quality has little effect on particle handling mechanisms, 3. diet quantity has significant effects on particle handling mechanisms, and 4. particle selection is confined to the labial palps. Studies such as these will lead to a better understanding of pallial organ function in mussels, and allow us to better model the critical limiting factors that mediate particle-feeding in bivalves and ultimately affect the trophic dynamics of benthic ecosystems.

HARMFUL ALGAL BLOOMS

EFFECTS OF CLAY, USED TO CONTROL HARMFUL ALGAL BLOOMS, ON JUVENILE HARD CLAMS, *MERCENARIA MERCENARIA.* Marie-Claude Archambault* and **Jon Grant,** Oceanography Dpt, Dalhousie University, Halifax, NS B3H 4J1; **Monica Bricelj,** Institute for Marine Biosciences, National Research Council of Canada, Halifax, NS B3H 3Z1; **Don Anderson,** Biology Dpt., Woods Hole Oceanographic Institution, Woods Hole, MA 02543.

Increased interest in using ecologically inert clays to mitigate harmful algal blooms at nearshore mariculture sites has prompted studies on the effectiveness of this method on prolific U.S. blooms, such as Florida's neurotoxic blooms of *Karenia brevis*. Potential repercussions of this control strategy revolve around the increasing flux of suspended particles to the benthos. Juvenile suspension feeding bivalves are potentially vulnerable as they could suffer burial and a decrease in clearance rates and/or increase in pseudofeces production in response to suspended clay, leading to reduced growth and delay in attaining size refuge from predators. The main objective of the research was to assess lethal and sublethal effects on juvenile hard clams, *Mercenaria mercenaria*, in a two-week flume application of phosphatic clay (by-product of phosphate mining) to a simulated bloom of a non-toxic dinoflagellate (*Heterocapsa triquetra* or *Prorocentrum micans*). Flow regimes simulated two extreme conditions, representing end members of a continuum expected in the field, a) where low flow allowed complete settling and formation of a sediment layer, and b) where high flow maintained complete particle resuspension. No clam mortalities occurred in either treatment. The sedimentation treatment showed variable growth inhibition in shell and/or tissue, but effects were not significant compared to controls (no sediment layer), and clams rapidly resumed siphon contact with the overlying water column. In contrast, a highly significant growth effect (~90% reduction in shell and tissue growth) occurred in trials with suspended clay compared to no-clay controls. Analysis of particle size-spectra, using a sampling method designed to maintain the integrity of flocs, showed evidence of clay flocculation, such that clay particles were found above the 100% retention efficiency size limit of the clam gill. These results suggest that repeated clay applications in the field are likely more detrimental to clams under flow conditions leading to prolonged *in situ* resuspension of clay than under conditions that promote rapid sedimentation.

HISTORY, SOME RECENT HAB EVENTS, AND THEIR IMPACTS ON SHELLFISH AND FINFISH IN WASHINGTON STATE. **Rita A. Horner,*** School of Oceanography, University of Washington, Seattle, WA 98195-7940.

Harmful algal blooms and their effects, especially paralytic shellfish poisoning (PSP), have a long history in the Pacific Northwest starting in June, 1793, when one of Captain George Vancouver's crew members died and four others became ill after eating mussels in central British Columbia. The next known occurrence was in May, 1942, when three people died and eight became ill from eating clams or mussels in Barkley Sound, British Columbia, and three others died near Port Angeles, Washington, after eating clams. Investigations suggested that only beaches on the Strait of Juan de Fuca and the open Pacific coast were affected and the Washington Department of Fisheries issued an annual closure of these areas from April through October. Since then, PSP has spread to all of western Washington's inland marine waters, except Hood Canal, and is also present along the open Pacific coast and in coastal estuaries. Beach closures are frequent and human illnesses continue to occur, albeit infrequently, even with increased monitoring. In 1991, domoic acid was found in razor clams and Dungeness crabs on Washington's Pacific coast, where it continues to be found sporadically in razor clams with consequences for their harvest. The causative organisms, several species of the diatom genus *Pseudo-nitzschia*, are sometimes present in bloom concentrations in inland waters of Puget Sound and in coastal estuaries, but if domoic acid occurs, levels are low and no closures have been required. No confirmed cases of amnesic shellfish poisoning (ASP) caused by domoic acid have been reported in the state. Other potentially harmful phytoplankton species, including *Heterosigma akashiwo* and *Chaetoceros* spp. occur here, primarily affecting finfish in net pens. Reactive oxygen species and possibly an unknown toxin are associated with *Heterosigma*, while *Chaetoceros* effects are mechanical. Thus, western Washington waters harbor a variety of potentially harmful marine phytoplankton species that continue to plague shellfish and finfish growers in more places and usually without warning.

EFFECTS OF THE TOXIC DINOFLAGELLATE, *KARENIA BREVIS*, ON LARVAL MORTALITY AND JUVENILE FEEDING BEHAVIOR IN THE BAY SCALLOP, *ARGOPECTEN IRRADIANS*. **Jay R. Leverone*** and **Norman J. Blake,** College of Marine Science, University of South Florida, St. Petersburg, FL 33701.

Florida populations of the bay scallop, *Argopecten irradians*, occur in areas prone to recurring blooms of the toxic dinoflagellate, *Karenia brevis* (=*Gymnodinium breve*). These blooms can have serious consequences for bay scallop recreational fisheries, aquaculture and restoration activities. Surprisingly, there are few published data regarding the effects of *K. brevis* on any aspect of bay scallop biology or ecology in Florida. A series of laboratory experiments was conducted to observe the direct effects of exposure to *K. brevis* on early life stages of the bay scallop, *A. irradians*. For bay scallop larvae, an LC_{50} of 900 cells/ml *K. brevis* was calculated after a seven-day exposure. Larval development was delayed and metamorphosis inhibited at concentrations above 500 cells/ml. Larvae responded similarly when exposed to either "whole" or sonicated "lysed" cultures of *K. brevis*. Clearance rates of juvenile scallops (5–15 mm shell height) were size dependent and significantly ($p = 0.05$) reduced at *K. brevis* concentrations of

50 cells/ml and higher. At 100 cells/ml, reduced clearance rates resulted in slower growth and reduced weight gain after one-week exposure. At 500 cells/ml, clearance rates were irreversibly affected even 20 hours after a spiked exposure. Findings will be discussed in light of recent efforts to restore bay scallops along the west coast of Florida.

DETRIMENTAL EFFECTS OF A RECENT *PRYMNESIUM* ISOLATE FROM BOOTHBAY HARBOR, MAINE (USA) UPON JUVENILE BAY SCALLOPS, *ARGOPECTEN IRRADIANS.*

Gary H. Wikfors* and **Jennifer H. Alix**, NOAA Fisheries, NEFSC, Milford, CT 06460 USA; **Roxanna M. Smolowitz**, Marine Biological Laboratory, Woods Hole, MA 02543; **Lacey Wallace**, Southampton College of LIU, Southampton, NY 11968; **Hélène Hégaret**, École Nationale Supérieur Agronomique de Rennes, Rennes, France.

The brackish-marine flagellate *Prymnesium* has been known to be toxic to finfish for decades. Symptoms in fish exposed to toxic *Prymnesium* include copious mucuous production and bleeding from the gills, and death by asphyxiation. Two strains of *Prymnesium*, isolated recently by Dr. R.R.L. Guillard were tested for ichthyotoxicity using cunner (*Tautogolabrus adspersus*); we confirmed toxicity and histopathology of one isolate, strain 97-20-1. Further, we determined and documented effects of the toxic *Prymnesium* strain (97-20-1) to bay scallops, *Argopecten irradians.*

In one exposure experiment, 10-mm scallops were placed in basins of ultrafiltered seawater, and cultured (bacteria-free) *Prymnesium* 97-20-1 were added to achieve a cell density of 10^5 cells/ml. Controls were scallops given an equivalent quantity of *Isochrysis* sp, (strain T-ISO) or an equivalent volume of algal culture medium. Scallops given T-ISO fed normally and produced fecal pellets; whereas, scallops exposed to *Prymnesium* twitched violently, produced pseudofeces and abundant mucous, and eventually displayed valve gape that was not responsive to stimulation. Within 24 hours, all scallops exposed to *Prymnesium* were moribund or dead. Histopathological analysis revealed severe, acute, total or near-total necrosis of the digestive gland and ducts, gill, and other tissues in scallops exposed to *Prymnesium* and normal tissue histology in controls.

In a later experiment, larger scallops (50-mm) were exposed to *Prymnesium* 97-20-1 (again with T-ISO as the control), and hemolymph samples were removed after 2.5 hours for analysis of hemocytes by flow-cytometry. Significantly more dead hemocytes were seen in scallops exposed to *Prymnesium* than to T-ISO, but no differences in aggregation and adherence or phagocytosis of plastic microbeads were detected. We believe this to be the first report of HAB effects upon the bivalve immune system. These experiments revealed effects of an ichthyotoxic microalga upon scallops ranging from behavioral to histopathological and immunological, and may serve as a template for studies of additional HAB-bivalve trophic interactions.

SHELLFISH BIOLOGY

BURROWING-INDUCED INTERNAL FRACTURES AND EXTERNAL ABRASION IN SHELLS OF THE HARD CLAM *MERCENARIA MERCENARIA* FROM RARITAN BAY, NEW JERSEY.

Richard R. Alexander,* Department of Geological and Marine Sciences, Rider University, Lawrenceville, NJ 08648; **Robert M. Baron**, Institute of Marine and Coastal Studies, NOVA Southeastern University, Fort Lauderdale, FL 33004.

The hard clam *Mercenaria mercenaria* from Raritan Bay, New Jersey, reburrowed once a month into sediment in flow-through tanks for a period of one year. Twelve specimens, between 34 and 38 mm dorsal-ventrally, reburrowed in each of five different textured sediments, namely 100% mud, 100% sand, admixture of 25% by volume ground shell hash and 75% mud, admixture of 25% shell hash and 75% sand, and admixture of 50% sand and 50% mud. Lacking tidal flushing through the tanks, mean shell growth, dorsal-ventrally, was one mm/yr. Shell accretion was statistically significantly depressed in sand and shell-sand vs. mud, and mud-shell substrata, a disparity attributed to repeated abrasion of valve margin's during monthly reburrowing in coarser textured sediments. Clams in sand and shell-sand had more concentric, fine ornament or lamellae worn smooth relative to specimens in mud and mud-shell mixtures. Upon sacrifice, 30 clams revealed "stuccoed," internal fractures initiated at the shell margin. Five linear fractures radiate dorsally. Seven cracks curve or diagonal from the shell margin. Three show right angle deflections, twice through adductor muscle scars. Four merge or branch from the margin. In 12 specimens, fractures are in both valves. In ten specimens, fractures were faintly visible on valve exterior. In four and two specimens, respectively, fractures were initiated at the posterior or anterior margin. Repaired fractured shells are randomly distributed among sediment textures. Nevertheless, fractures were probably induced and/or exacerbated by the reburrowing process.

INFLUENCE OF ENVIRONMENT AND FOOD SUPPLY ON SURVIVAL OF *CRASSOSTREA GIGAS* LARVAE: A MODELING STUDY.

Eleanor A. Bochenek* and **Eric N. Powell**, Rutgers University, Haskin Shellfish Research Laboratory, 6959 Miller Avenue, Port Norris, NJ 08349; **John M. Klinck** and **Eileen E. Hofmann**, Center for Coastal Physical Oceanography, Old Dominion University, Norfolk, VA 23529.

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 it: 1) defines larvae in terms of their protein, neutral lipid, polar lipid, carbohydrate, and ash content, 2) tracks weight separately from length to follow larval condition index, and 3) includes genetic variation in growth efficiency and egg quality to better simulate cohort population dynamics. Simulations show that departure of temperature, salinity or food content from optimum levels reduces larval cohort survival, generally either because some larvae fail to metamorphose successfully or because an increase in larval life span increases losses to predation. Also, different food compositions produce widely varying survivals at the same food concentration. The simulations suggest that the ratio of the combined carbohydrate and lipid pools to protein may best describe the overall quality of the food. In simulations emphasizing genetic variability within the cohort, larvae with high growth efficiency originating from large eggs outperform other egg quality-growth efficiency combinations for most environmental variables, including temperature, salinity, and food content. In contrast, whereas the simulations suggest that the influence of suboptimal temperature, salinity, or food content is to compress genetic variation by uniformly favoring high growth efficiency and large eggs, the simulations with food quality provide evidence of a mechanism that would expand genetic variation, because variations in food quality favor a much broader range of genetic types. The simulations support the supposition that food quality is an important variable controlling larval cohort success.

GROWTH CHARACTERISTICS OF *ARGOPECTEN GIBBUS* JUVENILES REARED IN TWO SUSPENDED CULTURE SYSTEMS.

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Grow-out of hatchery reared calico scallops (*Argopecten gibbus*) is conducted in Bermuda's inshore waters using square Irish scallop trays and triangular Japanese pearl nets. Both enclosures have advantages and disadvantages in both maintenance and handling throughout the grow-out phase. To assess optimum technology, yielding minimum labour demand and maximum scallop growth and survival, a comparative study was performed.

A pool of juvenile scallops was distributed in triplicate in 6.0 mm pearl nets, and in scallop trays lined with 6.4 mm black polyethylene "pouches". Scallop growth and survival were monitored monthly in trays and pearl nets of comparable mesh size and stocking densities ranging from 0.20 scallops-cm^{-2} to 0.10 scallops-cm^{-2}. At this time, sub-samples ($n = 50$) were collected from the pool of triplicates and both shell and tissue growth of juvenile scallops was recorded.

Preliminary results revealed that scallops cultured in pearl nets achieved higher growth rates than scallops in trays from juvenile to market size. Results also imply that the limiting effect of trays on scallop growth is immediate above the initial height and weight used in this experiment and increases until scallop growth nearly ceases. Comparisons for scallops grown in pearl nets and trays are made using Statview statistical package. Evaluation of trays and pearl nets as grow-out enclosures is discussed in terms of optimal grow-out strategy.

GROWTH AND DISPERSAL STUDIES OF *MYA ARENARIA* USING A NUMERICAL FLOW MODEL.

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Growth: Current velocities averaged over the flood tide were estimated by a numerical flow model and by clod cards for locations in an Eastern Maine bay and were compared to the annual shell size increment of clams collected at the same locations. Statistical models including initial shell size, year of sample, high-low current category estimated by clod cards or the numerical model and interactions explained 57–58% of the variability in growth increment after a difference transformation. High current simulated growth, although the effect on growth increment was less than that of sample year or initial size. The adjusted least squares mean for the growth increment at the sites with low flow, as identified by clod cards that averaged 4.35 ± .37 cm/s was 9.56 ± .247 mm, and low flow that averaged 2.99 ± .43 cm/s using the numerical model was 9.51 ± .274 mm. High flow sites averaging 5.86 ± .62 cm/s using clod cards had estimated growth increments of 11.90 ± .323 mm and high sites averaging 5.84 ± .46 cm/s using the numerical model had estimated growth increments of 11.70 ± .33 mm.

Dispersal: Clam populations, particularly in eastern Maine, are prone to recruitment failure of larvae onto intertidal flats due to the large tidal amplitude and resulting high flushing rates. Larvae move offshore during a larval development period that is extended in cool waters in the summer along the eastern coast. High variability in ME landings, mudflat sampling of settlement, spat bag studies of larval distributions are consistent with this theory. Presently, studies using Eulerian and LaGrangian numerical flow models are being utilized to determine the effect of tidal magnitude, length of dispersal period and variation in coastal geology on larval dispersal.

ESTIMATION OF INGESTION AND BIODEPOSITION RATES OF THE PACIFIC OYSTER, *CRASSOSTREA GIGAS*, IN A COASTAL LAGOON OF NW MEXICO.

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Four short-term (6–8 h) experiments were carried out at San Quintin Bay (SQB) in 1999 (June, October) and 2000 (January, April) in order to estimate "in situ" ingestion rates (IR) of the Pacific oyster, *Crassostrea gigas*. Experiments were carried out by pumping seawater directly into experimental trays containing adult oysters (94 ± 1.7 cm shell height, 3.64 ± 0.45 g). Total and organic particulate matter (TPM and POM, respectively) were measured every 1.5 h in the feces and pseudofeces produced by oysters and in the input seawater. Clearance and ingestion rates were calculated by using the ash balance method. In situ POM/TPM ratio

(0.15 to 0.30) and oyster clearance rates (2 to 7.5 L h^{-1}) were inversely related to TPM concentration (2–14 mg L^{-1}), while feces (7.9–13.7 mg TPM $oyster^{-1}$ h^{-1}) and pseudofeces production (4.8–13.2 mg TPM $oyst^{-1}$ h^{-1}) were directly proportional to TPM concentration. Net organic ingestion rates (3–4.2 mg OM h^{-1}) were not dependent on total particle concentration. It is suggested that particle resuspension plays an important role in regulating the variability of food quality in SQB, but such variability does not affect the net amount of organic matter ingested by *C. gigas* throughout the year.

EFFECT OF DIETARY PROTEIN/ENERGY RATIO ON GROWTH AND METABOLISM OF JUVENILE GREEN ABALONE (*HALIOTIS FULGENS*).

Laura E. Gómez,* FES-Cuautitlán UNAM, México; **Ma. Teresa Viana** and **Zaúl García-Esquivel,** UABC, BC, México; **Armando Shimada,** UNAM, México; **Louis R. D'Abramo,** MSU, USA.

Juvenile green abalone (initial size = 11.84 ± 1.003mm) were fed different practical diets formulated to contain different P/E ratios (mg/kcal) ranging from 62 to 108 mg/kcal. The dietary protein level increased from 25.8 to 44.1% while the level of energy remained constant (4056 to 4154 cal/g). After 61 days in a flow through system at 21C, growth of abalone fed diets with a P/E ratio of 100 and 108 mg/kcal (SGR = 2.42 ± 0.11 and 2.51 ± 0.10) was significantly greater than that of abalone fed the other diets. Food intake and therefore ingested energy per g of abalone were similar for all dietary treatments. Protein and energy digestibility, amonia excretion and oxygen consumption were determined after the growth experiment was terminated. Intake of digestible energy was different among dietary treatments, but intake of digestible protein did not change. Abalones ingest food to satisfy their energy requirements, and digestibility of protein and energy decreases as the P/E ratio increases. The most efficient diet for growth probably consists of a 100 P/E ratio with lower levels of dietary protein and energy.

MODELING THE GROWTH OF THE HARD CLAM, *MERCENARIA MERCENARIA*.

John Kraeuter* and **Eric N. Powell,** Rutgers University, Haskin Shellfish Research Laboratory, 6959 Miller Avenue, Port Norris, NJ 08349; **Eileen E. Hofmann** and **John M. Klinck,** Center for Coastal Physical Oceanography, Old Dominion University, Norfolk, VA 23529; **Ray Grizzle,** Jackson Estuarine Laboratory, 85 Adams Point Road, Durham, NH 03824; **Monica Bricelj,** Institute for Marine Biosciences, National Research Council, 1411 Oxford Street, Halifax, NS, B3H 3Z1, Canada; **Stuart Buckner,** 23529 Town of Islip, Environmental Control, 401 Main Street, Islip, NY 11751.

A physiologically-based model that simulates the growth of the hard clam, *Mercenaria mercenaria,* in response to environmental conditions of temperature, salinity, and food supply has been developed. The processes included in the clam model are those that are responsible for changes in clam soft tissue weight, length, and condition. Changes in clam soft tissue weight over time result from the difference in assimilation and respiration. Changes in clam condition are determined from a length-weight relationship that is reflective of average clam growth. Changes in clam length (growth) occur only when condition index is greater than zero, which happens when the clam has attained excess weight for a given length. No change in length occurs if condition index is zero (mean case) or negative (less weight than expected at a given length). Initial simulations with the clam model, that used allometric relationships to relate weight and length, resulted in growth rates that did not match those observed for hard clams over a range of weights and lengths. However, when weight and length are related using a third-order polynomial the simulated growth rates match observed rates. The implication of this result is that small clams are heavier for a given length and that large clams are longer for a given weight. This approach represents a change in the way that models are formulated for growth of clams and potentially other bivalve species. Additional simulations illustrate the effect of changing salinity and food environments and genetic variation in growth efficiency and respiration on hard clam growth and survival.

INFLUENCE OF SHORT TERM VARIATIONS IN FOOD SUPPLY AND CRITICAL PERIODS ON SURVIVAL OF *CRASSOSTREA GIGAS* LARVAE.

Eric N. Powell* and **Eleanor A. Bochenek,** Rutgers University, Haskin Shellfish Research Laboratory, 6959 Miller Avenue, Port Norris, NJ 08349; **John M. Klinck** and **Eileen Hofmann,** Center for Coastal Physical Oceanography, Old Dominion University, Norfolk, VA 23529.

A biochemically-based model was developed to simulate the growth, development, and metamorphosis of larvae of the Pacific oyster, *Crassostrea gigas.* The model defines the larvae in terms of protein, lipid, carbohydrate, and ash content and includes genetic variation in growth efficiency and egg quality. This model is used to investigate the premise that certain periods of larval life are more critical than others with respect to the availability of food and that food quality is as important as food quantity. Simulation results indicate that critical periods in larval life do exist. However, the critical portion of larval life depends on the structure of the food that the larvae encounter. Overall, the most critical time is late in larval life as the larvae approach metamorphosis. Increased protein at this time always improves larval survival. Increased lipid has the most effect about midway in larval life, but also exerts a positive impact late in larval life. However, exposure to certain types of food early in larval life can dramatically change cohort survival. Additional simulations show that larvae with high growth efficiency are more successful, as are larvae coming from large eggs. Changes in food quantity influence larval survival primarily by varying the length of larval life. Changes in food quality, however, by restricting the range of genotypes in the cohort that survive, as well as by varying larval life span, produce large changes in survivorship. The simulations support the adaptive advantage of larval cohorts with a relatively wide range of genotypes and suggest the important influence of variations in food quality in maintaining genetic variability.

POPULATION STRUCTURE OF THE HARD CLAM, *MERCENARIA MERCENARIA*, IN HAMPTON ROADS, VIRGINIA.

Melissa J. Southworth,* Juliana M. Harding, and **Roger Mann,** Department of Fisheries Science, Virginia Institute of Marine Science, Gloucester Point, VA 23062.

The hard clam, *Mercenaria mercenaria*, supports one of the most valuable fisheries in the Virginia Portion of the Chesapeake Bay. In recent years with the displacement of oystermen moving to clam harvesting as an alternative source of income, there has been a gradual decline in catch per unit effort in the clam fishery. Despite growing concern for the decreasing catch and the increasing pressure of proper management there has only been one survey in recent years that focused on stock assessment for management purposes (Wesson, 1995). Preceding this the last substantial surveys were prior to and just after Hurricane Agnes in the early 1970's. As such a survey of the Hampton Roads area was carried out in the summer of 2001. The survey documented the distribution of the current demographics including size and age structure of the hard clam stocks. The current distribution of hard clams exists over a cline of differing salinity and bottom types. From this we compare site versus size, age, and the frequency of recruitment. The frequency of recruitment, size, and age is then compared to the environment. Comparisons of historic data and current survey data show changes over decadal time scales that are useful indicators of long term environmental change in the Hampton Roads area.

AN EXAMINATION OF POTENTIAL CONFLICT BETWEEN HARD CLAM AQUACULTURE AND SAV IN THE LOWER CHESAPEAKE BAY.

Helen Woods,* Ken Moore, and **Carl Hershner,** Center for Coastal Resources Management, Virginia Institute of Marine Science, Gloucester Point, VA 23062.

The Commonwealth of Virginia wishes to promote both hard clam (*Mercenaria mercenaria*) aquaculture and the growth of submersed aquatic vegetation (SAV) along its shallow subaqueous bottom. Conflict can arise in areas where aquaculturists plant clams in SAV habitat and/or when SAV colonizes an area being used for aquaculture. This project examined the issue of bottom use conflict on the Eastern Shore of Virginia involving clam aquaculturists and SAV interest groups. This issue was examined by studying historic SAV and aquaculture trends, creating spatial GIS models to predict areas suitable for the growth of SAV and the development of clam aquaculture, and examining the current laws in Virginia and neighboring states which affect this issue. Results suggest that a management approach which annually defines a 50 meter buffer surrounding existing SAV beds would adequately protect SAV while minimizing areas legally unavailable but otherwise suitable for clam aquaculture, thus minimizing conflict.

SCALLOP BIOLOGY AND CULTURE

POSTLARVAL DEVELOPMENT OF THE GILLS AND IMPLICATIONS FOR FEEDING IN THE SEA SCALLOP, *PLACOPECTEN MAGELLANICUS*.

V. Monica Bricelj,* Anne Veniot and **Céline Barré,** Institute for Marine Biosciences, National Research Council, 1411 Oxford St., Halifax, NS B3H 3Z1, Canada; **Peter Beninger,** Laboratoire de Biologie Marine, Faculté des Sciences, Université de Nantes, 44322 Nantes, France.

Hatchery-reared bivalves often experience poor growth and survival during post-settlement stages, yet limited information is available on postlarval morphogenesis and feeding, especially in scallops. Our research uses scanning electron microscopy to document critical changes during the ontogeny of pallial feeding organs (gills, mantle, foot, lips and labial palps) in sea scallops (and recently in bay scallops, *Argopecten irradians*) and their correlation with size, between 0.3 and 14 mm in shell height (SH). We present here the results concerning gill development in *Placopecten magellanicus*. Pronounced changes in morphology of the filibranch gill produce a transition from a homorhabdic gill basket to the heterorhabdic, W-shaped, plicate gill characteristic of adults. Suspension-feeding is probably rather ineffective prior to reflection of the inner demibranch (occurring at ~1 mm SH and followed by accelerated proliferation of gill filaments), and formation of the outer demibranch (completed at ~2 mm SH). The onset of the heterorhabdic condition via differentiation of principal filaments, which allows bidirectional particle transport and thus the potential for selection and ingestion volume regulation on the gill, occurs fairly late in development, at ~3.3 to 5.0 mm. Full plication of the gill is only achieved at ~7 mm SH. This protracted development in *P. magellanicus* contrasts with that in oysters, which undergo more rapid gill differentiation and metamorphosis. We thus demonstrate that the gills are relatively undifferentiated at 1–2 mm SH, when sea scallops are commonly transferred from the hatchery to field nurseries by commercial growers. We are currently investigating the relationship between these morphological changes and evolving mechanisms of food capture and ingestion, in order to better define stage-specific nutritional requirements and growth performance during nursery culture of this commercially important species.

THE EFFECTS OF STOCKING DENSITY IN PEARL NETS ON SURVIVAL, GROWTH, AND REPRODUCTIVE POTENTIAL OF THE BAY SCALLOP, *ARGOPECTEN IRRADIANS IRRADIANS*.

Maureen Davidson, New York State Department of Environmental Conservation, Bureau of Marine Resources, 205 North Belle Mead Road, East Setauket, NY 11733.

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. 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 resources restoration should be held at moderate densities in pearl nets and low densities in lantern nets to maximize survival and reproductive potential.

DEVELOPING A COASTAL MAINE SEA SCALLOP ENHANCEMENT PROGRAM.

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Increasing the stock of wild scallops along the Maine coast by capturing wild seed at spatfall, holding them captive through what would be a time of high mortality in the wild and then releasing them has captured the imagination of many Maine scallopers. This concept builds upon a Japanese methodology that was exported successfully to New Zealand several years ago. We are working collaboratively with several groups of fishermen with assistance from a New England Consortium grant. The project is currently into its third season of spat collection and millions of scallop seed were released this last fall in several locations along the coast from last years spatfall.

Future work will involve continued collection of spat, defining the best locations for spat collection, monitoring survival of released seed, looking at increasing survival through holding the seed beyond the first year, addressing the issues of ownership and adjusting harvest practices to maximize yield.

This paper will focus on current results and near future efforts.

GROWTH AND MOVEMENT OF SEA SCALLOPS IN THE SOUTHERN PART OF THE GREAT SOUTH CHANNEL ON GEORGES BANK: A TAGGING STUDY.

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A rotational fisheries management strategy is being considered for the sea scallop, *Placopecten mangellanicus*, fisheries of the northeast United States. To implement a rotational management strategy site-specific information on sea scallop population dynamics is required. For example, the sea scallop is the best swimmer of the 400 known species of scallops, and can move as much as 15 km on Georges Bank. This could influence the size of the rotational areas closed or open to harvesting. Further, sea scallop growth rates show substantial variability over their geographic range. This could influence the time period rotational areas are open or closed. To begin the development of a site-specific data set, a sea scallop growth and movement experiment was conducted in the southern part of the Great South Channel of Georges Bank. Approximately 13,000 scallops were tagged and released in May 2001. Presently, 677 tagged shells have been returned showing movement of up to 7 km. and growth spanning more than 9 months. This preliminary data illustrates the need for further site-specific movement and growth experiments over the sea scallops range.

INVESTIGATIONS WITH TRIPLOID ATLANTIC SEA SCALLOPS, *PLACOPECTEN MAGELLANICUS*.

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Bivalve aquaculture has benefited from the introduction of triploids, but successful commercial production of triploid sea scallops, *Placopecten magellanicus*, remains elusive. Experiments were conducted to determine the optimum combination of incubation temperature (8°C and 14°C) and Cytochalasin B (CB) concentration (0.25 mg/l and 0.50 mg/l) for inducing triploidy in scallops. Two replicate spawning lots of larvae were produced (Lot 1; one female parent, one male; Lot 2: two females, two males), and ploidy analyses were performed via flow cytometry on Day 4 to determine the initial levels of triploid induction. In the 8°C incubation treatments, more triploids were found amongst the larvae from Lot 1 (~24% in both CB treatments) than in Lot 2 (13% in the 0.25 mg/l CB treatment, and 16% in the 0.50 mg/l CB treatment), numbers similar to those found in the untreated controls (16% in Lot 1 and 20% in Lot 2). Among embryos incubated at 14°C, both levels of CB produced ~60% triploids in Lot 1, while in Lot 2 more triploids were produced in the 0.50 mg/l CB treatment (61%) than in the 0.25mg/l CB treatment (39%). Many triploids were found in the 14°C controls (41% in Lot 1 and 22% in Lot 2). Studies comparing feeding rates between diploid and triploid sea scallop larvae will also be discussed.

A COMPARISON OF MICROALGAL DIETS FOR ENHANCED PRODUCTION OF *PLACOPECTEN MAGELLANICUS* POSTLARVAE.

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Little is known concerning stage-specific diets that optimize hatchery production of the sea scallop, *Placopecten magellanicus*, especially during vulnerable, post-settlement stages. Poor growth and survival of these developmental stages may be related to nutritional deficiencies. Therefore, our primary objectives were to identify high-performance algal diets, involving a minimum number of species, for cost-effective implementation in commercial hatcheries, and to determine algal properties (e.g., size, biochemical composition) which may contribute to differences in scallop growth. We also compared the lipid composition (including essential fatty acids) of postlarvae and their diets. To this end, *P. magellanicus* postlarvae (initial mean shell height = 388 μm) were exposed for 28 days at 14°C to five mixed microalgal diets, each consisting of one diatom and one flagellate, (at a constant, volume-equivalent concentration of 40 T-Iso cells μl^{-1}) in 400 L mesocosms. Diets consisted of one of three diatom species (*Thalassiosira weissflogii*, *Chaetoceros muelleri*, or *Fragilaria familica*), and one of three flagellates [*Pavlova lutheri*, *Pavlova* sp. (CCMP 459), or *Tetraselmis striata* (PLAT-P)]. Shell growth trajectories, determined by video imaging, showed that *F. familica*, a previously untested isolate from Mahone Bay, Nova Scotia, was the diatom yielding the highest growth rate (22.6 μm/day). *Pavlova* sp. (CCMP 459), which is known to support excellent growth of sea scallop larvae, yielded the highest growth rate (28.1 μm/day) of the three flagellates, perhaps due to its high levels of ARA (20:4n6; arachidonic acid). Although *T. striata* is known to be an excellent diet for oyster (*Crassostrea virginica*) spat, it resulted in the lowest scallop growth rate for both shell height (5.5 μm/day) and ash-free dry weight, providing a final mean size almost half that of scallops fed the highest performing diet. Poor performance of this diet may be partly related to the very low levels of DHA (22:6n-3; docosahexaenoic acid; as a % of total fatty acids) in its two algal constituents. Sea scallop growth rates on the two highest performing diets were comparable to maximum literature values for this developmental stage using 4 to 6-species diets.

IMPACTS AND CONTROL OF THE SPIONID POLYCHAETE, *POLYDORA WEBSTERI* ON THE SEA SCALLOP, *PLACOPECTEN MAGELLANICUS* IN MIDWATER CULTURE.

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Biofouling on culture structures and species is a normal phenomenon to shellfish growers in all countries, however, some fouling species are more damaging than others. In 1997, we initiated an experiment to look at the full-cycle grow-out characteristics of the sea scallop at a site in Passamaquoddy Bay that was judged to be the best oceanographically for culture based on food and circulation. Animals were grown at a depth of 5m and 20m from the surface in either pearl nets or lantern nets depending on the size of the scallops at the time. Nets were changed monthly during the period of highest biofouling (late spring to mid fall) and as required outside of that window. The results of the 20m treatment revealed that the shells were severely perforated by the polychaete *Polydora websteri* causing death. We postulated that the growing of the scallops in midwater effectively isolates them from their normal ecosystem with regular interspecific interactions (such as grazers) and allows commensal/parasitic organisms to grow out of control. Therefore, in the summer of 2001, we initiated a second phase to the project where we introduced juvenile green sea urchins grazers (*Strongylocentrotus droebachiensis*) to some experimental cages. Initial results indicated that the sea urchins were effective in reducing the density of *Polydora* on the scallop shells. Further results will be discussed during the talk as the study is still in progress.

DEVELOPMENT OF A STOCK ASSESSMENT PROGRAM FOR WEATHERVANE SCALLOPS IN ALASKA.

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Guideline harvest levels for Alaska's weathervane scallop (*Patinopecten caurinus*) fishery are currently based on historic average catches. Although vessels participating in the fishery are subject to a mandatory observer program that monitors catch and effort, research indicates that these statistics cannot reliably index changes in scallop abundance. In 2000, the Alaska Department of Fish and Game began work aimed at development of fishery-independent stock assessment methods that could ultimately be used to manage the fishery based on estimates of exploitable abundance. To date, we have made four research cruises that surveyed known scallop beds with a video drop camera, a towed video sled, and a New Bedford offshore survey dredge. Results indicate that the drop camera does not survey enough area to provide good density estimates. The video sled appears to be a more promising tool, but reviewing tapes to obtain scallop counts is time consuming. We are now experimenting with using the video sled to estimate dredge efficiency. A combination of video and dredge survey methods may ultimately prove to be the best choice for assessing weathervane scallop populations in Alaska.

BIOCHEMICAL INDICATOR OF GIANT SCALLOP *PLACOPECTEN MAGELLANICUS* QUALITY: LARVAL GROWTH, COMPETENCY AND SETTLEMENT.

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The purpose of this study was to monitor the lipid class content of larvae over the entire cycle, to verify the potential effect of varying feeding regimes on larval lipid content, quality, growth and survival, to evaluate the potential use of lipid class ratios to forecast larval growth and survival, and finally, to examine the effect of larval quality on settlement behavior and success. When larvae were able to feed from exogenous sources at day 4, three diet treatments were applied. Larvae were periodically sampled for lipid class analysis, growth measurement and survival assessment. Behavior of pediveliger larvae for each diet treatment were recorded with a videotape and endoscope set-up during settlement period. During the course of experiment, lipid class composition of the three diets were different in terms of triacylglycerol (TAG) rich in saturated and monounsaturated fatty acids. Our study show that TAG level in larval food was positively correlated with growth rate, TAG content and as a consequence, larval quality—as expressed with TAG-sterol (ST) or TAG-phospholipid (PL) ratios—prior to settlement. A positive relation between number of competent larvae produced and larval quality at day 8 has been found, suggesting that survival at competency was partly explained by the recovery efficiency of energetic reserves after embryogenesis. Larval quality was correlated with settlement success in the way that higher quality larvae has lead to poor settlement but explore the same time whatever the age, whereas low quality larvae decrease exploration time with age. As a consequence, the low settlement success observed in our experiment with high physiological condition larvae might be the effect of metamorphosis delay in response to poor environmental conditions.

EXAMINATION OF SEA SCALLOP, *PLACOPECTEN MAGELLANICUS*, AGGREGATIONS USING A VIDEO SURVEY IN CLOSED AREAS OF GEORGES BANK.

Kevin D. E. Stokesbury* and **Brad Harris,** School for Marine Science and Technology, University of Massachusetts Dartmouth, 706 South Rodney French Boulevard, New Bedford, MA 02744-1221.

Georges Bank is the world's largest natural scallop resource. During the summer months of 1999/2000/2001 SMAST in association with the scallop industry developed and conducted 23 video surveys on Georges Bank. These surveys produced a series of maps of the sea floor of Georges Banks containing high aggregations of sea scallops. The video survey detailed the distribution of substrate, depth, number of live and dead scallops, and macroinvertebrates (sponges, starfish, filamentous fauna). The video technique allows a previously unattained precise, accurate measure of these variables and allows correlation analyses between them. Further, the closed areas of Georges Bank have scallop densities higher than any previously observed. For example, the three areas surveyed in 1999 (1940 km^2) contained approximately 652 million scallops representing approximately 17 million kilograms (32 million lbs worth approximately $161 million) of harvestable scallop meats. This research addresses scallop stock assessment and the critical regional and national issue of the effects of mobile fishing gear on the marine benthic community. It has direct implications for rotational fisheries management, on an appropriate spatial scale (km), under consideration by the New England Fishery Management Council.

PARASITE AND HOST DEFENSES

THE EFFECT OF PH ON THE KILLING ACTIVITY OF HEMOCYTES IN THE PACIFIC OYSTER, *CRASSOSTREA GIGAS.*

Steven M. Allen* and **Louis Burnett,** Grice Marine Laboratory, University of Charleston South Carolina, 205 Fort Johnson Rd., Charleston, SC 29412.

In recent years there has been an increase in the occurrence of summer mortalities of the commercially important Pacific oyster, *Crassostrea gigas.* These mortalities occur during the late summer when water and air temperatures are at their highest. *C. gigas* are grown intertidally and are, therefore, air exposed for hours at a time. An oyster closed during air exposure depletes the oxygen stores within the shell and builds up CO_2 acidifying the tissues. The average pH of hemolymph from an oyster which is submerged in well aerated water (18°C) and ventilating is 7.52 (0.04 SEM; N = 35). The average pH of of hemolymph from an oyster which is aerially exposed for 4h in 30°C air is 6.83 (0.02 SEM; N = 26). We hypothesize that stresses associated with air exposure inhibit the immune system of the oyster and contribute to the summer mortalities. The focus of the present study was to determine if the innate immunity provided by hemocytes was decreased by low pH. The ability of hemocytes to kill the bacterium *Vibrio parahaemolyticus* was assessed using an *in vitro* killing assay. Hemocytes were treated with low pH and challenged *in vitro* with *V. parahaemolyticus.* A tetrazolium dye reduction assay was used to quantify the number of viable bacteria, from which a killing index was calculated. No significant difference was found between the two treatments pH 7.6 and pH 6.6 ($p < 0.01$; N = 14). ODRP Grant No. NA96RG0488.

MUCOID SECRETIONS PROTECT QPX FROM ANTIMICROBIAL AGENTS.

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Quahog parasite unknown (QPX) has recently caused significant mortality in aquacultured *Mercenaria mercenaria* in Massachusetts. It secretes a viscous, mucoid substance which may protect the parasite from host defense mechanisms; it has been reported that clams injected with QPX washed free of the mucus coat did not develop infections or disease. In this study the antimicrobial activity of *M. mercenaria* serum was measured against QPX with, or washed free of, its secreted coat. Massachusetts QPX cultures were grown for 7 days in the presence or absence of clam serum in the medium; the parasites were still in log stage growth. When coat-free QPX were added to serum-containing medium, dose-dependent growth inhibition was seen in cultures with 10–50 µg/ml serum protein; lower concentrations were often slightly stimulatory, higher concentrations produced ~100% inhibition. Growth of cultured QPX, when fully enveloped by mucus, was not inhibited by clam serum proteins at all concentrations tested (<60 µg/ml). If aliquots of coat-free QPX were incubated for various time intervals (during which time they produced mucoid envelopes) prior to the addition of clam serum to the cultures, there was a time-dependent reduction of serum-mediated growth inhibition.

MEASUREMENT OF *VIBRIO TAPETIS* CYTOTOXIC ACTIVITY ON *RUDITAPES PHILIPPINARUM* HEMOCYTES BY FLOW CYTOMETRY.

Gwénaëlle Choquet,* Philippe Soudant, Christophe Lambert, and **Christine Paillard,** IUEM-UBO, LEMAR, Technopôle Brest-Iroise, 29280 Plouzané, France; **Jean-Louis Nicolas,** LPI, IFREMER, Plouzané, France.

Vibrio tapetis is the causative agent of Brown Ring Disease which affects the clam, *Ruditapes philippinarum*. After incubation with *V. tapetis*, hemocytes loose filopods and become rounded, indicating production of a virulence factor by the bacteria. To rapidly quantify this factor, a flow-cytometric test has been developed. This test is based on the capacity of *V. tapetis* to inhibit adhesion of clam hemocytes to plastic. Several bacteria/hemocyte ratios, other *Vibrio* spp. pathogenic to bivalves and various *V. tapetis* isolates have been tested. Inhibition of adherence is detectable with as few as five bacteria per hemocyte. The greater cytotoxic activity of *V. tapetis* compared to *V. splendidus, Vibrio* sp. (*strain S322*), and *V. pectenicida* suggests a specific pathogenicity of *V. tapetis* to *R. philippinarum* hemocytes. Though all *V. tapetis* isolates possess the capacity to inhibit adhesion, significant variations of cytotoxicity among isolates has been demonstrated. These results are in agreement with *in vivo* pathogenicity tests. The identification and characterisation of the genes involved in *V. tapetis* cytotoxicity is in progress.

CHEMOTAXIS OF HEMOCYTES OF THE HARD CLAM, *MERCENARIA MERCENARIA*, TO QUAHOG PARASITE UNKNOWN (QPX) AND OTHER MICROORGANISMS.

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Quahog Parasite unknown (QPX) is a protist pathogen in the phylum Labyrinthulomycota affecting the hard clam, *Mercenaria mercenaria*. It causes a disease that has impacted hatchery and broodstock clam populations in Canada and the United States. Chemotaxis is the directed migration of cells in a chemical gradient, and is a well-documented immune response of hemocytes. It is postulated that there is a correlation between pathogenicity and chemoattraction; invading organisms able to escape detection by hemocytes would be better able to colonize their host. *M. mercenaria* hemocytes are known to exhibit chemotaxis in response to live *Escherichia coli* bacteria and to cell-free *E. coli* culture fluid. This study is the first investigation into host chemotactic response to QPX or other Labyrinthulomycota species. The chemotactic effects of several pathogenic and non-pathogenic organisms were compared. There was no chemotaxis by clam hemocytes toward QPX cells devoid of their mucofilamentous secretions, but positive chemotaxis for spent QPX media. There was positive chemotaxis for *Bacillus megaterium*, a non-pathogenic bacterium. Other organisms investigated included a non-pathogenic Labyrinthulomycota, a non-pathogenic Protist, and a pathogenic bacterium. Comparisons yielded a profile of *M. mercenaria* chemotactic response for a broad range of parasitic challenges.

EFFECT OF DIETARY FATTY ACID COMPOSITION ON LIPID PROFILES OF HAEMOCYTE MEMBRANES IN OYSTERS AND CLAMS AND ITS IMPACT ON IMMUNE FUNCTIONS.

Maryse Delaporte,* Jeanne Moal, and **Jean-françois Samain,** LPI, Ifremer de Brest, 29280 Plouzané, France; **Philippe Soudant, Gwénaëlle Choquet, Christophe Lambert,** and **Christine Paillard,** LEMAR, IUEM, 29280 Plouzané, France.

This study was designed to assess the influence of micro-algal diets on the fatty acid profile of haemocyte membranes and on immune functions. The oyster *Crassostrea gigas* and the clam *Tapes philippinarum* were fed three diets with varying PUFA composition.

The fatty acid composition of haemocyte and gill membranes of both bivalves was greatly influenced by the diet. Nevertheless, a selective retention of certain specific PUFA was observed in the analysed tissues: 22:6(n-3) for clam and 20:5(n-3) for oyster. Immune parameters were also affected. Indeed, a 20:5(n-3) and 20:

4(n-6) enrichment appeared to increase the phagocytic rate and the metabolic activity of clam haemocytes. A smaller positive effect of 20:5(n-3) on metabolic activity of oyster haemocytes was observed. Interestingly, when oyster haemocytes were incubated one hour at 18°C or 30°C, a positive correlation between the 22:6(n-3) content of haemocytes and the phagocytic rate was noticed.

ACTIVATION OF OYSTER DEFENSES BY ENVIRONMENTAL CONTAMINANTS. William S. Fisher* and **Leah M. Oliver,** U. S. Environmental Protection Agency, National Health and Environmental Effects Research Laboratory, Gulf Ecology Division, Gulf Breeze, FL 32561.

Four field studies performed on eastern oysters *Crassostrea virginica* support a hypothesis that Cu, Zn, and perhaps butyltins and polycyclic aromatic hydrocarbons (PAH) can stimulate hemopoiesis, hemocyte locomotion and hemocyte bactericidal capacity. The first study found circulating hemocyte numbers and locomotion were positively associated with contaminated sites in Tampa Bay, particularly those where pooled oyster tissues contained high concentrations of trace metal and PAH analytes. Subsequently, the relationships between these particular contaminants and hemocyte activities were extended to four additional bays (St. Andrew, Choctawhatchee, Pensacola, and Biscayne). A third study, which analyzed chemicals from individual oysters in Pensacola Bay, verified that circulating hemocyte numbers and bactericidal activity were positively correlated with Cu, Sn, Zn, butyltin, total metals, total polychlorinated biphenyls and total PAH. A fourth study showed that circulating hemocyte number and bactericidal activity were significantly elevated when oysters were moved from a relatively clean site to one with high concentrations of Cu, Zn, butyltins and PAH. These data provide a weight of evidence that certain chemical contaminants can stimulate defense-related hemocyte activity in oysters. Although chemical contaminants are generally suspected to suppress defense functions of oysters, these chemicals, for unknown reasons, appear to have the opposite effect.

IMPROVEMENT BY PROBIOTICS OF *PECTEN MAXIMUS* LARVAE DEFENSE CAPACITY, MEASURED BY CHEMILUMINESCENCE. Christophe Lambert* and **Christine Paillard,** LEMAR, IUEM-UBO, Place Copernic, Plouzané, France; **Jean-Louis Nicolas,** LPI, IFREMER, Plouzané, France.

Literature concerning evaluation of defense system capacity of bivalve larvae are scarce. It's why this work intended to adapt to oyster and scallop larvae a chemiluminescence (CL) test to measure oxygen intermediate synthesis (mainly H_2O_2) during phagocytosis of zymosan particles and inhibiting effect of *Vibrio pectenicida,* pathogenic for scallop larvae on this activity. As a result, activation of whole alive *Crassostrea gigas* and *Pecten maximus* larvae by zymosan particles have been shown to increase respectively 9.9 and 2.4 times the chemiluminescence activity. The peak level was obtained about one hour after activation. This CL test was used to compare CL activity of scallop larvae reared with antibiotic (Chloramphenicol 4 ppm), probiotic bacteria (available in our laboratory) or without treatment. The defense capacity of larvae reared with probiotic was higher than those with antibiotic or without treatment. Moreover, probiotic reared larvae were shown to be less sensitive to *in vitro* CL inhibition of *V. pectenicida.* This led to the conclusion that probiotics not only compete with bacterial flora but were also able to improve scallop larvae defense system (oxidative burst).

DEVELOPMENT OF A FLOW CYTOMETRIC MEASUREMENT OF OXIDATIVE METABOLISM PRODUCT FORMATION BY *CRASSOSTREA GIGAS* HEMOCYTES AND APPLICATION TO EVALUATE PATHOGENIC *VIBRIO* INHIBITING CAPACITY. Christophe Lambert,* Philippe Soudant, Gwénaëlle Choquet, and **Christine Paillard,** LEMAR, Laboratoire des Sciences de l'Environnement Marin, UMR6539, IUEM-UBO, place Nicolas Copernic, Plouzané, France.

A flow cytometric method to measure the production of oxidative metabolism products was adapted to *Crassostrea gigas* hemocytes. Measurement was based on the oxidation by hydrogen peroxide (H_2O_2) of intracellular 2′,7′-dichlorofluorescin (DCFH) in green fluorescent dichlorofluorescein (DCF). Activation by zymosan particles of the respiratory burst metabolic chain was shown to stimulate the DCFH oxidation in *C. gigas* hemocytes and a rate of 20 zymosan particles per hemocytes was found to be optimal. At the opposite, DCFH oxidation using phorbol myristate acetate (PMA) was not obtained. Anti-aggregant solution, used to avoid hemocytes clamping after bleeding, was shown to inhibit the respiratory burst measured by DCFH oxidation. Finally, the flow cytometric method developed during this work was used to evaluate and grade the DCFH oxidation inhibiting capacity of four *Vibrio* species, known or suspected to be pathogenic for bivalves.

PURIFICATION OF A NOVEL ANTIMICROBIAL PEPTIDE FROM THE EASTERN OYSTER (*CRASSOSTREA VIRGINICA*). Ann C. Mountz* and **Robert S. Anderson,** University of Maryland Center for Environmental Science, Solomons, MD 20688.

Oysters are routinely exposed to a variety of microbes; few of these microbes are pathogenic, however oyster diseases, such as Dermo and MSX, have lead to drastic reductions in oyster populations along the Atlantic coast of the United States. Understanding the oyster's immune system is an important element in determining how the remaining populations can best be protected or increased. Many aspects of the eastern oyster immune system have been characterized, including reactive oxygen species, phagocytosis, and lytic enzymes. Antimicrobial peptides are a more recently recognized component of the bivalve defensive capabilities, which have not yet been identified in the eastern oyster. A new antimicrobial peptide was purified from oyster serum via methanol precipitation, size-exclusion centrifugation, Sep-Pak filtration, and reverse-phase HPLC. Activity against the model Gram-positive bacterium was measured at all steps using a tetrazolium dye reduction assay. The purified peptide is 4.3 kDa based on mass spectrometry analysis and has preliminarily been named CVAP-1.

TEMPERATURE EFFECT ON IMMUNOCOMPETENCE OF CLAM *R. PHILIPPINARUM* AND ON *V. TAPETIS* CYTOTOXICITY. Christine Paillard,* Gwénaëlle Choquet, Christophe Lambert, and **Philippe Soudant,** IUEM-UBO, LEMAR Laboratoire de l'environnement marin, Technopôle Brest-Iroise, 29280 Plouzané, France; **Helen Reid** and **Harry Birbeck,** University of Glasgow, Division of Infection and Immunity, GLASGOW G12 8QQ, Scotland.

Adult clams, *R. philippinarum*, were conditioned for one month in the laboratory at three temperatures 7, 13 and 21°C, and then inoculated with *V. tapetis*. One month after *V. tapetis* challenge, clam phagocytic activity and *V. tapetis* cytotoxicity were measured. An increase of phagocytic activity was demonstrated in control clams maintained at the highest temperature. At 13°C, higher phagocytic activity was measured in *V. tapetis* inoculated clams compared to controls. Also, highest Brown Ring Disease prevalence and intensity was obtained at 13°C. Cytotoxic assays based on hemocyte-bacteria interactions were performed with bacteria grown at these three different temperatures. Generally, higher cytotoxicity was found when *V. tapetis* was grown at 13°C, which corresponds to the lower end of its optimal growth range. Further, cell rounding percentage depends on whether the clams were experimentally challenged with the bacterium and temperatures at which clam are maintained.

REPEATED HEMOLYMPH SAMPLING OF INDIVIDUAL CLAMS: WHAT DOES IT TELL US ABOUT SAMPLING PROCEDURES? Christine Paillard,* LEMAR, UMR 6539, IUEM-UBO, Plouzané, France, **Susan E. Ford,** Haskin Shellfish Research Laboratory, Rutgers University, Port Norris, NJ.

Hemocyte concentrations, protein levels, and enzyme activities are often used as measurements of the health status of marine bivalves and are frequently considered an index to assess their susceptibility to infectious agents. Yet these parameters are known to vary considerably on a seasonal, regional, and individual basis, and are likely to be affected by laboratory procedures. Sources of variation such as these add uncertainty to the interpretation of hemolymph assays. We repeatedly sampled the hemolymph of clams, *Ruditapes philippinarum*, in both laboratory and field conditions, to determine 1) how consistent measurements were over time for the same individual, 2) whether time of sampling affected results, and 3) how much repeated sampling itself altered the measurements. Most parameters were relatively consistent over time (individuals had consistently high or low values), but more so in the field than in the laboratory. There were significant day-to-day variations when sampling occurred over a several-day period, but, sampling time explained very little of the overall variation. Repeated sampling increased mortality and altered hemolymph constituents in the laboratory, but not the field, study.

THE ANTIOXIDANT SYSTEM OF THE PROTISTAN PARASITE *PERKINSUS MARINUS*. Eric J. Schott,* José A. F. Robledo, Wolf T. Pecher, Florence A. Okafor, and **Gerardo R. Vasta,** COMB, UMBI, University of Maryland, Baltimore, MD 21202.

Spread of the protistan parasite *Perkinsus marinus* within the oyster *Crassostrea virginica* is believed to be via engulfment and migration by phagocytic hemocytes. Because phagocytosis of killed *P. marinus* trophozoites elicits production of reactive oxygen intermediates (ROIs) by oyster hemocytes, but phagocytosis of live trophozoites does not, *P. marinus* appears to have mechanisms to prevent the accumulation or production of ROIs. We previously described two Fe-type SOD genes (*PmSOD1, PmSOD2*) from *P. marinus* and demonstrated the ability of their products to convert $O_2^- \cdot$ to H_2O_2. *P. marinus* is resistant to moderate levels of H_2O_2, suggesting that it also possesses a mechanism for H_2O_2 removal. However, significant efforts to detect *P. marinus* catalase activity and gene sequences have been unsuccessful. Instead, we have found that *P. marinus* trophozoites possess abundant ascorbate dependent peroxidase (APX) activity. We have partially purified *P. marinus* APX, which co-migrates with a 35 kD band on non-denaturing gels. Continuing genetic, biochemical, and cellular studies of *P. marinus* FeSODs and APX will contribute to further characterize the *P. marinus* antioxidant defense system. [Supported by Grant No NA06RG0101-5 from ODRP, NOAA, through the Maryland Sea Grant, to GRV].

SEASONAL AND CULTURE SITE EFFECTS ON THE PHYSIOLOGICAL, IMMUNOLOGICAL AND BROWN RING DISEASE STATUS OF THE MANILA CLAM *RUDITAPES PHILIPPINARUM*. Philippe Soudant,* Gwénaëlle Choquet, Christophe Lambert, Alain Marhic, and **Christine Paillard** LEMAR UMR 6539, IUEM-UBO, Place Nicolas Copernic, Plouzané, France.

To assess the effects of environmental conditions on Brown Ring Disease (BRD) expression, physiological and immunological status, juveniles of clams were distributed in four rearing sites selected for their varied ecological characteristics. Clams were sampled for analysis every three months for eighteen months in each sites. Significant site and seasonal effects have been established for the condition index, the growth rate, the haemocyte concentration and cytomorphology, and the enzymatic activities and protein content of the total hemolymph. Some significant relationships between immunological and physiological parameters were observed. The haemocyte concentration was correlated with size and mortality rate of haemocytes while their complexity was correlated with phenoloxidase activity. Also, correlation was shown between the haemocyte concentration and the size of clams while haemocyte size and hemolymph protein content were correlated to condition index. Surprisingly, the seeded clams showed very low BRD prevalence in all sites and for all seasons. Meantime, high prevalence was observed in natural stock from one of the site. This suggests hatchery seeded clams may be BRD resistant and claimed further studies.

RELATIONSHIPS BETWEEN SUMMER MORTALITIES AND DEFENCE MECHANISMS IN FAMILIES OF *CRASSOSTREA GIGAS* REARED IN DIFFERENT ENVIRONMENTAL CONDITIONS. P. Soudant,* C. Lambert, G. Choquet, S. Ford, and **C. Paillard,** LEMAR, IUEM-UBO, Place Nicolas Copernic, Plouzané, France; **L. Degremont, M. Delaporte, J. Moal, P. Boudry, P. Soletchnick, J.-P. Joly, M. Ropert, E. Bédier, A. Huvet,** and **J.-F. Samain,** LGP, LPI, LCs of IFREMER, France.

High individual variability is often encountered when measuring defence mechanisms in bivalves. Such variation is suspected to result from both environmental and genetic factors. Determine whether defence mechanisms of *C. gigas* are genetically based was thus part of a national program conducted by IFREMER and dedicated to understand the causes of summer mortalities in *C. gigas* juveniles. Fifteen bi-parental families, obtained from a nested half-sib crossing design, were reared four months in three sites. Six families were selected on their survival performance (three "good" and three "bad" ones) to compare their immunological status. As expected, most of immunological parameters tested were significantly different according to the culture site. More interesting is the significant differences measured between good and bad families. For instance, higher total haemocyte counts and lower oxidative metabolism were observed in good families. Moreover, incubation with pathogenic *Vibrio* sp. S322 inhibited significantly more the adhesion capabilities and oxidative metabolism of haemocytes from bad families.

PURIFICATION AND CHARACTERIZATION OF LYSOZYME FROM PLASMA OF EASTERN OYSTERS (*CRASSOSTREA VIRGINICA*). Qing-Gang Xue* and **Jerome F. La Peyre,** Cooperative Aquatic Animal Health Research Program, Department of Veterinary Science, Louisiana State University Agricultural Center, Baton Rouge, LA 70803; **Aswani K. Volety,** Division of Ecological Studies, Florida Gulf Coast University, Fort Myers, FL 33965; **Fu-Lin E. Chu,** Virginia Institute of Marine Science, College of William and Mary, Gloucester Point, VA 23062.

Lysozymes are antimicrobial proteins which are defined as 1,4-β-N-acetylmuramidases cleaving a glycosidic bound between N-acetylmuramic acid and N-acetylglucosamine of peptidoglycan. Lysozyme activity has been detected in the body fluids and tissues of many bivalve molluscs but lysozymes from only a few bivalve species have been purified. The molecular weights of the purified lysozymes range from 11 to 18 kDa. The analysis of N-terminal amino acid sequences, when determined, indicated that they all belong to a distinct type of lysozyme, the i-type. Lysozyme from plasma of eastern oysters was recently purified by a combination of ion-exchange chromatography on CM-Sepharose Fast Flow and gel filtration chromatography on a Superdex G-75 column. The final preparation showed a single band on SDS-PAGE gel with a molecular weight of 18.4 kDa. About 1.5 mg of lysozyme was purified from one liter of oyster plasma and the lysozyme specific activity increased 150 fold. The enzymatic properties, stability and antimicrobial activity of the purified plasma lysozyme are currently being determined.

SEA URCHIN BIOLOGY, PATHOLOGY AND CULTURE

SEA URCHIN MORTALITY IN MAINE, INITIAL CASE REPORT AND OVERVIEW. Ralph Elston, AquaTechnics/Pacific Shellfish Institute, PO Box 687, Carlsborg, WA 98324.

Compared to some other species of marine invertebrates, health parameters and disease conditions of sea urchins are less well studied. The purpose of this case report is to present initial observations that may suggest causes or significant factors related to sea urchin mortalities occurring in Maine in 1999 and 2000. These observations may be useful in indicating what further investigations are needed to make a more definitive diagnosis regarding cause of the losses.

Sea urchin (*Strongylcentrotus droebachiensis*) mortalities were observed in two areas of Frenchman's Cove, Maine in late October 2000. Previously, urchin mortalities were reported in August 1999 by divers and during an urchin transfer in August 2000. The samples from 1999 were examined bacteriologically and histologically by Dr. Paul Waterstraat of the Maine Department of Natural Resources. The presence of a purple exudate from the teste was reported. The urchins were also examined for the presence of a paramoeba, previously associated with urchin mortalities in Nova Scotia, and none of these parasites were found.

Two groups of urchins were received live and chilled on November 29, 2000. One group consisted of four urchins collected from Winter Harbor and the other group consisted of two urchins collected from Schoodic Point. All urchins were examined on receipt, and processed for necropsy and histological examination. The teste diameter/height ranged from 53/34 mm to 58/35 mm. Clinically, three of six urchins appeared healthy and exhibited mobility of the spines. Two urchins showed loss of spines, one from all except the oral surface and the second unilaterally in a band from the oral to aboral surface. Three were confirmed males and three were females.

Of six animals examined histologically, no lesions were found in two of the healthy appearing individuals. In both urchins with loss of spines, there were multifocal areas of eosinophilic intranuclear inclusion bodies in epithelium of the spines and their musculature and associated necrosis of epithelium and myocytes. These inclusion bodies were relatively common in one individual but rare in another. This finding corresponds to the loss of urchin spines noted by divers and other on site investigators. Such inclusion bodies may represent a viral infection but there are other non-infectious causes of inclusion bodies. Verification of a viral infection would require examination by transmission electron microscopy.

IMPORTANCE OF DIETARY MINERALS AND PIGMENTS FOR INCREASING SOMATIC GROWTH OF JUVENILE GREEN SEA URCHINS (*STRONGYLOCENTROTUS DROEBACHIENSIS*).

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Understanding the nutritional requirements of juvenile sea urchins is necessary for diet formulation and optimizing somatic growth. From Feb 2000 to July 2000, different mineral sources and concentrations (modified Bernhart-Tomerelli salt mix at 0%, 1.5%, 3%, 6%, and 15% and Shur-Gain at 3% and 6%) were incorporated in pigmented (beta-carotene) diets to determine the effect on juvenile sea urchin (14 mm to 15 mm initial test diameter) somatic growth. Non-pigmented diets were also used with 3% of each mineral source incorporated in the diet. After the 154-day experiment, the juveniles fed the non-pigmented diets were smaller (20 mm average TD) than those fed the 3% mineral pigmented diets (23 mm average TD) ($P<0.001$). The juveniles fed the 15% mineral diet were larger (24.3 mm average TD) than those fed the 0% and 1.5% mineral diets (22 mm average TD) ($P<0.001$). As well, the juveniles fed the pigmented diet with 3% Shur-Gain mineral (which lacked magnesium) were smaller (21 mm average TD) than those fed the diet with 3% Bernhart-Tomerelli mineral (which included magnesium) (23.5 mm average TD) ($P<0.001$). From Nov 2000 to April 2001, a pigmented (beta-carotene) prepared diet with 15% Bernhart-Tomerelli salt mix was compared to kelp (the natural sea urchin diet) to determine differences in juvenile somatic growth production between diets. After the 159-day experiment, the juveniles (1 mm to 2 mm initial average test diameter) fed the prepared diet were larger (7.4 mm average TD) than the kelp-fed juveniles (4.4 mm average TD) ($P<0.001$). Prepared diets require pigments and high mineral content to optimize juvenile sea urchin somatic growth.

SEA URCHIN DISEASE CONCERNS IN ATLANTIC CANADA.

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The green sea urchin (*Strongylocentrotus droebachiensis*) has been fished in Atlantic Canada since the 1950's with a commercially developed fishery emerging in the 1980's in the Bay of Fundy, New Brunswick and southwestern Nova Scotia (NS). In the 1990's, Newfoundland, Quebec and to some extent Prince Edward Island, also began fishing this species. The total Atlantic Canadian sea urchin landings in 2000 were 3,050 MT (live weight) with a value of $7.08 million.

The fishery, however, has not been without disease setbacks. Between 1980 and 1983 sea urchin mortalities in NS were estimated at 245,000 tons associated with a parasitic amoeba, *Paramoeba invadens*. Since 1995, a die-off associated with paramoebisasis of 50,000 to 100,000 tons has occurred representing an estimated 10 to 100 times the weight of urchins taken by the NS fishery. Currently, the Department of Fisheries and Oceans Canada lists *P. invadens* and Bald-Sea-Urchin disease as "regional concerns" with trematode metacercariae and turbellarian parasitism listed as "negligible significance". The objective of this presentation is to give an overview and update on the current disease concerns facing the green sea urchin fishing and aquaculture industry in Atlantic Canada.

PHOTOPERIOD, URCHIN "EYES" AND GAMETOGENESIS.

Michelle Moody and **Charles W. Walker,** Department of Zoology and Marine Biomedical Research Group, University of New Hampshire, Durham, NH 03824.

Shortening daylength in the fall results in the initiation of changes in two populations of cells within the gonads of both sexes of the green sea urchin, *Strongylocentrotus droebachiensis*. These cellular populations include spermatogonia in males and oogonia in females and somatic cells called nutritive phagocytes (NP) in both sexes. During the summer, gonial cells are amitotic and NP store nutrients; during the photoperiod changes in the fall, gonial cells begin mitosis and NP mobilize nutrients. Details of these processes can be addressed on the web page http://zoology.unh.edu/faculty/walker/urchin/gametogenesis.html The detectors for these changes in day-length are unknown, but might include tube feet, spines, the dermis or the gonads themselves. We are using western blots and immunocytochemistry to detect PAX6 (the complex eye master control switch gene) and rhodopsin proteins within each of these tissues.

It is also unknown whether the response to photoperiod occurs at the level of the NP, which then mobilize nutrients and thus cause gonial cells to divide, or whether both populations of cells are independently effectors for the photoperiod cue. In order to distinguish between these two possibilities, we are examining the expression of the *c-myc* protooncogene and of the SEAW1 stem cell controlling gene in both males and females during the months when the photoperiod que occurs (August—October).

Practical applications of this research for aquaculture might result from preventing the photoperiod and thus suppressing gametogenesis in the green sea urchin. Gonads in such sea urchins should contain principally or exclusively nutritive phagocytes and should be of superior size, taste, texture and firmness. Ovaries and testes containing fewer gametes relative to NP are actually preferred in most cultures that each sea urchin gonads and such conditions exist naturally in sea urchins during the summer.

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ABIOTIC AND BIOTIC THREATS TO SEA URCHIN HEALTH: WHAT PRICE CULTURE?

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Understanding how environmental factors affect urchin health will be important to ensure a consistent supply of urchins for human food resources; however, unlike other fisheries, our knowledge of their susceptibility to toxicants and pathogens has been limited and based on sporadic efforts. Sea urchin gamete fertilization and embryo survival have been developed into a standardized test to evaluate the toxicity of coastal sediments and used in research on mechanisms of toxicity suggesting that toxicants entering nearshore waters or culture facilities might have adverse effects. Mass mortalities of adult urchin populations have occurred because of bacterial, protozoal, or unknown pathogens, which might be influenced by temperature, water movement, and population densities, but the role of toxicants is unknown. No cases of neoplasia in urchins have been submitted to the Registry of Tumors in Lower Animals. Multidisciplinary approaches must be consistently applied in monitoring urchin health, with more research on the immune system and impacts of environmental factors. Funds to support these studies must be factored into the fishery equation.

GROWTH RESPONSE AND ACCLIMATION OF GREEN SEA URCHINS TO FLUCTUATING SALINITY.

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Echinoderms are osmoconformers—external salinity determines internal osmotic concentrations. Green sea urchins are exceptional among echinoderms because they occur in brackish-water environments. These habitats are among the most productive fishing grounds and populations in these areas often have some of the largest individuals. The states of Maine and New Hampshire have granted sea urchin lease sites in these habitats and leaseholders plan to seed these areas with hatchery-reared juveniles. However, previous workers have documented that larger sea urchins can tolerate bouts of low salinity of both greater intensity and duration than smaller individuals. I tested the growth response of juvenile sea urchins to periodic bouts of sub-lethal hyposaline conditions. I collected samples from an area not exposed to low salinity and divided them into two groups: treatment (bouts of hyposaline conditions) and control. After an initial acclimation period, the treatment group displayed the same growth rate as the control group despite repeated exposures to hyposaline conditions. The acclimation period was equivalent to 10 days of growth. These experiments demonstrate the importance and the feasibility of acclimating juveniles to hyposaline conditions before releasing them into lease sites.

URCHIN HEALTH ISSUES IN MAINE.

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Maine urchin harvesters have reported mortalities of green sea urchins, *Strongylocentrotus droebachiensis*, over the last three years. The urchin mortalities have been observed in at least 19 locations along the coast of Maine. Gross examination of urchins taken from areas of mortality revealed a progression of lesions ranging from the presence of an inflammatory exudates, loss of tube feet and spines to complete denudement of the urchin test. Examination of wet mount or squash preparations of coelomic fluid, organ samples, and culture failed to indicate the presence of *Paramoeba invadens*, the agent responsible for recurring mortalities of urchins in Nova Scotia. Elevated temperature, mortality from draggers, and crashes of significant phytoplankton blooms have each been implicated as a cause of mortality, but no consistent etiology has yet been determined for the outbreaks. Attempts at restoration by transplanting juvenile urchins or reseeding beds have likewise met with limited success. Given the multifactorial nature of aquatic disease, the occurrence of disease outbreaks in a fishery experiencing a significant declines in harvest landings, does not bode well for the sustainability of the population. Rather than attributing urchin mortalities to "natural causes", there is clear need to establish a mechanism for disease surveillance and control. Unfortunately, there appears to be a surprising lack of information about the pathobiology of sea urchins, despite the considerable use of sea urchins as a laboratory model.

LOBSTER BIOLOGY AND FISHERIES

THE EFFECTS OF HABITAT ADDITION AND STOCK ENHANCEMENT FOR AMERICAN LOBSTERS, *HOMARUS AMERICANUS* ON ARTIFICIAL REEFS IN RHODE ISLAND.

Kathleen M. Castro* and **J. Stanley Cobb,** University of Rhode Island, Kingston, RI 02881; **Richard Wahle,** Bigelow Laboratory for Ocean Sciences, Boothbay Harbor, ME 04575; **John Catena,** Restoration Center, National Marine Fisheries Service, Gloucester, MA 01930-2298.

Six experimental artificial reefs were established in Narragansett Bay, RI in February 1997. These have been monitored according to a before-after-control-impact design by means of visual surveys, traps, tag-recapture, photoquadrats and airlift sampling for 6 years. Juvenile and adult lobster density at the reef site increased from near zero to >1 lobster/m^2, significantly higher than before reef placement. Settlement of young-of-the-year lobsters significantly increased. We used microwire tags to mark hatchery reared lobsters and released them as 5th-6th stage lobsters onto three of the reefs at a density of 4.5/m^2 for three years. However, subsequent density of young-of-the-year lobsters on the enhanced treatment sites was not significantly different from the non-enhanced sites. Despite intensive sampling, only three of these tagged lobsters were recovered after placement. Placement methods may contribute to survival possibilities. Field and lab observations confirm behavioral differences between 4th stage and 5th stage hatchery-reared lobsters as compared to field-caught lobsters. Predation rates in the lab were significantly higher for the 4th stage hatchery-reared lobsters raised in the URI facility compared to the RI field-caught lobsters and hatchery-reared lobsters obtained from Maine.

CHARACTERIZATION OF MICROBIAL ASSEMBLAGIES INVOLVED IN THE DEVELOPMENT OF SHELL DISEASE IN THE AMERICAN LOBSTER, *HOMARUS AMERICANUS.*

Andrei Y. Chistoserdov* and **Feliza Mirasol,** Marine Sciences Research Center, SUNY at Stony Brook, Stony Brook, NY 11720; **Roxanna Smolowitz,** Marine Biological Laboratory, Woods Hole, MA 02543.

A combination of culture-based and molecular techniques was used to study the consortia of bacteria isolated from shell lesions and hemolymph of lobsters. Diseased lobsters used in this study were collected in the Eastern Long Island Sound, NY (ELIS), Buzzards Bay and Vineyard Sound, MA. Preliminary denaturing gradient gel electrophoresis (DGGE) data indicate that up to five independent phylotypes of bacteria are present in lobster lesions. At least two of them were found in all lobsters from ELIS. This is consistent with the five to six morphotypes of bacteria which were cultured on a marine agar. All isolated bacteria were either aerobic or facultatively anaerobic. No strictly anaerobic or microaerophilic bacteria were isolated from lesions. Only two different types of fastidious chitinolytic bacteria were isolated on a medium containing chitin. One of the two is a motile bacterium forming bright yellow colonies, which was present in all analyzed lobsters (from Buzzards Bay, Vineyard Sound and ELIS). The second is a bacterium forming white colonies. It was isolated from only two Buzzards Bay lobsters. No correlation has been found between the presence or severity of shell disease and the presence of bacteria in hemolymph.

CHANGES IN LOBSTER POPULATIONS IN NARRAGANSETT BAY, RHODE ISLAND, 1959–2000.

J. S. Cobb and **M. Clancy,** Department of Biological Sciences, University of Rhode Island, Kingston, RI 02881.

The past 35 years have seen an extraordinary increase in the catch of lobster (*Homarus americanus*) along the northeast coast of North America. Rhode Island landings have tripled since the late 1960s. We explored a fishery-independent data set of lobster abundance developed from weekly research otter trawls made at two locations in Narragansett Bay, RI by the University of Rhode Island on a continuing basis since 1959. The patterns of abundance over time differed substantially at the two stations; one of the stations was highly correlated with the NMFS catch data set for the same time period. Water temperature, which has increased steadily since 1960, was significantly correlated with commercial landings and with abundance at one of the stations. Lobster abundance at both stations and the commercial landings were significantly correlated with the NAO winter index. At one of the stations the timing of greatest abundance in the trawl samples shifted to earlier in the summer. It seems likely that there have been effects of climate on lobster abundance, however the effect appears to have acted differentially at the two stations in Narragansett Bay.

THE ROLE OF *VIBRIO FLUVIALIS* AND OTHER BACTERIAL SPECIES IN LOBSTER MORTALITIES IN MAINE.

Cem Giray* and **Deborah A. Bouchard,** Micro Technologies, Inc., 41 Main Street, Richmond, ME 04357.

Weak lobsters (*Homarus americanus*) and mortalities have been reported by pounds in Maine for several years with *Vibrio fluvialis* implicated as the etiological agent responsible. Bacterial screening of moribund lobsters was performed by Micro Technologies, Inc. through a contract with the Maine Department of

Marine Resources. The majority of isolates collected were putatively identified as *Hyphomicrobium indicum* through 16S rRNA sequencing while *V. fluvialis* was not isolated from any of the lobsters. Further characterization demonstrated that *H. indicum* could grow at a temperature range from 3°C to a higher limit between 16–20°C while *V. fluvialis* grew best between 16–30°C with minimal growth at 8°C. The virulence of *H. indicum* and *V. fluvialis* was tested by injection into lobster hemolymph at various dosages. Both isolates resulted in 75–100% mortality within 24 hours when injected at 10^8 colony-forming units (CFU), but only *H. indicum* resulted in mortalities (12.5%) when injected at 10^6 CFU/lobster. *V. fischeri* was used as a negative control while *Listonella anguillarum* and *Aerococcus viridans*, both indicated as lobster pathogens, were utilized as positive controls. The identification of *H. indicum* as the primary isolate collected from moribund lobsters and the observation of lobster mortalities mainly during lowered water temperatures suggest that the isolate responsible could be *H. indicum* but not *V. fluvialis*.

SHELL DISEASE PREVALENCE AND SEVERITY IN OFFSHORE AMERICAN LOBSTER POPULATIONS. **Diane Kapareiko, Richard A. Robohm, John J. Ziskowski, George R. Sennefelder,** and **Anthony Calabrese,** National Marine Fisheries Service, Milford Laboratory, 212 Rogers Avenue, Milford, CT 06460.

During the period 1990–1992, 15,004 lobster from 146 commercial catches at nine offshore canyon sites surrounding the 106-Mile Sewage-Sludge Disposal Site were examined for signs of shell disease. Overall, 1,184 lobster (7.9%) had lesions. Females were more affected by this condition than males. Shell-lesion occurrence was independent of carapace length (CL), but strongly related to location (proximity to the 106-Mile Dumpsite as well as to the 12-Mile Dumpsite). Data collection for the shell-disease study included not only evaluation of presence or absence of disease, but also measurements of lesion size and carapace length. This was done in anticipation of developing a method that would determine the percentage of total surface area of each lobster affected by shell disease; this percentage is the basis of a Disease Severity Index (DSI). An estimate of lobster surface area could be derived mathematically from carapace length, for both male and female lobster in our database, using the formula $y = 1.1034 + 1.9677 * \log(CL)$. The percentage of surface area covered by shell lesions, multiplied by 10^3, provides a DSI that may allow better statistical correlations between mean disease severity and site of lobster collection.

Regression Tree analysis of this multi-variate database indicated that, unlike prevalence, the most important variable affecting the DSI was carapace length. Overall, DSI's for smaller lobsters ($CL < 95$), were significantly higher ($p < 0.02$) than larger lobsters, regardless of sex or location. Small females had significantly higher DSI's ($p < 0.04$) than large females. Males showed no significant differences when similarly compared. A complete nonparametric regression analysis of our DSI in relation to proximity to the 106-Mile Site, may indicate whether sewage sludge dumping had any effect on the severity of shell disease lesions in offshore American lobster populations.

DEPTH-RELATED PREDATION PRESSURE ON LARVAL LOBSTERS (*HOMARUS AMERICANUS*) PRIOR TO SETTLEMENT. **Wendy Norden** and **J. Stanley Cobb,** University of Rhode Island, Kingston, RI 02880.

Prior to settlement, lobster postlarvae swim near the surface of the water for several days. Settlement behavior begins between two and six days after metamorphosis from the third larval stage. During settlement, postlarvae dive from the surface to the benthos to seek out suitable habitat. This potentially exposes the postlarvae to a variety of predators not present at the surface. Understanding where in the water column predation pressure is the greatest will help in understanding behavioral strategies used by lobster postlarvae when seeking appropriate habitat. We tethered postlarvae at various depths to experimentally test the potential predation pressure throughout the water column. Tether lines were deployed over mud and cobble substrate types to look at the difference in predation pressure as it relates to habitat with four depths (0.5m, 1.5m, 2.5m, 4m) above substrate. Higher predation occurred over cobble substrate than over mud. Most of the predation occurred within 1.5m of the bottom and was probably primarily due to the cunner, *Tautogolabrus adspersus*. Laboratory observations with epibenthic fish species confirmed their ability to consumer postlarvae.

THE EFFECT OF TEMPERATURE ON SEXUAL MATURITY IN THE FEMALE LOBSTER, *HOMARUS AMERICANUS*. **Susan A. Little*** and **Win Watson,** University of New Hampshire, Zoology Department, Durham, NH 03824; **Bonnie Spinazzola,** Atlantic Offshore Lobstermen's Association, 114 Adams Road, Candia, NH 03034.

The purpose of this study was to determine if female American lobsters (*Homarus americanus*) inhabiting areas with dissimilar annual temperature profiles (measured in degree days >10°C), reached sexual maturity at different sizes. We compared the size at maturity of lobsters captured in 3 different offshore locations (two southern and one northern), 1 inshore site (Isles of Shoals, NH) and one estuarine site (Great Bay Estuary, NH). Lobsters inhabiting the Great Bay Estuary experienced the warmest temperatures (1320 degree-days), followed by the southern offshore sites (876; 906), the northern offshore site (669), and the Isles of Shoals (416). Lobsters that migrate could gain a considerable thermal advantage. For example, southern offshore lobsters would increase their degree-days from 216 to 906. For this reason temperature exposure for the offshore lobsters was calculated assuming a seasonal migration. The maturity of female lobsters was determined using a combination of abdominal width/carapace length ratios, cement gland stages, and egg stages. As expected, lobsters that experienced the most degree-days above 10°C reached sexual maturity at smaller sizes. The size at which 50% of the lobsters were mature was 84 mm CL for the Great Bay estuary (n = 71), <83 mm CL for the two southern offshore sites (n = 80 lobsters), 91 mm CL for the northern offshore site (n = 40), and 89 mm CL for the Isle of Shoals (n = 33). This difference in size at maturity was also manifested in the average size, and range of sizes of berried females captured in each location. These data suggest that regional differences in size at maturity should be taken into account when managing the fishery. However, it is important to fully understand all the behavioral, developmental and oceanographic processes that influence size at maturity before implementing any new regulations. This project was funded by the Northeast Consortium.

ENVIRONMENTAL MONITORS ON LOBSTER TRAPS.

James Manning, Northeast Fisheries Science Center, Woods Hole, MA, **Bonnie Spinazzola,** Atlantic Offshore Lobster Association; **Patrice Farrey,** Maine Lobstermen Association; **David Casoni,** Mass Lobstermen Association; **Clare Grindal,** Downeast Lobstermen Association.

Beginning in early 2001, electronic temperature probes were distributed to over 70 New England lobstermen by the four largest associations. These units are recording hourly temperatures at fixed locations throughout the Gulf of Maine including many offshore canyons sites. The objective is to occupy these same locations year-after-year for documenting interannual variability of the deeper bottom water. Phase II of the project, just getting underway in 2002, will deploy several salinity sensors around the region. The primary objective is to characterize the water mass and to assess the influence of remote source water. Participating lobsteren are asked to record catch wherever probes are attached. Understanding the biological significance of the physical variability is a secondary but a potentially worthwhile bi-product of the study. Details are posted on the project website http://www.nefsc.nmfs.gov/~jmanning/emolt.html.

LESIONS ASSOCIATED WITH RECENT EPIZOOTIC SHELL DISEASE IN *HOMARUS AMERICANUS* ON THE NORTHEAST COAST.

Roxanna Smolowitz,* Andrea Hsu, and **Erin Summers,** MBL, 7 MBL St., Woods Hole, MA 02543; **Andrei Christoserdov,** Marine Sciences Research Center, SUNY, Stony Brook, NY 11794.

Shell disease is a commonly recognized problem in lobsters held in impoundments during winter months. However, recently, the economically devastating disease has reached high proportions in free-living populations along the coast from Eastern Long Island Sound, New York, to the Vineyard Sound, Massachusetts. The disease is characterized by multifocal to confluent, shallow to deep erosions, primarily of the dorsal carapace, often resulting in thin, easily compressed shells and, rarely, ulcerations. Grossly, lesions usually show marked melanization, but no obvious pathogen.

Histopathologically, carapace erosions are of variable depth; but deeply extensive erosions are common. Carapace matrix in the erosions is usually missing, but in some instances, unlike lesions noted in impoundment disease, pillars of carapace matrix still remain attached in the eroded tissue. Inflammation is composed of increased numbers of hemocytes in the underlying connective tissues and accumulations of usually necrotic hemocytes between layers of carapace. A second type of inflammation consists of variable amounts of carapace proliferation produced by the intact hyperplastic epithelium underlying the eroded site. Various organisms are identified in the erosions, but the predominate organism found at the interface of necrotic and live shell are bacteria. Work is underway to identify the pathogenic bacteria at the lesion interface.

WHAT CAN DATA FROM INDUSTRY CONDUCTED TAG-RECAPTURE PROGRAMS TELL US? A STUDY OF THE AMERICAN LOBSTER (*HOMARUS AMERICANUS*).

Barbara A. Somers,* and **Kathleen M. Castro,** University of Rhode Island, Fisheries Center, East Farm, Kingston, RI 02881; **John Sorlien,** Rhode Island Lobstermen's Association, Box 421, Wakefield, RI 02880; **Tom Angell,** Department of Environmental Management, Coastal Fisheries Lab, Wakefield, RI 02880.

Tag-recapture data for the American lobster (*Homarus americanus*) compiled from a two year experimental tag-recapture program done by the Rhode Island Lobstermen's Association (RILA) in cooperation with Rhode Island Sea Grant and the Rhode Island DEM is being used to determine migration and life-history characteristics for the Southern New England lobster stocks. Twenty boats participated in the program; number of lobsters tagged and recaptured varied among fishing areas, taggers and recapture reporters. A total of 11,964 lobsters were tagged and released over a 16-month period. The majority of the lobsters were tagged in August and September of 2000 and the months with the highest recapture rates were July, August and September of 2000 and 2001. 982 lobsters have been recaptured to date giving a recapture rate of 8.2%. Recaptures were received by phone and by mail with a total of 91 fishermen returning tags. Biological data estimating growth, egg frequency, shell disease and movement will be reported as well as perceptions about cooperative research from the project participants.

OYSTER REEFS AND RESTORATION

A CHARACTERIZATION OF IDEAL HABITAT STRUCTURE FOR THE STRIPED BLENNY *CHASMODES BOSQUIANUS*. **Elizabeth M. Flynn*** and **Kennedy T. Paynter, Jr.**, Department of Biology, University of Maryland, College Park, MD 20742.

Historically, oyster reefs dominated the trophic interactions of the Chesapeake Bay. Besides improving water quality and forming an integral link in the food web, oysters provided the physical structure that facilitated the development of a complex benthic community. In the past 150 years, disease, overfishing, and pollution have decimated the oyster population and destructive harvesting methods have reduced complex, high relief reef structures to flat "footprint" bars. The impact of this systemic loss of vital habitat on the small demersal reef fish has gone relatively unstudied. The objective of this study is to separate and identify the structural components of shell aggregations that constitute desirable habitat for the striped blenny, *Chasmodes bosquianus*.

Disarticulated oyster shells were arranged in a 30-gallon glass aquariumin various vertical, horizontal and angled arrays to tested for spatial preferences. Individuals were released into the tank and remotely observed via overhead video for one hour. Mean perch duration, perch frequency, minimum and maximum perch durations, and total duration of association with the shell arrays were analyzed. The results indicate that *C. bosquianus* prefer 1–2 cm NN distances. *C. bosquianus* similarly preferred 60 degree arrays as compared to 90 degreeswith regard to total association, maximum perch duration, and frequency. The short-term behavioral patterns analyzed here suggest that purely structural elements of oyster reefs are critical factors influencing demersal fish population distributions.

A COMPARISON OF ACOUSTIC TECHNIQUES, VIDEOGRAPHY, AND QUADRAT SAMPLING FOR CHARACTERIZING SUBTIDAL OYSTER REEFS. **Raymond Grizzle,*** **Larry Ward**, and **Jamie Adams**, Jackson Estuarine Laboratory, University of New Hampshire, Durham, NH 03824; **Semme Dijkstra**, Center for Coastal & Ocean Mapping, University of New Hampshire; **John Nelson**, New Hampshire Fish & Game Department, Marine Fisheries Division, Durham, NH 03824.

Acoustic techniques, videography, and quadrat sampling were used to characterize several subtidal oyster reefs in the Great Bay Estuary in New Hampshire and to compare their effectiveness, with the long-term goal being a general protocol for reef mapping and monitoring. The acoustic techniques consisted of single beam, multibeam, and sidescan sonar. Preliminary analysis of the acoustics data indicated that reef boundaries could readily be mapped. Videography was conducted by systematically imaging each of 40 sampling cells in a grid covering the approximate area of each reef. A single drop was made in each cell and a 5 to 10-s recording made of a 0.25 m^2 area. A still image was produced for each of the 40 cells and all were combined into a montage that revealed the approximate boundaries of the reef. Five to ten cells on each reef were randomly chosen and sampled by divers using a 0.25 m^2 quadrat; all live oysters were measured (shell height) to nearest millimeter using calipers. Oyster counts were also made directly from each video image and compared to the quadrat data from the same photo; preliminary analyses showed good correlations between these counts. Overall, our results to date indicate that acoustic techniques generally can delimit the boundaries of oyster reefs, as has been demonstrated in other studies. Their potential for inferring other reef characteristics (e.g., oyster densities), however, is being assessed. Thus far, the major finding is that videography may be a powerful and relatively inexpensive tool for detailed reef mapping, including inferring oyster densities and perhaps other characteristics.

PREVALENCE OF ENTERIC MICROORGANISMS IN THE EASTERN OYSTER (*CRASSOSTREA VIRGINICA*) AND THEIR OVERLYING WATERS AT REPRESENTATIVE SITES OF AN OYSTER GARDENING PROGRAM IN MOBILE BAY, ALABAMA. **Kimberly A. Hamilton,*** **D. LaDon Swann**, and **William Burkhardt, III**, Department of Fisheries and Allied Aquacultures, Auburn University, Auburn, AL 36849 and U.S. Food and Drug Administration, 1 Iberville Dr., Dauphin Island, AL 36528-0158.

An oyster gardening program for the restoration of the eastern oyster, *Crassostrea virginica*, in Mobile Bay, AL began in May 2001. Thirty study locations were established in Mobile and Baldwin counties along Mobile Bay. Of these sites three from Mobile county and two from Baldwin county for a total of five sites were purposefully selected in July 2001 to determine if a relationship exists between enteric microorganisms in oysters and their overlying waters at different geographic locations along Mobile Bay. The oysters were suspended under piers inside Eastfields floats (surface area of 0.35 m^2) which positioned the oysters directly below the surface of the water. Oyster and water samples were taken from each site on a monthly basis and levels of fecal coliforms, *Escherichia coli*, and male-specific bacteriophage (an enteric viral simulant) were quantified using pre-established protocols.

The results have shown intermittent high levels of coliforms in the shellfish, but levels in their overlying waters were low. The bacteriophage levels have been consistently lower than the level of detection; thus indicating that human wastewater treatment source is not a likely cause of fecal coliforms. A high number of birds and their droppings have been observed at some of the study sites from which the Eastfields floats are suspended. These results may have implications for future site selection of the gardening program. Findings from these analyses will be used to inform local communities and regulatory agencies of any impacts water quality may have on shellfish quality.

USE AND VALUE OF OYSTER REEFS AMONG RECREATIONAL FISHERMEN IN LOUISIANA.

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Oyster reefs serve a wide variety of purposes. Until recently, the majority of research has focused on the relationship between the quantity and quality of reefs and commercial harvests derived therefrom. Recreational fishermen in Louisiana, however, also make extensive use of oyster reefs. This study serves two purposes. First, it provides an examination of catch rates and species diversity among inshore recreational fishermen fishing over oyster reefs in comparison to inshore fishermen not fishing over oyster reefs. Second, it provides an estimate of 'willingness to pay' among individual recreational fishermen for the privilege of fishing over oyster reefs. This study employed a telephone survey of Louisiana anglers to generate an estimate of the value of oyster reefs in Louisiana to the recreational fishing sector. Overall, the value is found to be significant, gives a justification for maintaining oyster reefs in addition to commercial harvest.

RELAYING AS A METHOD TO REMOVE HOOKED MUSSELS FROM OYSTERS PRIOR TO REHARVEST FOR SALE.

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The hooked mussel, *Ischadium recurvum*, is considered a native species of the northern Gulf of Mexico, but introduced in the Chesapeake. It is a small bivalve that can be found in great numbers attached to subtidal oysters located in low salinity environments. In the northern Gulf of Mexico, oystermen have traditionally relayed mussel-laden oysters to higher salinity waters with the expected results of killing them, and thereby removing them, before reharvest of the cleaned oysters for sale.

Results of laboratory, small-scale field and two commercial-scale field experiments indicate that removal of mussels due to transplanting to higher salinity waters is less a function of physiological salinity stress and more a function of the transplanting process itself. The hooked mussels were crushed, dislodged or stressed to the point of permanent gaping during the commercial-scale relaying operations. This resulted in an immediate 32–34% mussel mortality, with a corresponding negligible oyster mortality. The dead and dying mussels attracted predators, such as the blue crab, *Callinectes sapidus*, and the southern oyster drill, *Stramonita haemastoma*, which resulted in continued mussel losses in the ensuing weeks.

In the more coastal location, where salinity is relatively high, mussel-fouled oysters were cleaned by the third week after a summer transplant. The dilemma is that oysters in the higher salinity waters may also become more vulnerable to predators and the oysterman must consider reharvest in a short time. In the more inland location, where salinity is more intermediate, and perhaps with less abundant oyster predators present, mussel removal was a longer process with 76% removed by the fifth week. Results of these experiments strongly suggest that salinity is a key element in the removal of mussels from oysters, but more in the role of a habitat response and less as a physiological response to salinity.

MODELING THE INFLUENCE OF FILTRATION BY OYSTER STOCKS ON TURBIDITY AND SEAGRASS GROWTH.

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Chesapeake Bay has undergone severe ecological changes. Oysters are almost completely absent due to overharvest and diseases and seagrass beds either are in decline or have disappeared due to high water turbidity reducing light availability. Oyster reefs and seagrass beds tended to improve water quality by actively filtering particles out of the water, reducing wave energy, and minimizing sediment resuspension. In part because of the loss of these ecosystem functions water quality is poor and seagrass restoration not always successful. We hypothesized that the reestablishment of oyster stocks, either via restoration of reefs or aquaculture, could benefit seagrass populations. Results from laboratory experiments and field studies were used to paramterize a model predicting the extent to which oysters and seagrasses can affect water quality and enhance the chances of survival and expansion of seagrasses. Oysters were capable of increasing light penetration due to high summer filtration rates. The ability of seagrass beds to minimize resuspension was a function of water depth and their capacity to attenuate waves. Large reproductive seagrass plants that occupied the entire water column were more effective at minimizing resuspension than small vegetative plants. Our model results suggest that increasing oyster stocks can dramatically enhance light penetration and thereby increase the area of Bay bottom where seagrasses can grow.

THE EFFECT OF STOCKING DENSITY ON BENTHIC COMMUNITY DEVELOPMENT AND SHELL HEIGHT IN THE EASTERN OYSTER, *CRASSOSTREA VIRGINICA*.

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The eastern oyster, *Crassostrea virginica*, is a gregarious, reef-forming organism. Oyster populations that once dominated the Chesapeake have declined significantly and interest has recently arisen to restore the economic and ecological benefits of native oyster populations. Understanding the ecological importance of oysters and oyster reefs is critical to the restoration of the estuary's ecosystem as a whole. Oyster densities on most Maryland reefs are very low (about 3 oysters/m^2), however, natural reefs formed in other areas are comprised of high densities of oysters (>500/m^2). In order to maximize the effectiveness of oyster restoration, it is important to determine how oyster density may affect oyster growth, parasite prevalence and the formation of reef habitat utilized by the benthic community.

In the fall of 1999, twelve 0.2-acre experimental plots were constructed in the Patuxent River by placing fossil oyster shell on a barren natural oyster bar. The plots were assigned one of four treatments, zero, 124, 247, 494 oysters/m^2, in a randomized design. Oyster growth was 0.12 (± 0.004 SEM) mm/day for the 2000 season and slowed to 0.07 (± 0.006) mm/day in 2001. The data did not show any effect in shell height due to density of oysters. Colonization of the oyster reefs with fouling organisms was related to density of oysters. In summer and fall of 2000, barnacle density declined with increasing oyster density.

EVALUATING THE CONTRIBUTION OF COMMERCIAL OYSTER AQUACULTURE TO RECRUITMENT.

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Commercial oyster (*Crassostrea virginica*) aquaculture operations concentrate large numbers of potentially reproductive animals. Such aggregations may function to produce large numbers of larvae that subsequently recruit to natural beds. Anecdotal observations have suggested the enhancement of recruitment in the vicinity of commercial aquaculture operations; however, confirmation of such an effect has generally not been possible, as new recruits derived from the cultured stock generally cannot be distinguished from those resulting from the spawning of wild oysters. Recent stocking of commercial leases in North Carolina with oysters produced in a hatchery in Louisiana has provided an opportunity to evaluate the potential for the enhancement of local recruitment due to the spawning of cultured stock. Oysters from the Gulf coast and Atlantic coast have been previously shown to exhibit genetic differences in mitochondrial DNA (mtDNA) haplotype. PCR (polymerase chain reaction) amplification and DNA sequencing of 409 base pairs of the 16s ribosomal gene are being used to screen recent recruits collected from 7 natural beds surrounding 1.5 acres of leased bottom (stocked with ~70, 000 Gulf oysters), as well as a sample of the culture stock. Sequence data will be analyzed to determine what proportion of recent settlers exhibited mtDNA haplotypes consistent with the hypothesis that they were produced by the spawning activity of the culture stock. Preliminary analyses revealed some recruits exhibit mtDNA haplotypes identical to those exhibited by the culture stock, suggesting that there is some local recruitment resulting from reproduction of commercial aquaculture stock.

RECRUITMENT OF THE OYSTER *CRASSOSTREA VIRGINCA* ON INTERTIDAL REEFS IN AREAS WITH INTENSE BOATING ACTIVITY IN THE INDIAN RIVER LAGOON, FLORIDA.

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Productivity, diversity and survival of estuaries are threatened by explosive coastal population growth and associated recreational activities. One major area of recreational growth has been the number of people motoring in small pleasure craft at high rates of speed. In counties bordering Mosquito Lagoon (northernmost section of the Indian River Lagoon system, east coast of central Florida), there were 51,000 registered boaters in 1998. Numbers have increased 10% annually since 1986 and continue to grow. In areas of Mosquito Lagoon with intense boating activity, intertidal reefs of *Crassostrea virginica* with dead margins commonly occur. The dead margins consist of mounds of disarticulated shells. The cause(s) of the reef die-offs is unclear. However, the disarticulated shells may be reducing reef sustainability if these surfaces are unavailable for oyster recruitment. Recruitment trials were run on eight reefs (4 impacted, 4 healthy) in two eight-week trials in Summer 2001 and Winter 2001–2002. Sediment loads, temperature and water motion at all sites were monitored. In the Summer 2001 trial, no significant differences were found between or within sites for settlement or recruitment. However, temperatures reached over 40°C on portions of the dead reef. Data of this type is needed to identify causes of reef declines, habitat-specific management protocols and appropriate restoration techniques.

OFFSHORE FISHERIES

INTEGRATING VESSEL TRACKING, CATCH DATA, AND DEPLETION MODELS TO ESTIMATE COMMERCIAL SCALLOP DREDGE EFFICIENCY. Todd Gedamke* and **William DuPaul,** Virginia Institute of Marine Science, School of Marine Science, College of William and Mary, Gloucester Point, VA 23062.

In June of 1999, Georges Bank Closed Area II was opened to the United States sea scallop fleet after a five-year multi-species fishing closure. During the five-month opening, nearly six million pounds of scallop meats were harvested, catch-per-unit effort data were collected from over 1,000 commercial tows, and the fine-scale distribution of fishing effort was recorded hourly by mandatory vessel monitoring systems. A spatial analysis of both catch and effort data was performed to locate areas consistent with the DeLury model assumptions. Gear efficiency was estimated to be 45% utilizing a combined maximum likelihood analysis of CPUE declines in all suitable regions. An additional independent estimate of efficiency was generated from survey stations that were sampled before and after the opening. A kriging analysis was used to determine mean catch rates and the index-removal method was applied to compare the change in catch rates to the total landings reported for the opening. Dredge efficiency was estimated to be 54%.

The results of this study suggest that the 25% efficiency estimate used in calculations prior to the opening resulted in the overestimation of absolute biomass and the setting of a quota that exceeded target exploitation levels. In addition, the results of this study suggest that information from the vessel monitoring systems, now in use on many commercial fleets, can provide the fine scale spatial details necessary to successfully apply depletion models to open-ocean commercial fishing operations.

THE USE OF A 4-INCH (101 MM) SEA SCALLOP (*PLACOPECTEN MAGELLANICUS*) RING DREDGES IN THE CONTEXT OF AN AREA MANAGEMENT STRATEGY. Kevin D. Goff, William D. DuPaul,* and **David B. Rudders,** Virginia Institute of Marine Science, College of William and Mary, Gloucester Point, VA 23062.

Early attempts to manage the sea scallop (Placopecten magellanicus) fishery focused on establishing age-at-entry controls. Since 1994, the primary management strategy shifted to an effort control program and an increase in scallop dredge ring size to 3.5 inches (88 mm) from 3.0 inches (76 mm), with the intent of increasing yield per recruit.

Dramatic increases in scallop biomass in three areas of Georges Bank closed to mobile fishing gear in 1994 and two areas in the mid-Atlantic closed in 1998 to protect concentrations of small (<80 mm) scallops have stimulated interest in developing an area wide management strategy for sea scallops.

During commercial openings of these areas during 2000–2001, the performance of a 4-inch (101 mm) ring scallop dredge was evaluated against the standard 3.5-inch (88 mm) ring dredge. Detailed catch data from 208 tows on eight commercial trips showed a reduction in scallop harvest of up to 42.5% for scallops <90 mm and increases in harvest efficiency of up to 12.9% for scallops >115 mm. These results are closely related to the size frequency distribution of the scallop resource. When the size frequency distribution of the scallop population has a modal shell height of greater than 110 mm, the use of a 4-inch ring dredge may play a significant role in realizing the benefits of area-based management.

REBUILDING SEA SCALLOP (*PLACOPECTEN MAGELLANICUS*) STOCKS USING AREA CLOSURES AND ROTATIONAL FISHING. Deborah Hart* and **Paul Rago,** NOAA Fisheries, Northeast Fisheries Science Center, 166 Water St., Woods Hole, MA 02543.

In the seven years since the closure of three large areas on Georges Bank to trawling and dredging in December, 1994, sea scallop biomass on Georges Bank has increased by more than a factor of twenty. Limited fishing in the closed areas in 1999 and 2000 yielded about 11 million lbs of scallop meats while still allowing for increases in biomass in these areas. Dramatic increases in biomass have also been seen in the Mid-Atlantic Bight after two areas there were closed to scallop fishing for three years in 1998. These experiences indicate that area closures can be an effective way to rebuild shellfish stocks and alleviate growth overfishing. Strong recruitment observed since the closures suggests that the closed areas have also become important sources of scallop larva. Both theory and practice indicate that rotational management, where areas are successively closed and then opened to fishing, can increase both yield and spawning-stock biomass in the sea scallop fishery.

FLEET DYNAMICS OF THE ATLANTIC SEA SCALLOP FISHERY. Paul J. Rago and **Deborah R. Hart,** NOAA Fisheries, Northeast Fisheries Science Center, 166 Water St., Woods Hole, MA 02543.

The sea scallop (Placopecten magellanicus) supports the second most valuable commercial fishery in the northeast USA. Closure of large areas of Georges Bank to scallopers in 1994 not only resulted in rapid increases in scallop biomass but also altered the spatial distribution of fishing effort. Detailed electronic information on the hourly position of each vessel was used to study the behavior of the fishing fleet for the period 1998 to 2000. Additional information from at-sea observers corroborated the analyses of vessel monitoring data. Limited re-openings of the closure areas in 1999 and 2000 induced marked shifts in fishing effort. Fine-scale changes in fishing patterns can be related to the scallop density, size composition and economic value as well as the presence of bycatch species. The scallop fishery is conducted over an area of about 12,000 nm^2, but more than 75% of fishing activity is concentrated within an area of about 3,000 nm^2. When scallop density is low, however, fishing vessels disperse more widely. Such variations in the concentration of fishing activity have important implications for impacts on habitat and finfish bycatch and provides insights into management strategies for bivalve fisheries.

POPULATION STRUCTURE OF THE INDIAN SQUID, *LOLIGO DUVAUCELII* ORBIGNY, 1848, IN IRANIAN WATERS OF THE OMAN SEA. **Farhad Rajabipour,*** Offshore Fisheries Research Centre, Chabahar, Iran.

The population biology of the Indian squid *Loligo duvauceli* Orbigny, 1848 was described from monthly sampling, December 1997 to January 1999. Samples were caught as by-catch of bottom fishing trawlers from continental shelf area of Oman Sea at southeast of Iran. 1281 samples including 694 males and 587 females with the mean length of dorsal mantle and weight of 109.8 ± 34.95 mm and 48.32 ± 36.23 gr for males, and 110.6 ± 22.4 mm and 54.34 ± 28.35 gr for females, were collected. Maximum length (ML) of dorsal mantle in males and females was observed in January and June. Length-weight relationship was acquired. It is reversed at 72 mm length of dorsal mantle in logarithmic model. ML50 of males and females is 15.4 cm and 8.7 cm. There is no significant difference between females and males frequencies at the beginning of summer and ending of winter. NGI and GSI indices detected that the pick of reproduction occurs at the beginning of summer and winter. Males have three length classes, two recruitment peaks at beginning of summer (17.4%) and winter (82.6%), but females have only one length class and two recruitment peaks at beginning of winter (58.97%) and summer (41.3%); (t0 = 1.4/year). Age and mortality indices were measured. Catch rate of *L. duvauceli* in Iranian waters of Oman Sea was 0–100 kg/hour.

RECRUITMENT DYNAMICS OF NORTHERN SHRIMP (*PANDALUS BOREALIS*) IN THE GULF OF MAINE. **Anne Richards,* Michael Fogarty,** and **David Mountain,** NOAA Northeast Fisheries Science Center, Woods Hole, MA 02543; **Mirta Teichberg,** Boston University Marine Program, Woods Hole, MA 02543.

The western Gulf of Maine is the southern limit of distribution for northern shrimp *Pandalus borealis.* Previous studies showed that recruitment of northern shrimp in the Gulf of Maine is significantly affected by spring surface temperatures as well as spawning stock biomass. The temperature effect corresponds to the period of planktonic larval development. The purpose of this study was to extend these analyses by examining the influence of additional environmental factors (freshwater runoff, wind patterns, and climate indicators) on recruitment and to consider mechanisms for the spring surface temperature effect. Runoff and wind patterns strongly affect circulation within the western Gulf of Maine and thus may affect recruitment through effects on retention and/or transport of larvae. Results to date indicate no significant effect of mean river discharge, timing of peak discharge, other temperature effects, or the NAO winter index; however, the spring surface temperature effect continues to be significant. The match-mismatch hypothesis is a possible explanation for this effect, and we are evaluating the hypothesis by developing estimates of timing of onset of the spring phytoplankton bloom along with estimates of timing of the shrimp hatch.

DEEP SEA RED CRABS OFF SOUTHERN NEW ENGLAND: HAS THERE BEEN A FISHERY IMPACT ON THE POPULATION? **James R. Weinberg*** and **Charles Keith,** NMFS, NEFSC, 166 Water St., Woods Hole, MA 02543.

A directed trap fishery for the deep-sea red crab, *Chaceon* (formerly *Geryon) quinquedens* Smith, has taken place off the coast of southern New England since the mid-1970s. These slow-growing crabs occur from about 250–1000 m, and males grow larger than females. The National Marine Fisheries Service conducted a survey of population size-structure in 2001 and measured the relationship between carapace width and body weight. This information had not been updated since the late 1970s. Given that the fishery targeted large males, we examined whether there was a decline over time in the percentage of large males in the population and in the individual weights of large males. Overall, the percentage of large males in 2001 was lower than in 1974, whereas the fraction of large females either stayed the same or increased slightly during the same time period. Data collected between 1973 and 2001 suggested that male body weight, for a given body size, has declined over time. There was no trend over time in female body weight. Thus, compared to the 1970s, the population appears to have a lower percentage of large males in 2001, and these males have lower biomass for their carapace size. The results might be due to harvesting of large, heavy males over time; however, the surveys are too widely spaced in time, and too few in number, to draw strong conclusions about causality.

DISEASES OF SHELLFISH: CONTRIBUTED SESSION

MORTALITIES OF CULTURED ABALONE, *HALIOTIS IRIS*, INFECTED BY A NOVEL HAPLOSPORIDIAN. B. K. Diggles, P. M. Hine, V. L. Webb, and **E. W. Maas,** National Institute of Water and Atmospheric Research, Kilbirnie, Wellington, New Zealand; **J. Nichol** and **S. Wakefield,** School of Medicine, University of Otago, Wellington South, New Zealand; **R. Roberts,** Cawthron Institute, Nelson, New Zealand; **C. S. Friedman,** School of Aquatic and Fishery Sciences, University of Washington, Seattle, WA; **N. Cochennec-Laureau,** Laboratoire de Genetique et Pathologie, IFREMER, La Tremblade, France; **K. S. Reece** and **N. A. Stokes,*** Virginia Institute of Marine Science, Gloucester Point, VA.

Chronic mortalities of juvenile abalone, *Haliotis iris,* occurred in a commercial culture facility in New Zealand during the Austral summer and autumn of 2000 and 2001. Histopathology of moribund abalone showed heavy systemic infections of a uni- to multinucleate protozoan parasite associated with severe tissue damage. Heavily infected abalone exhibited lethargy, loss of righting reflex, and weak surface adherence. Mortality levels reached 90% in the affected raceways. The parasite was identified as a haplosporidian based on TEM and molecular analyses. Ultrastructural characteristics of the parasite included the presence of multinucleate plasmodia, lipid droplets, an anastomosing endoplasmic reticulum and the production of haplosporosome-like bodies from nuclear membrane-bound golgi apparatus that matured into haplosporosomes. Molecular confirmation of the TEM identification was accomplished by performing in situ hybridization (ISH) and by PCR-amplifying and sequencing the parasite's small subunit ribosomal RNA (SSU rRNA) gene. A DNA probe specific for several members of the haplosporidia exhibited hybridization to the plasmodia in ISH of infected animals. The SSU rRNA gene sequence was novel, but phylogenetic analyses strongly supported grouping this parasite with the haplosporidians. The parasite was at the base of the phylum Haplosporidia, ancestral to *Urosporidium crescens* and the *Haplosporidium, Minchinia,* and *Bonamia* species.

LOW SALINTY TOLERANCE IN MANILA CLAMS *VENERUPIS PHILIPPINARUM.* Ralph Elston, AquaTechnics/Pacific Shellfish Institute, PO Box 687, Carlsborg, WA 98324 USA; **Dan Cheney, Brian MacDonald,** and **Andrew Suhrbier,** Pacific Shellfish Institute, Olympia, WA.

The manila clam, *Venerupis philippinarum,* is the second most important product of molluscan aquaculture on the west coast of the United States and is grown on several other continents. We evaluated the tolerance and the low salinity response in this clam species using experimental exposures of 17 populations of manila clams. Ambient seawater (30 psu) and freshwater were first temperature conditioned, then mixed using aperture controlled flows into incremental salinity head tanks that supplied the test chambers. Water supplied to the test tanks was supplemented with algal feeds. Low salinity tolerance of clams was determined by performing exposures of up to four weeks in duration at constant salinities, followed by a recovery period. Clams were tested in salinity increments of 2.5 psu (= ppt).

Tolerance to low salinity events in clams is both a function of survival response (duration of shell closure) and the physiological adaptability of the clams to exist in low salinity environments. All individual clams from all sources were tolerant to 15 psu but populations showed variable proportions of individuals with tolerance to 12.5 psu and none were tolerant to long term exposure to 10 psu. Variable tolerance to 12.5 psu appears to be a function of the effectiveness and duration of the shell closure response. All clams could withstand 6 days at 5 psu, but complete group mortality occurred by 12 days at 5 psu. All clams could withstand 8 days exposure to 10 psu but complete group mortality occurred by 14 days at 10 psu. Little effect of temperature was found on the clams' tolerance to marginal low salinities. The low salinity exposures resulted in alterations in the digestive gland epithelium including loss of granulation, cellular swelling and cellular sloughing. We concluded that candidates to select for low salinity tolerance are available in most populations but some populations are enriched with individuals with tolerance to the marginal salinity (12.5 psu).

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A SPECIFIC PATHOGEN FREE CULTURE SYSTEM FOR *C. GIGAS* LARVAE AND SPAT. Sean E. Matson* and **Christopher Langdon,** Hatfield Marine Science Center, Oregon State University, Newport, OR 97365.

The Molluscan Broodstock Program (MBP), a selective breeding program for the Pacific oyster, *Crassostrea gigas,* uses a Specific Pathogen Free culture system for all production and maintenance of larvae, spat, broodstock and microalgae. This system is necessary to exclude infectious agents of *Haplosporidian costale* (Seaside Organism, SSO), which has been found in Pacific oysters grown in Yaquina Bay, Oregon, where the program draws its seawater, and for the safe outplanting of MBP spat in field test sites along the West coast (USA). All seawater entering MBP facilities is filtered through sand, diatomaceous earth, and a series of 20, 5, and 1 μm cartridge filters. Seawater to mass algal cultures and the nursery is also irradiated with UV-light at >30,000 micro-

Watts-sec/cm^2 (MWS) as a back-up precaution. Since the system's inception, no MBP spat have been identified as being contaminated with SSO, or any other infectious agent. A series of laboratory experiments was performed to assess the effects of UV water on larval growth and survival, spat growth and survival, and microalgal culture density. Experiments with oyster larvae indicated that both the micro-filtration system and UV water treatment had a significant negative effect on larval growth ($p = 0.0001$). A significant reduction in growth was evident at UV intensities as low as 10,000 MWS ($p < 0.05$). Methods that have significantly improved larval growth, survival, speed to metamorphosis and spat growth within the SPF culture system include substituting a 0.2μm filter and charcoal for a UV filter when rearing larvae, and the addition of calcium bentonite (2mg/ml/day) or calcium montmorillonite (5mg/ml/day) to larvae and spat cultures ($p < 0.05$).

INTRAMITOCHONDRIAL CRYSTALS WITHIN THE HAEMOCYTES OF MUSSELS (*MYTILUS EDULIS*) EXPERIENCING UNEXPLAINED MORTALITIES.

Gary R. Meyer,* and **Susan M. Bower,** Department of Fisheries and Oceans, Pacific Biological Station, Nanaimo, British Columbia, V9R 5K6, Canada.

Persistent mortality (having a cumulative total of about 75%) was observed between August 1999 and April 2000 amongst a stock of cultured blond mussels (*Mytilus edulis* selectively bred for their blond coloured shell trait) grown near Quadra Island, British Columbia, Canada. During this period, 7 samples of mussels ($n = 30$ per sample) were examined. Up to 27% had macroscopic lesions (orange pustules) and up to 50% had histopathology consisting of multifocal and or diffuse haemocyte infiltration that contained abundant phagocytic cells and necrotic cellular debris usually in the mantle/gonad and digestive gland. However in some instances, this pathology was also observed in the adductor muscle, gills, kidney or heart tissue. No etiological agent was evident. Intracellular mycobacteria-like organisms were observed within a few haemocytes in up to 20% of the mussels examined however their role in the disease remains unknown. During ultrastructural examinations, intramitochondrial crystalline arrays were commonly observed within necrotic or lysed haemocytes. These crystals were not observed "free" within the cytoplasm of affected cells and never observed in adjacent "healthy-looking" cells. The chemical composition and significance of these crystals is unknown. However, we speculate that they are likely composed of condensed protein. Similar crystals have been reported from the mitochondria of a wide variety of organisms and were usually affiliated with an abnormal situation such as disease, pathological process, or response to cell damage.

ENVIRONMENTAL DETECTION OF THE RICKETTSIALES-LIKE PROKARYOTE CAUSING WITHERING SYNDROME IN ABALONE.

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A gastrointestinal Rickettsiales-like prokaryote (WS-RLP) was recently identified as the causative agent of Withering Syndrome, a chronic, fatal disease of wild and cultured California abalone. Using a polymerase chain reaction (PCR) diagnostic method developed to detect the WS-RLP in abalone gut tissue samples, we have amplified WS-RLP DNA from necrotic tissue held at room temperature for up to 7 days, from samples fixed and embedded in paraffin, and from seed abalone as small as 2mm. A non-destructive WS-RLP sampling method is desired since both wild and farmed abalone are highly valued. We developed a method involving tangential flow filtration that concentrates particles > 0.1 μm from large volumes of seawater, allowing purification of DNA for PCR from 15+ liter samples. Although sample processing is laborious, using this method we have detected the pathogen DNA in effluent from abalone holding units in both experimental and culture facilities. The WS-RLP is present in feces from infected animals, and PCR using fecal material appears to be useful for efficiently detecting the WS-RLP in various stages of farm production. We are currently investigating an hypothesis that filter-feeding organisms concentrate the WS-RLP from endemic waters and can, thereby, be used to monitor WS-RLP presence in natural populations. Initial attempts to amplify WS-RLP DNA from gill or gut tissue of mussels (*Mytilus* spp.) being held with WS-RLP positive abalone have been unsuccessful. Yet it is clear that molecular-based tools will significantly aid management of this devastating disease. Supported, in part, by California Sea Grant College and the Marine Region, California Department of Fish and Game.

VIBRIO ASSOCIATED WITH JUVENILE OYSTER DURING SUMMER MORTALITY IN FRANCE.

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Among the hypotheses advanced to explain summer mortalities of oyster in France (Morest program), the bacterial disease appeared one of most relevant. Indeed, recent works of Lacoste (2001) and Waechter (thesis 2001) showed that of *Vibrio splendidus* (I and II) strains were able to kill the juvenile or adult oysters by bath or injection. To verify this hypothesis, oysters were sampled along the French coasts and analysed during mortality period. Only the half of moribund oysters were invaded by dominant bacteria. Haemolymph of other diseased oysters as well as healthy oysters was often colonised by more or less abundant and diversified bacterial flora. The isolation of a same dominant bacterial strain on several oysters of the same batch, occurred only in hatchery and on only one oyster park. The taxonomic studies (by ARDRA of gyrase B, and sequences of 16S DNAr) made it possible to classify in 3 species these opportunist bacteria: *V aestuarianus* (3 types), *V. splendidus* (II) and *V anguillarum*. Except V. *aestuarianus* (type II). Phenotypical characters of these strains were fickle and could not be used to identify the bacteria.

FURTHER MOLECULAR CHARACTERIZATION OF *PERKINSUS ANDREWSI* AND RELATED ISOLATES.

Wolf T. Pecher, José A. F. Robledo, Cathleen A. Coss, and **Gerardo R. Vasta,** COMB, UMBI, University of Maryland, Baltimore, MD 21202.

We previously described ultrastructure, behaviour in culture, and the rRNA locus of a *Perkinsus* species isolated from the baltic clam *Macoma balthica*. The morphological characterization did not reveal features remarkable enough to clearly indicate that the isolate was a distinct *Perkinsus* species (Coss et al. 2001a). However, the degree of difference of all rRNA genes and intergenic regions examined was comparable to or greater than differences between accepted *Perkinsus* species, which lead to its designation as *P. andrewsi* n. sp. (Coss et al 2001a, b). Here we report further molecular characterization of *P. andrewsi* and the characterization of an additional isolate from the hard clam *Mercenaria mercenaria*. Based on the molecular characterization both isolates are closely related. Their relationship with other recognized *Perkinsus* species will be discussed. [Supported by Grant No. NA06RG0101-5 from ODRP, NOAA, through the Maryland Sea Grant College, to GRV].

IN VITRO CULTURE OF *PERKINSUS ATLANTICUS* FROM CLAMS *TAPES DECUSSATUS:* CHARACTERIZATION OF THE rRNA GENE.

José A. F. Robledo* and **Gerardo R. Vasta,** COMB, UMBI, University of Maryland, Baltimore, MD 21202; **Patrícia A. Nunes** and **M. Leonor Cancela,** Molecular Biology and Biotechnology, CMS-CCMar, University Algarve-UCTRA, Campus Gambelas, 8000-810 Faro, Portugal.

Perkinsus atlanticus cultures were derived from fresh hemolymph and gills, and from tissues incubated in fluid thioglycollate medium from infected clams *Tapes decussatus* from Algarve (South of Portugal). *P. atlanticus* cultures were established in the medium developed and optimized for *P. marinus*. Proliferating cells were cloned by limiting dilution, and the species identity was established by applying two PCR-based *P. atlanticus*-specific diagnostic assays and by sequencing the RNA locus. We provide for first time the complete sequence of the rRNA locus of a *P. atlanticus* and compare by alignment with those partically characterize rRNA loci from other *Perkinsus* species. Actively growing cultures exhibited considerable size heterogeneity with a large proportion of clusters constituted by small cells. Behavior of *P. atlanticus* in culture resembles that of *P. marinus*. Optimization of the culture conditions for *P. atlanticus* is underway. Established clonal cultures will be useful for genetic characterization of this species, and to increase our understanding of its pathogenicity. [Supported by Grant NA06RG0101-5 from ODRP, NOAA, through the MD Sea Grant College, to GRV, and the Portuguese Government PERKLAM Program to MLC].

GENE DISCOVERY IN *PERKINSUS MARINUS* USING EXPRESSION SEQUENCE TAGS (EST).

José A. F. Robledo,* Eric J. Schott, Adam G. Marsh, and **Gerardo R. Vasta,** COMB, UMBI, University of Maryland, Baltimore, MD 21202.

Since the late 1940s the protistan parasite *Perkinsus marinus* has been associated with mass mortalities of the eastern oyster, *Crassostrea virginica*. Based on decades of research, several approaches have been proposed to control "Dermo" disease including early disease detection, selective breeding of disease-resistant oysters, and treatment with anti-parasitic agents. However, none of these approaches has proven effective. In recent years virtually all fields of biology have benefited from the information generated by genomic approaches. We are building a *P. marinus* EST database sequencing cDNAs from two *P. marinus* Lambda ZAP libraries constructed using *P. marinus* propagated in standard culture medium and in medium supplemented with *C. virginica* serum. From the first 300 ESTs two findings are evident: there are many expressed genes whose products are known to be involved in virulence of other parasites and there are notable differences in the classes of genes expressed in control versus serum-supplemented cultures. As the number of ESTs increases, so will prospects for identifying new targets for therapy and for understanding the biological basis of parasite virulence, infectivity, and pathology.

THE NRAMP GENE AND COMPETITION FOR AVAILABLE IRON BETWEEN *CRASSOSTREA VIRGINICA* AND *PERKINSUS MARINUS*.

José A. F. Robledo* and **Gerardo R. Vasta,** COMB, UMBI, University of Maryland, Baltimore, MD 21202.

In the past decades *Perkinsus marinus* has produced extensive damage to oyster bars with catastrophic consequences for shellfisheries and the health of coastal waters. Despite intense parasite pressure, no Dermo-resistant oysters have been identified so far in natural populations. The identification of genes that are directly linked to disease-resistance and assessment of their value as genetic markers, may facilitate the establishment of disease-resistant oyster strains. Most parasites have strong iron requirements and have developed efficient mechanisms for iron acquisition from their hosts. Reciprocally, most hosts have developed mechanisms to prevent pathogens from acquiring iron while maintaining availability for their own cells. Iron sequestration from the pathogen is also a non-specific host response to infection (nutritional immunity). The mammalian natural resistance-associated macrophage protein (Nramp) is a membrane transporter thought to be a determinant of resistance/susceptibility to intracellular pathogens. We have cloned Nramp from *C. virginica* and *P. marinus*, used them to screen genomic libraries, and examine their gene organization. The characterization of these genes and their products in both host and parasite will provide insight into their competition for iron, and yield information on the mechanisms underlying disease susceptibility [Supported by Grant No NA06RG0101-5 from ODRP, NOAA, through the Maryland Sea Grant College, to GRV].

CHARACTERISATION OF GENE EXPRESSION IN RESPONSE TO *PERKINSUS MARINUS* AND *HAPLOSPORIDIUM NELSONI* INFECTIONS IN THE EASTERN AND PACIFIC OYSTERS. Arnaud Tanguy,* Susan E. Ford, and **Ximing Guo,** Haskin Shellfish Research Laboratory, Institute of Coastal and Marine Sciences, Rutgers University, 6959 Miller Avenue, Port Norris, NJ 08349.

The eastern oyster *Crassostrea virginica* has two major diseases: Dermo caused by the parasite *Perkinsus marinus* and MSX by *Haplosporidium nelsoni*. Effects of infection in *C. virginica* range from reductions in condition index, hemolymph protein concentrations and lysozyme activity to decline in reproductive output, retarded growth and death. MSX-resistant strains of *C. virginica* have been developed at Rutgers University, and the Pacific oyster *C. gigas* appears to be resistant to both diseases. At present, however, we know little about molecular mechanisms of infection and host defense against the two parasites. We have begun a study searching for genes involved in host response to Dermo and MSX infections, using subtractive expression analyses. For Dermo, controlled artificial infections are conducted in both *C. virginica* and *C. gigas*. For MSX, infected wild oysters and uninfected resistant oysters, which were naturally exposed to the parasite, were used to construct the subtractive library. Nine subtractive libraries were made: six for Dermo and three for MSX exposures. About 70 clones were selected for sequencing from each library. Among the first batch of sequences analyzed, one-third matched with known genes in GenBank, and the other represent unknown genes. A detailed characterization of all sequences will be presented at the meeting.

BIVALVE HABITAT SUITABILITY AND THE ROLE OF BIVALVES IN ECOSYSTEMS

HOW COMMUNITY-BASED OYSTER RESTORATION CAN ENHANCE RESEARCH EFFORTS: EXAMPLES FROM SOUTH CAROLINA AND ALABAMA. Loren D. Coen,* Marine Resources Research Institute, SCDNR, 217 Fort Johnson Rd., Charleston, SC 29412; **Richard K. Wallace,** Auburn University Marine Extension and Research Center, 4170 Commanders Dr., Mobile, AL 36615; **Nancy Hadley,** Marine Resources Research Institute, SCDNR, 217 Fort Johnson Rd., Charleston, SC 29412.

In both South Carolina and Alabama, we have been utilizing community-based programs to construct and/or enhance larger scale oyster restoration efforts at selected sites using criteria based on science and best management policies. We are trying to use these sites as research platforms to test various restoration methodologies such as type of shell, alternative substrates, reef configurations, time/method of planting, and stabilizing meshes. In South Carolina we are actually placing 'habitat' (over 40 tons of shell in 2001), rather than oysters into tidal creeks across the state. Routine monitoring (chemical, physcial and biological parameters) is being conducted by both trained volunteers (students and adults) and researchers. In South Carolina, we have also meshed our reef monitoring efforts with HAB efforts through the NOS's South Carolina Phytoplankton Monitoring Network (SCPMN) and we are using the reefs also as 'living classrooms' for a variety of groups (e.g., one built adjacent to the SC Aquarium). In Alabama, oysters produced by volunteers using gardening techniques are being planted at relic reef sites that were already enhanced for sportfishing (as artificial reef fish habitat). This strategy creates opportunities for restoration research which can be supported by multiple interest groups. By constructing oyster reefs, we are improving the suitability of sites for oyster reestablishment, thereby improving habitat quality for reef residents and transient species.

OYSTER HABITAT SUITABILITY AS A COMPONENT OF RESOURCE MANAGEMENT. William S. Fisher,* U.S. Environmental Protection Agency, National Health and Ecological Effects Research Laboratory, Gulf Ecology Division, Gulf Breeze, FL 32561.

Economic and ecological issues have led resource managers to examine depletion of eastern oyster reefs along the U. S. Gulf of Mexico and Atlantic coasts. *Crassostrea virginica* is a lucrative commercial species (over $60M in 2000) that also supports ecosystem integrity by providing enhanced vigor, organization and resilience. These values are threatened by overfishing, habitat perturbation and land use changes that are also economically-driven, but diminish the survival of both oysters and reefs. Relevant and defensible scientific information is required to assess costs and benefits of healthy and productive oyster reefs and to establish management goals for their conservation, mitigation and restoration. Characterization of environmental conditions that support oyster survival and propagation is a requisite for this assessment. In this regard, habitat suitability indices (HSI) have been developed that can be used to identify potential sites for mitigation and restoration. However, greater attention is needed on conditions for spat settlement, a bottleneck life-stage for oyster populations. Although spat may settle on many substrates, success is greatest on bivalve shells and limited by even thin layers of sediment deposition. Better understanding of currents, turbidity, sediment deposition and other conditions that influence larval settling are needed to improve HSI evaluations and the potential for successful oyster reefs.

CONTINUOUS MONITORING OF PUMPING PRESSURE AND VALVE GAPE IN THE OYSTER *CRASSOSTREA VIRGINICA* IN RESPONSE TO CHANGES IN ENVIRONMENTAL PARAMETERS.

Dana M. Frank* and **J. Evan Ward,** University of Connecticut, Department of Marine Sciences, 1080 Shennecossett Road, Groton, CT 06340.

We have developed an optical biomonitor capable of continuous short and long-term recording of pumping pressure and valve gape in bivalve molluscs. 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 are able to examine the relationship between valve gape and pumping pressure, in response to measured changes in environmental parameters such as temperature, food availability, dissolved oxygen concentrations and current velocity. Laboratory trials are currently underway to establish responses to some of these variables with the goal of deploying the system, along with sensor arrays to measure these parameters, in the field. Using this method, we hope to expand the scope of our understanding about the compensatory responses of bivalve molluscs to changes in environmental parameters *in situ*. Additionally, it allows us to explore more thoroughly the mechanisms available to shellfish for controlling pumping rates. Results of laboratory trials have revealed multiple patterns of association between pumping and valve gape and have established that the system is able to record changes in these physiological parameters in real time.

A SIMPLIFIED SESTON UPTAKE MODEL FOR BIVALVES: PRELIMINARY FIELD TESTS.

Raymond Grizzle* and **Jennifer Greene,** University of New Hampshire, Jackson Estuarine Laboratory, Durham, NH 03824; **Mark Luckenbach,** Virginia Institute of Marine Science, Gloucester Point, VA 23062.

A spreadsheet-based model was developed to predict the percent of the total water column cleared of seston by suspension feeding bivalve molluscs on a daily basis:

$$\% \text{ Water Clearance} = (A \times B \times C)/(D \times E) \times 100$$

where A = mean bivalve density (# ind/m^2), B = mean individual clearance rate (m^3/individual/da), C = bottom area of reef (m^2), D = cross-sectional area of water column (m^2), and E = mean water flow speed (m/da). It is designed for use in estimating the impacts of shellfish reef restoration projects on water quality. The major simplifying assumption is a completely mixed water column, and mean clearance rates are based on literature values. We tested the model over a portion of one tidal cycle at two different sites, both blue mussel (*Mytilus edulis*) reefs, one natural and one constructed. On both occasions, upstream and downstream locations were sampled concurrently at 30-min intervals for 2 to 5 hr for in situ fluorometry and/or chlorophyll *a* from a fixed height ~10 cm above the bottom, and water depth and mid-depth water flow speed were measured. Replicate 0.16 m^2 quadrats were sampled on each reef, and mean density and size (shell length) of the live mussels were determined. The model predicted average clearance/seston uptake for the ambient water flow conditions, mussels densities, and other conditions measured on the constructed reef to be <2% of the total water flow. The field trial data corroborated the model predictions, showing no significant differences between mean upstream and downstream samples of chlorophyll *a*. For data from the natural reef, the model predicted a mean of 17.6% total clearance. During the field trial, measured chlorophyll concentrations were significantly lower downstream with a mean difference (representing seston uptake) of 16.3%. In situ fluorometry indicated an overall uptake of 28%. In conclusion, both preliminary tests showed good agreement with model predictions. Further testing is underway to determine the model's general applicability.

THE INFLUENCE OF REEF ARCHITECTURE AND SCALE ON SUCCESS OF OYSTER REEF RESTORATION.

Mark Luckenbach,* Janet Nestlerode, Paige Ross, Jr. and **Alan Birch,** Virginia Institute of Marine Science, College of William and Mary, Wachapreague, VA 23480.

Current efforts to restore oyster reefs in the Chesapeake Bay entail the placement of substrate on the shallow seabed to promote oyster settlement and reef development. Because oyster shell and other alternative cultch are in short supply and/or costly, it is important that we optimize our placement of this material to maximize restoration success. In several field experiments we have been investigating the role of three components of reef architecture—vertical relief, interstitial space and spatial scale—on the development of oyster populations and associated fauna on restored reefs. Our results indicate that modest differences in vertical relief and interstitial space have dramatic effects on early post-settlement survival of oysters and the development of viable reefs. To investigate the issue of scale and the role of reef size we have initiated a large-scale restoration experiment in the Chesapeake Bay. In a replicated block design, we have constructed high relief reef bases ranging in size from 400 m^2 to 8000 m^2 and are characterizing the development of oyster populations and associated assemblages on the reefs. Oyster recruitment and survival patterns from the first year of this study reveal significant effects of scale.

EFFECTS OF *CRASSOSTREA VIRGINICA* POPULATIONS ON SEDIMENTATION, PHYTOPLANKTON SPECIES COMPOSITION AND AMMONIA CYCLING IN EXPERIMENTAL MESOCOSMS. Jennifer Mugg Pietros and **Michael A. Rice,** Department of Fisheries, Animal and Veterinary Science, University of Rhode Island, Kingston, RI 02881.

To determine the effects of oyster populations on water quality, a mesocosm study was performed from June to October 2000. Mesocosms with a volume of 13,000 L were used, in which there was triplicate control tanks without oysters and triplicate experimental tanks each with 200 oysters (≈35 mm in valve height; nominally filtering about 55L day^{-1} ind^{-1}). Experiments were run sequentially in time for three week periods with water exchange rates ranging from 0% to 100% per day (0 to 13,000 L day^{-1}). Several parameters were measured and compared between the control and experimental tanks, including chlorophyll-a, particulate organic and inorganic matter, sedimentation rates, nitrate, ammonia, phytoplankton species and numbers, and oyster growth rates. There were no significant ($P < 0.05$) differences between tanks for most parameters, with the exceptions of rates of sedimentation and species composition of phytoplankton in the water column. Diatoms of the genus *Nitzschia* were predominant in mesocosms with oysters, and in control tanks, *Skeletonema* were dominant. Rates of ammonia excretion by oysters of various sizes was determined by the sensitive salicylate-hypochlorite method, allowing for rapid determination of excretion rates to minimize biases introduced by volatilization or tranformation of the ammonia over time. The excretion of ammonia by oysters can be described by the allometric equation $E = 50.65w^{0.6992}$, when E is the excretion rate in μg NH_3-N hr^{-1} and w is the dry soft tissue weight in g. Based upon these data, it would be expected in the three-week experimental period that an additional 470 $\mu g\ L^{-1}$ ammonia above the average control concentrations of about 40 $\mu g\ L^{-1}$ would be present in each mesocosm with oysters, but no significant difference was noted between the experimental and control tanks. This suggests that there is rapid cycling of ammonia, perhaps by uptake by the rapidly regenerating phytoplankton populations. This is publication number 3910 of the College of the Environment an Life Sciences at the University of Rhode Island, with support from the RIAES under project number H-886.

A MODIFIED HABITAT SUITABILITY INDEX FOR THE EASTERN OYSTER, *CRASSOSTREA VIRGINICA*. Thomas M. Soniat,* Department of Biology, Nicholls State University, Thibodaux, LA 70310.

A series of habitat suitability index (HSI) models have been developed for the U.S. Fish and Wildlife Service for use in environmental impact and planning studies. HSI models assess habitat quality for particular species; their output is a numerical index from 0 to 1, where 1 represents optimal habitat and 0 represents unsuitable habitat. An HSI for eastern oysters was developed by Cake (1983) and modified by Soniat and Brody (1988). The Cake model includes variables important to larval stages (presence of cultch, mean summer salinity and a gregarious settling factor, measurable as the mean abundance of living oysters) and post-settlement stages (historic mean salinity, frequency of killing floods, substrate firmness, oyster drill density and intensity of the parasite *Perkinsus marinus*). The modification of Soniat and Brody removes the gregarious settling factor from the model, simplifies model structure, and better accounts for the negative effects of high salinity, parasitism and drill predation on oysters. The modified HSI should be tested against a new, independently-collected data set.

USE OF OYSTER HABITAT BY REEF-RESIDENT FISHES AND DECAPOD CRUSTACEANS IN THE CALOOSAHATCHEE ESTUARY, FLORIDA. S. Gregory Tolley,* Aswani K. Volety, and **Emily C. Lindland,** Florida Gulf Coast University, Fort Myers, FL 33965; **James T. Winstead,** United States Environmental Protection Agency, Gulf Breeze, FL 32561.

Habitat suitability of oyster reefs for fishes and decapod crustaceans was examined monthly at three sites in the lower Caloosahatchee Estuary. At each site, 1-m^2 lift nets containing approximately 5 liters (volume displacement) of oyster clumps were deployed for a period of two weeks. Fishes and decapods sampled averaged 94 individuals m^{-2} or 23 individuals l^{-1} oyster cluster. Nine species of decapods and 18 species of fishes were identified: reef-resident fishes included *Gobiesox strumosus, Chasmodes saburrae, Gobiosoma robustum,* and *Opsanus beta;* the xanthid *Eurypanopeus depressus* and the porcellanid *Petrolisthes armatus* represented the dominant decapod crustaceans. Organism abundance ranged from 30–197 individuals m^{-2}, density ranged from 5.2–63.3 individuals l^{-1} oysters, species diversity (H′) ranged from 0.05–1.75, species richness ranged from 4–11 species per sample, and species dominance ranged from 24.8–97.7%. Species diversity, species dominance, and organism abundance all differed significantly among stations. Among-station differences in organism density (individuals l^{-1} oyster cluster) were found for the fishes *Chasmodes saburrae*, *Gobiesox strumosus*, and *Opsanus beta*, and for the crabs *Eurypanopeus depressus*, *Menippe mercenaria*, *Panopeus* sp., and *Petrolisthes armatus*. Of these, only *E. depressus* exhibited significant seasonal variation in density (Kruskal-Wallis test: n = 79, p = 0.00007), being more abundant during the warmer months.

EFFECTS OF FRESHWATER RELEASES AND SEASON ON OYSTERS (*CRASSOSTREA VIRGINICA*) IN CALOOSAHATCHEE ESTUARY, FLORIDA.

Aswani K. Volety* and **S. Gregory Tolley**, Division of Ecological Studies, Florida Gulf Coast University, Fort Myers, FL 33965; **James T. Winstead**, U.S. Environmental Protection Agency, Gulf Ecology Division, Gulf Breeze, FL 32561.

The influence of freshwater releases and season on disease prevalence and intensity of *Perkinsus marinus*, condition index, gonadal condition, recruitment potential, and growth of oysters was examined monthly at five locations along the Caloosahatchee estuary, Florida. Temperatures and salinities at the study sites ranged from 16–33°C and 3–39 ppt respectively. Higher temperatures and salinities favored *P. marinus*. While prevalence of *P. marinus* ranged from 0 (after heavy rainfall and/or freshwater water releases) –70% (during dry winter months), overall intensity was light (0–1.3). Comparison of mortality among sites indicated that juvenile oysters tolerated salinities of 5–38 ppt. Condition index of oysters was influenced by reproductive cycle and spawning events. Spat recruitment (1–5 spat/shell) and growth of juvenile oysters was higher at sudtidal than at intertidal locations, where sparse oyster distribution and swift currents appeared to limit growth and settlement success. Oysters were reproductively active between March and October, with peak reproduction occurring from June–October. Overall, results suggest that periodic short-term freshwater releases may benefit oysters by lowering the salinity and thus the intensity of *Perkinsus marinus*. Laboratory studies suggest that adult oysters can tolerate low salinities (3ppt) for 1–2 weeks. Given high salinities and infection intensities during winter months, it is recommended that freshwater releases take place during winter instead of current summer releases.

PARASITIC AND SYMBIOTIC FAUNA INHABITING OYSTERS (*CRASSOSTREA VIRGINICA*) AND MUD CRABS (*PANOPEUS HERBSTII*) SAMPLED FROM THE CALOOSAHATCHEE ESTUARY, FLORIDA.

James T. Winstead,* United States Environmental Protection Agency, Gulf Breeze, FL 32563; **Aswani K. Volety** and **S. Greg Tolley**, Florida Gulf Coast University, Fort Myers, FL 33965.

Oysters, *Crassostrea virginica*, inhabiting five sites in the Caloosahatchee River estuary were studied over a 13 month period to determine the suitability of oyster habitat in relation to their health and condition. Histological examination of 650 oysters (10 animals per station per month) revealed a varied parasitic and symbiotic fauna inhabiting these molluscs at all stations. Organisms found included protozoans (*Nematopsis* sp. and *Perkinsus marinum*), digenetic trematodes (*Bucephalus cuculus* and the first report from this area of what appears to be *Echinostoma* sp.), cestode larvae (*Tylocephalum* sp.) and a hydrozoan inquiline symbiont (*Eutima* sp.). In addition, a significant number of mud crabs, *Panopeus herbstii*, inhabiting oyster habitat at two sites were found to be parasitized by rhizocephalan barnacles, *Loxothylacus* sp. Prevalence and pathological consequences of these organisms and the role oyster-parasite relationships may play as an indicator of ecosystem complexity (biodiversity) is discussed.

POSTERS

HABITAT AND ECOLOGY OF GREEN MUSSELS, *PERNA VIRIDIS*, IN FLORIDA.

Patrick Baker,* Fisheries and Aquatic Sciences, University of Florida, Gainesville, FL 32611; **Amy Benson,** Florida Caribbean Science Center, USGS, Gainesville, FL 32653.

The green mussel, *Perna viridis*, was introduced to Tampa Bay, Florida, prior to 1999. It is abundant from Tampa Bay to Charlotte Harbor and has appeared elsewhere in Florida. As with invasive freshwater zebra mussels (*Dreissena polymorpha*), early green mussel sightings were in industrial water intake systems, and green mussels currently foul many industrial and municipal structures. Green mussels show other parallels to invasive zebra mussels, such as reaching densities of over 10,000 per m^2 in an ecosystem that lacks a close native analog. It was with zebra mussels in mind that we initiated a study to examine the habitat and ecological impacts of green mussels. Based on preliminary results, however, predictions of green mussel ecological impacts should not be modeled upon zebra mussel impacts, for several reasons. First, green mussels in Tampa Bay have been limited to artificial substrata, such as pilings and buoys. Contrary to expectations, they rarely appear on mangroves, nor do they overgrow benthic sediments. Second, green mussels have invaded a habitat that already has a high diversity of potential competitors, such as barnacles and oysters, and known molluscivores, including flatworms, crabs, stingrays, and fish. Third, there is no early evidence of negative impacts on native taxa, with the possible exception of oysters on artificial substrata. Green mussels increase the structural diversity of the fouling community on pilings, and both the shells and the interstitial space are occupied by numerous other taxa, both native and nonindigenous. At least five native bivalves commonly occur in green mussel aggregations, for example. Ecosystem impacts of this abundant invader are almost certain to develop, but may differ considerably from those of other mussel-like invaders.

Green mussels have survived several winters in Florida, and appear to reproduce more than once annually. Their initial spread southward is consistent with dominant coastal currents, which could eventually carry larvae around to Florida's east coast. Additionally, juveniles have become abundant in aquaculture systems in Charlotte Harbor, and culturists are concerned about both their impacts on seawater systems and their potential spread via human vectors. The northern (thermal) limit of green mussels has not yet been determined.

INTRODUCING THE CLAMMRS PROJECT: CLAM LEASE ASSESSMENT, MANAGEMENT AND MODELING USING REMOTE SENSING.

Shirley Baker,* **Ed Phlips,** and **David Heuberger,** Department of Fisheries and Aquatic Sciences, Institute of Food and Agricultural Sciences, University of Florida, Gainesville, FL 32653; **Clay Montague,** Department of Environmental Engineering Sciences, University of Florida, Gainesville, FL 32653; **Leslie Sturmer,** Cooperative Extension Service, Institute of Food and Agricultural Sciences, University of Florida, Cedar Key, FL 32625.

In cooperation with the Florida Department of Agriculture and Consumer Services, Division of Aquaculture, we are addressing the needs of the emerging Florida hard clam (*Mercenaria mercenaria*) aquaculture industry. The needs we are addressing include: 1) Risk of catastrophic crop loss; 2) Optimum farm management practices; and 3) Selection of new aquaculture areas. Adoption of remote sensing technologies in management practices will enhance the sustainable development of open-water clam farming by increasing production, farm efficiency, and profitability. Specific objectives are to: 1) Install remote water-quality and weather stations in clam aquaculture areas to provide growers with timely information important to their management decisions; 2) Create a water-quality database to be used by the pilot Cultivated Clam Crop Insurance program to document events associated with crop loss; 3) Develop new techniques to monitor changes in natural food abundance and quality, such as the use of satellite imaging; 4) Develop a clam production model to examine optimum management practices to increase production and profitability; and 4) Use the production model and remote water-quality monitoring in selection of new highly productive aquaculture areas in Florida.

HEMOCYTES OF *HOMARUS AMERICANUS* STAINED WITH A MODIFIED WRIGHT-GIEMSA STAIN: DESCRIPTION AND COMPARISON TO CURRENT CLASSIFICATION SCHEMES.

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Transmission electron microscopy and phase contrast light microscopy are common methods used to examine crustacean hemocytes. Using these techniques, three types of hemocytes are currently recognised in *Homarus americanus* and many other decapod crustaceans: Large Granule, Small Granule, and Hyaline hemocytes. These procedures are, however, either time consuming, costly or, do not provide a permanent record of the results. Hemocytes of *H. americanus* were classified, based on cytoplasmic and nuclear morphology, after staining cytocentrifuged preparations with a modified Wright-Giemsa stain. Up to 11 different hemocytes, Types 1 to 11, could be identified in some samples. Morphologic similarities to the Large Granule and Small Granule hemocytes were observed. Based on similarities in appearance and alterations in number during *Aerococcus viridans* infections, the Type 1 hemocyte is considered the morphologic and functional equivalent of hemocytes in the Small Granule hemocyte category with the Type 6 hemocyte being its immediate precursor. Types 2, 7, and 8 may be subsets of hemocytes included in the Large Granule hemocyte category. A counterpart of the hyaline cell has yet to be conclusively identified. This technique should prove useful in a research setting when alterations in number and types of hemocytes are being evaluated for assessment of lobster health.

IMPACT OF ATRAZINE ON ANEUPLOIDY IN THE PACIFIC OYSTER, *CRASSOSTREA GIGAS*.

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Aneuploidy has previously been reported in the Pacific oyster, *Crassostrea gigas,* and has been shown to be negatively correlated with growth. This is especially important since high variability of growth rate is one of the major problems in the aquacultural production of the species. The present study investigated the effect of an environmental factor on the level of aneuploidy. *Crassostrea gigas* animals at adult and spat stages were subjected to different concentrations of atrazine representing peak values found in the Marennes-Oleron Bay (A: 0.01 mg/l) and a value ten times higher (B: 0.1 mg/l). Although atrazine did not show any effect on the oyster mortality, significant differences in aneuploidy level were observed between the different treatments (8% for control, 16% for treatment A and 20% for treatment B). Moreover, the same level of response was observed at adult and spat stages. Finally, the offspring of oysters previously exposed did not show any difference in larval growth but the hatching rate differed (74% for control, 62% for treatment A and 55% for treatment B). This is the first evidence for an environmental cause of aneuploidy in the Pacific oyster, *Crassostrea gigas*.

STATUS OF THE *MYTILUS EDULIS* STOCK WHICH SUPPLIES HIGH-QUALITY SPAT TO MUSSEL GROWERS IN ILES-DE-LA-MADELEINE (GULF OF ST. LAWRENCE).

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Mussel growers in Iles-de-la-Madeleine rely mostly on Bassin du Havre-Aubert for spat supply. This small (~3 km^2) and shallow (max. depth = 3.5 m) basin have only a restricted opening to the sea and mussels spawn earlier than in any other local areas while larval growth is rapid. Thus, spat reaches sleeving size in early September. These mussels have a higher mean heterozygosity than all other local stocks and they show rapid growth and higher resistance to stress (including summer mortalites). This basin is dedicated only to spat collection and no grow-out is allowed. An unknown but possibly important harvest of wild mussels by local residents arose questions about the status of this stock which produces larvae for spat collection. In a 1997 survey, the low abundance of young mussels added worry about the future of this population. Surveys were repeated in 1999 and 2001. A very limited recruitment was observed in 1999 while large numbers of adults were moved to a new area possibly by the action of currents. However, the overall population remained stable at about 11 million individuals. In 2001, the area covered with mussels expanded considerably and recruitment was abundant. The near future of this population, and thus spat collection, seems not at risk in terms of potential for larvae production.

PREDATOR INDUCED PHENOTYPIC RESPONSE BY THE COMMON PERIWINKLE (*LITTORINA LITTOREA*).

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An organism's phenotype depends upon both genetics and environmental influences. The relative importance of these factors is of great importance to the field of evolutionary biology. Phenotypic plasticity, the ability to provide a single generational response to an environmental pressure, has been used to describe this relationship between genetics, the environment and phenotype. Northern Yellow Periwinkles (*Littorina obtusata*) exhibit intraspecific variance in shell thickness when exposed to predation by Green crabs (*Carcinus maenus*). This response is believed to give Periwinkles a competitive advantage compared to other prey not exhibiting phenotypic plasticity. It is unknown if the Common Periwinkle (*Littorina littorea*) can alter shell morphology when exposed to predatory stress. This study examines the morphological response of Common Periwinkles to the threat of Green crab predation. Periwinkles (n = 96) were assigned in equal numbers to one of three treatment means. Mass, operculum diameter, and shell length, width and thickness were compared among Periwinkles exposed to ambient seawater (negative control), seawater containing crab effluent, or seawater with crabs and their effluent. Initial data analysis indicates differences in growth rates among the treatments. Periwinkles exposed to crabs and their effluent grew more rapidly than those in the other treatments, suggesting that rapid growth may be a response to the threat of predation.

CONTINUOUS *IN VITRO* CULTURE OF *PERKINSUS ATLANTICUS*, PARASITE OF THE CARPET SHELL CLAM *TAPES DECUSSATUS*.

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Continuous *in vitro* cultures of the clam *Tapes decussatus* parasite *Perkinsus atlanticus* were established from infected clam gill fragments, infected clam haemolymph and parasite hypnospores isolated from infected clam gill fragments incubated in Ray's fluid thioglycollate medium (RFTM). No continuous cultures could be initiated from *P. atlanticus* zoospores. The highest success rate in establishing continuous cultures was obtained with cultures initiated from hypnospores (100%), followed by cultures initiated from gill fragments (93%) and from haemolymph (30%). The source of parasite also influenced the time taken to establish cultures and the size of cultured cells. *In vitro* proliferation of parasites was mostly by vegetative multiplication. Zoosporulation yielding motile biflagellated zoospores was observed in low proportion (<1% of dividing cells) in every culture. Morphology of cultured parasites corresponded to that of *P. atlanticus* found in clam tissues. Cultured parasites enlarged in RFTM and stained blue-black with Lugol's solution, which are characteristic of *Perkinsus* spp. DNA sequences of the internal transcribed spacer region of the ribosomal RNA gene complex matched those of *P. atlanticus*. All cultures were established in a medium designated JL-ODRP-2A which was similar in composition to the culture medium JL-ODRP-1 originally used to propagate *Perkinsus marinus in vitro*. The commercial culture medium (1:2 v/v) DME:Ham's F-12 with fetuin supported the proliferation of *P. atlanticus in vitro*.

A R&D PROGRAM TO DEVELOP *MYA ARENARIA* CULTURE IN ILES-DE-LA-MADELEINE (GULF OF ST. LAWRENCE).

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A 5-yr R&D program started in summer 2000 to develop a profitable soft-shell clam culture in the lagoons of Iles-de-la-

Madeleine. This program looks at a scenario based on the transfer of undersized clams from an unexploited population to an aquaculture site for grow-out until legal size (>51 mm) is reached. We have focused mainly on the potential of this population to provide an annual harvest of 5 million clams. It should not be a problem considering its >265 million individuals of 15–45 mm and high recruitment. Further, the low predator abundance and the absence of pathologies (e.g. neoplasia) suggest it should not suffer heavy losses. The small hydraulic device used to resuspend the top surface layer and ease collection seems not to have negative impacts. The large size of these clams should minimize predation and net protection over seeded areas could possibly be avoided. Work has been initiated to optimize storage of large quantities of clams during the extended periods between harvesting and subsequent seeding. Experiments were also initiated to define the optimal seeding period and optimal density according to shell length.

LOSS OF EYE PIGMENTATION IN TWO GAMMARIDEAN AMPHIPODS FROM THE BIOSPHERE. Vania R. Coelho, Columbia University, Biosphere 2 Center, Oracle, AZ 85623; **Jeffrey D. Shields,*** Virginia Institute of Marine Science, Gloucester Point, VA 23062.

During an ecological assessment of amphipod populations in the Biosphere 2, several specimens of *Ceradocus rubromaculatus* and *Leucothoe spinicarpa* were observed with alterations in their eye pigmentation. For *C. rubromaculatus,* 1768 specimens were examined and 64 (%) presented with some degree of alteration. For *L. spinicarpa,* 150 specimens were examined and 91 (%) presented with some degree of alteration. Grossly, the eyes appeared either completely white with the individual retinas unpigmented, or the eyes were partially pigmented with pigmentation loss surrounding a pigmented core within the ommatidia. Histologically, the optic nerves showed a range of pathologies from minor losses of pigmentation, especially in the centrally-located ommatidia, to complete loss of pigmentation. In all cases, there was little to no damage associated with the optic nerves or the ommatidia. There was also no indication of increased infiltration of hemocytes into the optic nerve complex or the ommatidia. Pigment cells can be negatively affected by contaminants or by the lack of phytopigments in the crustacean diet. For example, crustaceans lack the ability to synthesize carotenoids and must obtain them from their diets. We speculate that the loss of pigmentation in the eyes of amphipods from the Biosphere may be caused by the lack of certain dietary phytopigments.

SHELL REPAIR RATES IN SURGICALLY DAMAGED VALVES OF THE BLUE MUSSEL (*MYTILUS EDULIS*) AND THE RIBBED MUSSEL (*GEUKENSIA DEMISSA*) FROM NEW JERSEY. Tricia L. Cranmer, Department of Marine and Environmental Studies, University of San Diego, San Diego, CA 92110; **Daniela Zima,*** Richard Stockton College of New Jersey, Pomona, NJ 08240; **Richard R. Alexander,** Department of Geological and Marine Sciences, Rider University, Lawrenceville, NJ 08648.

The blue mussel *Mytilus edulis* and the ribbed mussel *Geukensia demissa* were collected ($n = 120$) from the salt mashes of Tuckerton, New Jersey. Equal numbers of specimens ($n = 15$) were notched at, or had holes drilled near, the posterior or ventral margin of the valves of each species. These shell regions bear scars from shell-crushing crabs and holes drilled by moonsnails. Repair progresses through four stages: 1) tissue plugging the shell gap or hole, 2) extension of the periostracum over exposed tissue, 3) calcification beneath the periostracum, and 4) valve-thickening, pigmentation, and expression of a ny ornamentation. Shell repair was completed between two to seven weeks. Average repair rates are statistically significantly faster for epibyssate *M. edulis* relative to the endobyssate *G. demissa* (Mann-Whitney U test; $p < 0.05$) pooled for all types and location of shell damage. Correspondingly, field surveys show that shell repair frequency is greater among blue mussels (19% of all specimens) than ribbed mussels (10% of all specimens). Mantle tissue repaired posterior shell notches faster than the ventral margin in *M. edulis,* although shell repair rate is statistically indistinguishable between ventrally and posteriorly notched shells of *G. demissa.* Correspondingly, field surveys reveal that 96% of all shell repairs in *M. edulis* are located near the posterior margin. Mortality before completion of shell repair was significantly greater for experimentally drilled vs. notched shells of either species. Mantle tissue was damaged by penetrative shell-drilling, but not inevitably in valve margin breakage.

OVER-WINTERING OF *CRASSOSTREA ARIAKENSIS* IN LAND-BASED SYSTEMS IN VIRGINIA. Alan J. Erskine and **Standish K. Allen, Jr.,** Aquaculture Genetics and Breeding Technology Center, Virginia Institute of Marine Science, Gloucester Point, VA 23062.

Decimation of the *Crassostrea virginica* industry in Virginia has been well documented. Recent interest has turned to non-native *C. ariakensis* culture for experimentation. We hypothesized that it was beneficial, biosecure and cost effective for industry to over-winter *C. ariakensis* inside their hatcheries. Triploid *C. ariakensis* were deployed in raceway and upweller systems at four sites in Chesapeake Bay from November 2000 to April 2001. Three size classes, 4–6mm, 6–8mm and 8–12mm, were deployed in replicate at a maximum carrying capacity of 2000 grams total wet weight. Twenty-five random oysters were sampled monthly and shucked wet weights and hinge to lip shell height measurements were recorded. A low salinity site (10–12ppt) on the bayside of Chesapeake Bay and a mid salinity site (22–25ppt) on the seaside had the highest growth at 30 mm and 3 grams. This experiment shows that over-wintering juvenile *C. ariakensis* could be incorporated as a step in the technology of non-native oyster culture.

IRRADIATION OF OYSTER PRIMARY CELL CULTURES WITH ULTRAVIOLET-LIGHT TO ELIMINATE BACTERIAL AND PROTOZOAL CONTAMINANTS.

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Microbial contamination of oyster primary cell cultures is an obstacle to cell line development. *Perkinsus marinus* and *Vibrio vulnificus* are two prevalent contaminants of oyster cell cultures. Studies have shown that ultraviolet-light (UV) effectively kills certain aquatic protozoa and bacteria. Our objective was to develop a procedure to eliminate *P. marinus* and *V. vulnificus* from primary cell cultures by UV irradiation, while retaining the viability of oyster cells. Oyster heart cells, *P. marinus* and *V. vulnificus* cultures were irradiated with 50,000, 100,000, 201,000, and 402,000 μjoules/cm^2 of UV light. Two days post-irradiation, oyster cell viability was determined by measuring their metabolic activity using the MTS/PMS assay. *Perkinsus marinus* growth, 2, 4 and 6 days after irradiation, and *V. vulnificus* growth, 3, 6, and 24 hours post-irradiation, were both assessed by measuring turbidity and metabolic activity (MTS/PMS assay). Results indicated that oyster cells viability decreased significantly ($P < 0.0001$) with increasing UV energy levels. *Perkinsus marinus* growth on days 2 and 4 were significantly inhibited by all UV levels, while growth on day 6 was inhibited with UV levels above 100,000 μjoules/cm^2. *Vibrio vulnificus* growth, 3 and 6 hours post-irradiation, was inhibited ($P < 0.05$) by all UV levels, but growth rebounded 24 hours after irradiation. This study suggests that UV irradiation has a very limited ability to eliminate *P. marinus* or *V. vulnificus* without negatively affecting oyster cell primary cultures.

IN VITRO EFFECTS OF HEAVY METALS AND ATRAZINE ON PACIFIC OYSTER, *CRASSOSTREA GIGAS*, HAEMOCYTES.

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In the last decades shellfish culture has developed in a significant way around the world. However, culture areas are often subjected to recurring pollutions. The recrudescent use of herbicides in agriculture, including atrazine, implies pollutant transfer towards the aquatic environment in estuarine areas. Moreover, industrial wastes are sources of heavy metal contamination. It is appropriate to consider the harmful effects of pollutants in marine species, particularly in cultured molluscs. Bivalves, including mussels and oysters, have been suggested as ideal indicator organisms because of their way of life. They filter large volumes of seawater and may therefore accumulate contaminants within their tissues. The development of techniques allowing analysis of the effects of such compounds on bivalve biology may lead to monitoring of pollutant transfer in estuarine areas. In this context, the effects of atrazine and the effects of various heavy metals on defence mechanisms were analysed in Pacific oysters, *Crassostrea gigas*. Pollutant effects were tested *in vitro* on oyster haemocytes. Cell viability and activities were monitored by flow cytometry. Enzymatic phenoloxidase-like activity was also evaluated by spectrophotometry. Atrazine induced no effect on oyster haemocytes under the conditions tested. On the contrary, mercury caused a significant mortality of haemocytes maintained *in vitro*. Aminopeptidase and phenoloxidase-like activities were also modified in the presence of this pollutant.

KEY WORDS: Pacific oyster, *Crassostrea gigas*, haemocytes, flow cytometry, heavy metals, atrazine, phenoloxidase, cellular activities, toxicity

INDUCIBLE ANTIBACTERIAL ACTIVITY IN OYSTER (*CRASSOSTREA VIRGINICA*) HEMOLYMPH.

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Oyster (*Crassostrea virginica*) tissues resist infection by marine bacteria, but the basis of this is unclear. Bacteria invading internal spaces are quickly agglutinated and phagocytosed by hemocytes; however, areas where hemocytes are not active (e.g., epithelial surfaces) also resist heavy colonization. Oyster lysozyme kills bacteria, but is thought to be only weakly effective against the most common marine species (Gram-negatives). Soluble bacteriolytic peptides found in many organisms, including the mussel *Mytilus edulis*, may supplement phagocytosis and the activity of lysozyme in oysters as well.

Our objectives were to determine whether or not anti-Gram-negative bacterial activity could be induced in the hemolymph of *C. virginica*, and if so, to isolate and characterize its source. Lipopolysaccharide (LPS), laminarin, or dH$_2$O control was injected into the adductor muscles of wild *C. virginica* (N = 6 oysters/treatment) collected locally. Hemolymph was collected from the adductor muscles at 12 and 24 h and pooled by time interval and treatment type. Soluble protein from cell-free hemolymph plasma was desalted using a C_{18} column, eluted with 50% acetonitrile, dried down and reconstituted in dH_2O, and spotted on a plate lawn of *Escherichia coli*. Plasma protein samples from LPS-challenged (12 and 24 h post-challenge) and laminarin-challenged (24 h) oysters inhibited *E. coli* growth, while plasma protein from unchallenged oysters did not.

The induced antibacterial activity passed through a 10-kilodalton (kDa) cutoff membrane. Further separation by reversed phase high performance liquid chromatography (HPLC) and analysis by matrix-assisted laser desorption ionization mass spectrometry (MALDI-MS) revealed several peptides around 3–7 and 9.5 kDa to be candidates for its source. These candidate peptides are currently being sequenced.

CONSTRUCTING SHELLFISH REEFS IN A POLLUTED, URBAN ESTUARY: SCIENTISTS JOIN WITH THE COMMUNITY TO WORK FOR A COMMON GOAL. **Jennifer Greene,* Raymond Grizzle, David Burdick, and Larry Ward,** Jackson Estuarine Laboratory, University of New Hampshire, Durham, NH 03824; **Ann Reid,** Great Bay Coast Watch, Sea Grant Cooperative Extension, Durham, NH 03824.

South Mill Pond, a 7-hectare estuarine embayment in the City of Portsmouth, New Hampshire has been receiving combined sewer overflows since the late 1800s. A tide gate controls water flow into and out of the Pond and there is a road causeway with box culverts that separates the Pond into inner and outer sections. The City has a major sewer re-construction project underway that will, when completed, reduce inputs of sanitary sewer wastes. Scientists joined with the City, students from local schools, and local volunteers to begin ecological restoration of the Pond in 2001. Six shellfish "mini-reefs" (each 2 × 5 m in surface area) were constructed in May using mussels (*Mytilus edulis*) transplanted from a nearby natural reef. Mortalities as high as 80% occurred on the three reefs in the inner pond within the first 2 months, likely as a result of elevated temperatures caused by extended closure of the broken tide gate during June and July. Hence, three additional mussel reefs were constructed in the inner pond in October 2001. Oysters (*Crassostrea virginica*) remotely set at Jackson Estuarine Laboratory were added as 3-month old spat to the three new reefs in the inner pond and the three original reefs in the outer pond in October, resulting in six mussel/oyster reefs. Eighth grade students participated in construction of the mussel reefs and third graders participated with the oysters. Community volunteers also participated in reef construction and post-construction monitoring. Three quadrat ($0.16\ m^2$) samples per reef showed initial mean mussel densities on the six original reefs constructed in May ranged from 150 to 930 m^{-2}. After 6 months (November), mussel densities had dropped to 50 to 115 m^{-2} on the six original reefs but size-frequency plots showed an average increase in shell length of 5 mm in the largest size class of mussels over the first 3 months, indicating good individual growth rates. Recruitment into the smallest size classes also occurred on some reefs. Using the same sampling protocol, initial mussel densities on the three new reefs ranged from 430 to 1376 m^2. Future work will include continued monitoring of all nine reefs, construction of abutting saltmarsh, and environmental monitoring.

THE EASTERN OYSTER AS AN INDICATOR SPECIES TO ESTABLISH RESTORATION TARGETS IN SOUTHWEST FLORIDA ESTUARIES. **Rashel V. Grindberg,* Erin C. Rasnake, Michael Savarese, and Aswani K. Volety,** Florida Gulf Coast University, College of Arts & Sciences, 10501 FGCU Blvd South, Ft. Myers, FL 33965.

Water management practices within Southwest Florida have drastically altered natural water quality conditions within estuaries. The ecological and physiological responses of oysters, *Crassostrea virginica*, were compared among pristine (Blackwater River), semi-altered (Henderson Creek) and altered (Faka-Union canal) estuaries with historically similar hydrologic conditions. 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 productivity are displaced seaward relative to pristine estuaries. Henderson Creek, an estuary receiving pulses of nutrients and freshwater due to weir design, has oyster populations with higher mean productivities and higher living densities. While upstream locations in all three estuaries have lower disease prevalence among adult oysters, juveniles experience heavy mortality due to freshwater releases. Overall growth rates are higher at upstream locations. Spat recruitment occurred between June and October in all estuaries.

Faka-Union and Henderson Creek estuaries are scheduled for restoration. The patterns of oyster distribution and physiology will help establish target restoration conditions and provide a foundation for the monitoring of restoration effectiveness in Southwest Florida estuaries. Henderson Creek is presently undergoing restoration through the retrofitting of its upstream weir to permit dynamic control of freshwater release. Sheet flow should be restored to Faka-Union in order to re-establish salinity levels favorable for maximum reef development, living density, oyster growth, recruitment, and productivity. Changes in oyster ecological and physiological conditions will be monitored over subsequent years to gauge restoration effectiveness.

LONG-TERM CHANGES IN INTERTIDAL OYSTER REEFS IN A FLORIDA LAGOON POTENTIALLY CAUSED BY BOATING ACTIVITIES: AN ANALYSIS OF AERIAL PHOTOGRAPHS FROM 1943–2000.

Raymond Grizzle* and **Jamie Adams**, Jackson Estuarine Laboratory, University of New Hampshire, Durham, NH 03824; **Linda Walters**, Department of Biology, University of Central Florida, Orlando, FL 32816.

Previous research in the late 1990s had shown that some intertidal oyster (*Crassostrea virginica*) reefs in that portion of the Mosquito Lagoon within the Canaveral National Seashore, Florida had dead margins consisting of mounded up, disarticulated shells. It was hypothesized that boating activities were the cause of the damage because all the reefs were adjacent to major boating channels. We characterized the history of the appearance of dead margins (and other reef changes) using aerial photographs taken between 1943 and 2000. Imagery analyzed included prints (black & white, color, or color infrared) from 1943, 1951, 1963, 1975, 1988, and 1995, and digital imagery from 2000 (USGS 1:12,000 digital ortho-quarterquads), at scales from 1:6,000 to 1:24,000. Prints were scanned at a resolution sufficient to yield 1-m pixels. After scanning, each set of images was referenced to the year 2000 imagery using ArcView and ArcInfo GIS software. All reefs found to have dead margins based on 1995 and 2000 aerials were visited in November 2001 to confirm the presence and extent of dead areas. This provided a general ground-truthing for the "signature" (a highly reflective, light-colored area adjacent to darker-colored live reef) to be used to detect the appearance of dead margins in the historical aerials. The earliest appearance of dead margins was in the 1975 aerials on reefs adjacent to the intracoastal waterway (ICW), a major boating channel. The total number and areal extent of dead margins increased with time. Our current preliminary estimate is that between 10 and 20% of the reefs in the Seashore have been damaged. The most dramatic changes have occurred in reefs along the ICW, some apparently migrating away from the channel as much as 50 m and with empty shells mounded up a meter above the high water line. This historical analysis provides strong (although only correlative) evidence that boating activity has had dramatically detrimental effects on some oyster reefs in the study area. Ongoing studies are aimed at further testing this hypothesis and elucidating the actual mechanisms involved.

AN OYSTER (*CRASSOSTREA VIRGINICA*) REEF RESTORATION EXPERIMENT IN NEW HAMPSHIRE INVOLVING CROSBREED STOCK AND NATIVE TRANSPLANTS.

Raymond Grizzle, Jennifer Greene, and **Stephen Jones**, Jackson Estuarine Laboratory, University of New Hampshire, Durham, NH; **Mark Luckenbach** and **Roger Mann**, Virginia Institute of Marine Science, Gloucester Point, VA.

The first documented MSX epizootic in New Hampshire occurred in 1995, resulting in dramatic declines in oyster abundances on some reefs. The present project is the first experimental scale effort in the State aimed at development of a long-term program to mitigate the effects of disease. In 1999, studies were initiated in the Salmon Falls River to: (1) characterize a disease-decimated reef, (2) restore portions of the reef using spat from CROSBreed stock (an MSX and dermo-resistant line) and transplanting of native oysters, and (3) determine the effects of the restored reef on water quality. Here we report on the first two objectives. Diver surveys, tonging, and underwater videography indicated the overall bottom area probably dominated by oysters before 1995 extended along the main channel a total distance of 350 m with an average width of 15 m. Quadrat sampling in 2000 and 2001 showed this area was numerically dominated by the ribbed mussel (*Geukensia demissa*) with a mean density of 40 m^2; oysters were 20 m^2, and the blue mussel (*Mytilus edulis*) 5 m^2. Maximum, mean horizontal free-stream flow speed measured over portions of two tidal cycles with an acoustic Doppler velocimeter was 32 cm s^{-1}. In May 2000, approximately 230 bushels of native oysters, dredged from the Piscataqua River about 1 km south of the study site, were deposited in an area measuring 10 × 30 m on the natural reef bottom. Spat from CROSBreed stock were remotely set in June of 2000, held in bags for 4 months, then transferred to a 10 × 20 m area on the natural reef. In October 2001, after 1 year of development, the CROSBreed reef area had a mean density of 226 oysters m^2, compared to 90 m^2 on the native transplant reef, and 36 m^2 on the natural reef. The CROSBreed oysters have shown good growth, increasing from a mean size of 26.8 mm shell height when put out in October 2000 to 48.6 mm in October 2001, and no measurable mortality. Natural spat set in 2000 was greatest on the CROSBreed reef, suggesting a "minimum threshold density" for successful natural recruitment to restored reef areas.

WHAT CERTAIN 19TH AND EARLY 20TH CENTURY NAVIGATIONAL AND SPECIAL PURPOSE SURVEY CHARTS REVEAL ABOUT CHANGES IN THE OYSTER REEF MORPHOLOGY OF THE LOWER CHESAPEAKE BAY.

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The decline of commercial oyster production in the lower James River is reflected in the reduction of oyster-reef dimensions over time. Employing navigational hydrographic survey charts made by the U.S. Coast Survey in 1854-55, 1871-73, and 1940s and oyster survey charts made in 1878 and 1909 by the USCGS and the old U.S. Fish Commission, we examine changes in the dimensions of the natural oyster reefs of the lower James River, Chesapeake Bay, and discuss the likely cause of those changes.

MORPHOLOGY OF A CHESAPEAKE BAY OYSTER REEF SYSTEM IN 1871–1873.

William J. Hargis, Jr., Helen E. Woods,* Rebecca Arenson, Elizabeth Mountz, Marcia R. Berman, and **Sharon Dewing,** Center for Coastal Resources Management, Virginia Institute of Marine Science, Gloucester Point, VA 23062.

During research on oyster reef evolution in the Chesapeake Bay it became apparent that those once prominent benthic features must have influenced their surrounding significantly. To investigate the extent of that influence on the geomorphology, hydrodynamics, and other ecologically important features, we developed a 3 dimensional presentation of the once highly productive oyster reef system of the James River estuary. Charts of the study are drawn from soundings made by the U.S. Coast Survey in 1871–73 were employed. Soundings were digitized on a Numonics 2200 digitizing tablet interfaced with ArcInfo running in a UNIX operating environment. The coordinate grids were projected to NAD27. A 3-D TIN (Triangulated Irregular Network) model generated the picture of the bottom of the study area extant in 1871–73. The 3-D presentation, center-piece of our presentation, clearly depicts the oyster reef system of 1871–73. Extending into the intertidal and acting as a massive system of weirs and baffles, the upthrusting reefs (Hargis, In Press) must have affected deposition, scouring and erosion, not only near field but more distantly, even into the shallows and adjacent shorelines. Undoubtedly, they influenced biological processes too.

GEOGRAPHIC VARIATION IN NUCLEAR GENES OF THE EASTERN OYSTER *CRASSOSTREA VIRGINICA*.

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Studies of genomic DNA in the eastern oyster, *Crassostrea virginica*, have uncovered genetic discontinuities in population structure. Like most benthic marine invertebrates, *C. virginica* has a planktonic larval stage, which is in principle capable of widespread dispersal. If extensive dispersal occurs, one would expect to observe genetic homogeneity across the geographic range of the species. However, studies have found geographic genetic heterogeneity in both mitochondrial DNA (mtDNA) and nuclear DNA (nDNA) of *C. virginica*. The break in mtDNA has prompted the division of the *C. virginica* into Atlantic and Gulf coast subpopulations. However, unlike mtDNA, the degree of nDNA differentiation is still poorly known. This study examines nDNA from Atlantic and Gulf coast oyster populations for genetic polymorphisms using restriction endonuclease fragment length (RFLP) analysis, denaturing gradient gel electrophoresis (DGGE), and direct sequencing. Nuclear primers are currently being screened for polymorphisms in a panel of organisms representing the geographic range from Canada to Mexico. This work will improve on previous research by examining additional nuclear markers and will help clarify the question of large-scale variation between Gulf and Atlantic coast oyster populations as well as shed light on smaller-scale regional patterns of variation.

CHARACTERIZATION AND MANIPULATION OF SEX STEROIDS AND VITELLOGENIN IN FRESHWATER MUSSELS.

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The characterization and manipulation of sex steroids and vitellogenin in freshwater mussels, is critical for the development of artificial culture procedures and the evaluation of reproductive health of populations. The current study included an evaluation of reproductive cycles and vitellogenin in *Elliptio buckleyi*. In addition, female *Elliptio buckleyi* and *Lampsilis teres* were exposed to exogenous estradiol to induce spawning. Body tissues were collected from adult mussels and standard RIA procedures were utilized for androgen, and estrogen analyses. Vitellogenin, an egg yolk protein produced under the influence of sex steroids, was determined using an indirect method developed by Blaise et al. (1999). Sex steroid concentrations were found to be closely correlated to reproductive activities and spawning. Tissue concentrations of both vitellogenin and estrogen were significantly elevated following exposure to exogenous estradiol. Histological examination of gonad tissue also indicated changes in reproductive status. The development of these procedures for use with freshwater mussel species will be critical to the elucidation of potential habitat and contaminant effects on reproductive function, as well as the culture of endangered species.

A SPECIFIC ASSOCIATION OF DOCOSAHEXAENOIC FATTY ACID WITH CARDIOLIPINS OF SOME MARINE BIVALVES.

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A cardiolipin (CL) class, also termed diphosphatidylglycerol, was isolated by high performance liquid chromatography from lipid extracts of *Pecten maximus, Crassostrea gigas* and *Mytilus edulis*, and characterized by analytical and spectroscopic methods. The fatty acid (FA) composition of this CL represents a specific association with docosahexaenoic acid [22:6(n-3), DHA], which accounted for more than 90% w/w of the total FA of this class. The structural determination by nuclear magnetic resonance spectrometry and positive ion electrospray mass spectrometry verified a configuration having four identical 22:6(n-3) molecules. This specific composition is different from those already reported in other eukaryotes. Indeed, CL acyl chains reported in the literature are mainly composed of monounsaturated or diunsaturated chains with

16 or 18 carbon atoms. The DHA-enriched CL may reflect a specific adaptation in bivalves that enhances the structural and functional mechanisms of biomembranes in response to environmental variations (temperature, salinity, emersion).

HEMOCYTE-MEDIATED DEFENSE RESPONSES OF THE LOBSTER *HOMARUS AMERICANUS*. **Brenda S. Kraus,* Amy E. Beaven,** and **Robert S. Anderson,** Chesapeake Biological Laboratory, University of Maryland Center for Environmental Science, P. O. Box 38, Solomons, MD 20688.

Lobster hemocytes held in primary culture avidly phagocytosed untreated yeast cells labeled with fluorescein succinimidyl ester. However, serum-treated yeast cells were less readily ingested by the hemocytes. Serum agglutinins can serve to facilitate phagocytosis of certain foreign particles (opsonization). Although anti-yeast agglutinins were present in lobster serum they seemed to inhibit phagocytosis, perhaps by blocking sites involved with recognition by hemocytes. Bacterial agglutinins have also been measured in lobster sera; their role as opsonins is under study. After appropriate stimulation by phagocytosis or membrane perturbation, hemocytes can produce cytotoxic reactive oxygen species (ROS) which contribute to host defenses by destroying microorganisms. Luminol-augmented chemiluminescence (CL) was used to quantify ROS generation by lobster hemocytes. Phorbol myristate acetate (PMA), a protein kinase C activator, was shown to elicit ROS in hemocytes withdrawn from healthy lobsters, based on hemolymph protein concentration. However, phagocytosis of *Listonella anguillarum* infrequently (~25%) produced a ROS activity in PMA-responding animals. Opsonization of *L. anguillarum* with serum did not enhance CL; this was not unexpected because no anti-*L. anguillarum* agglutinin activity was detected in lobster serum. Studies related to the role of agglutinins in recognition and effector mechanisms of lobster hemocytes using other biotic and abiotic test particles are in progress.

POTENTIAL ELIMINATION OF THE PROTOZOAN PATHOGEN *PERKINSUS MARINUS* FROM EASTERN OYSTERS BY FRESHET EVENTS. **Megan K. G. La Peyre,*** U.S.G.S. Louisiana Cooperative Research Unit, School of Forestry, Wildlife and Fisheries, Louisiana State University, Baton Rouge, LA 70803; **Amy D. Nickens** and **Jerome F. La Peyre,** Cooperative Aquatic Animal Health Research Program, Department of Veterinary Science, Louisiana State University Agricultural Center, Baton Rouge, LA 70803.

Environmental conditions have long been held to be critical controls on host-parasite interactions but little attention has been paid to the effects of short-term events on host-parasite interactions. We tested the hypothesis that freshet events may be detrimental to *P. marinus* while having only minimal impacts on *C. virginica* survival. Research based predominantly on environmental averages has led to the consensus that environmental conditions such as salinity and temperature control *P. marinus* infection in *C. virginica*. In contrast, salinity records from the Gulf coast indicate that there is considerable variation in salinity, and several studies have suggested that freshet events may be related to low *P. marinus* infection intensities of oysters in certain areas. In order to determine the effects of freshets on *C. virginica* and *P. marinus*, we initiated a controlled laboratory experiment. In April, July and December, 2001, oysters collected from Grand Isle, LA were divided equally between a control system (maintained at 20 ppt) and a treatment system (salinity lowered from 20 ppt to 0-1 ppt over 48 hours). Thirty oysters were sampled weekly from each tank and infection intensities determined. Oyster mortality was monitored daily. Treatment oysters did have reduced *P. marinus* body burden, but actual success was highly dependent on the season. *Crassostrea virginica* was not adversely affected by freshet events in cool temperature, spring and winter experiments, but experienced high mortality during a summer freshet event. Since most freshet events occur in winter-spring months, the use of controlled freshwater events might present a potential management tool for reducing *P. marinus* infection in oysters.

A FIELD INVESTIGATION OF THE EFFECTS OF V-NOTCHING ON THE HEALTH AND SUSCEPTIBILITY TO INFECTION OF OVIGEROUS FEMALE AMERICAN LOBSTERS. **Jean Lavallée,*** AVC Lobster Science Centre, University of Prince Edward Island, 550 University Avenue, Charlottetown, PE, Canada, C1A 4P3; **Donald J. Rainnie,** AVC Inc., 550 University Avenue, Charlottetown, PE, Canada, C1A 4P3.

V-notching is a fishery management practice consisting of marking ovigerous lobsters by punching a V shaped notch in the tail before returning it to the seawater. This study investigated some of the possible effects of V-notching on the health of ovigerous lobsters during the 2000 fall fishing season on Prince Edward Island. One hundred and thirty ovigerous lobsters were physically examined and assigned to two treatment groups. Hemolymph samples were collected from 15 lobsters from each treatment group and analysed for total protein (TP), total hemocyte counts (THC) and for the presence of *Aerococcus viridans, Anophryoides haemophila* and *Vibrio spp.* Lobsters were housed individually in cages containing 2, 3 or 4 compartments. Lobsters from the treated group were V-notched immediately prior to returning the cages to the sea bottom. Monitoring for mortality, and vigor status was conducted at least weekly. Hemolymph was taken on 15 lobsters of both treatment groups for TP and THC at the mid-point in the study. At study termination (55 days), all lobsters were re-examined and hemolymph was sampled for determination of TP, THC and presence of *A. viridans, An. haemophila* and *Vibrio spp.* Additionally, 2 V-notched lobsters were sampled on Days 1, 3, 6, 13, 27 & 55 for presence of *A. viridans, An. haemophila* and *Vibrio spp.* and for histological assessment of the wound. Mortality during the course of the study was limited to one lobster, and reflected an act of cannibalism. There was no significant difference ($p > 0.05$) between the two treatment groups for any parameter monitored. Histological examination of the V-notch wound revealed that the wounds appeared well sealed off to the environment by the rapid infiltration of hemocytes by 24 hours.

A PRELIMINARY LINKAGE MAP FOR THE PACIFIC OYSTER *CRASSOSTREA GIGAS*, CONSTRUCTED WITH RAPD AND AFLP MARKERS.

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The development of genetic markers and linkage maps is an important step toward the identification and potential improvement of commercially important traits in oysters. The construction of a linkage map requires a large number of molecular markers, which have been a challenge in oysters. Microsatellites are probably the best markers for linkage mapping, but they are expensive to develop and not readily available to many labs. Therefore, we developed relatively inexpensive markers such as the random amplified polymorphic DNA (RAPD) and amplified fragment length polymorphism (AFLP) markers for linkage mapping in the Pacific oyster *Crassostrea gigas* Thunberg. Selected markers were used for linkage mapping in a reference family with 75 progenies. The reference family was a backcross of an interstrain hybrid to one of the parental strain, ((Miyagi × Hiroshima) × Miyagi). In this study, 110 RAPD primers were screened with the parents and five progenies, and 25 primers with at least one reliable and segregating band were selected for mapping analysis. The 25 primers generated 259 bands, of which 102 (39%) were polymorphic in the two parents. The number of segregating RAPD markers was 41 in the mother and 17 in the father. Using ten AFLP primer combinations, 802 peaks were obtained, of which 253 (32%) are polymorphic. The number of segregating AFLP markers was 81 in the mother and 50 in the father. A preliminary female linkage map was constructed with 122 RAPD and AFLP markers covering all ten chromosomes, and details of the map will be presented at the meeting.

GONADAL MATURATION OF TRIPLOID SCALLOPS *ARGOPECTEN PURPURATUS* LAMARCK, 1819.

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It is expected that in triploid organisms the energy normally used for reproduction would be allocated to growth. However, not all triploid molluscs are completely sterile; and in some cases even gametes are produced. The aim of this study was to assess the gonadal development in the native scallop *Argopecten purpuratus* induced to triploidy.

A. purpuratus is a functional hermaphrodite, the male gonad being creamy-white, located proximal to the foot, and the distal female gonad is bright orange-red. They were induced to triploidy with 6-dimethylaminopurine (6-DMAP). Treated (=induced) and control scallops were processed for histology using routine methods. At the age of 11 months, when the control scallops were mature, some treated scallops had a gonad which showed a uniform brown colour. These were true triploids as evaluated through chromosomal counts. They showed the tendency of reducing the female gonad, only few pre-vitelogenic oocytes were observed, in otherwise empty acini. The male gonad was relatively more developed, but no gametes further than secondary spermatocytes or early spermatids with no flagella were detected.

DETECTION OF THE WHITE SPOT SYNDROME VIRUS (WSSV) IN CARCINOLOGIC FAUN ASSOCIATED TO SHRIMP CULTURE OF SINALOA, MEXICO, USING POLYMERASE CHAIN REACTION (PCR) AND *IN SITU* HYBRIDIZATION.

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One of the most important sanity problems in penaeid shrimp populations of trade important in the world, is originated by virus infection. Recently in our country was detected the White Spot Syndrome Virus (WSSV), that produce high mortality in the population of cultured shrimps, mainly in the states of Nayarit, Sinaloa and Sonora. For this research, we collected samples in summer of 2001. The samples were from different species associated to culture farms, (*Litopenaeus vannamei, L. stylirostris, Callinectes sapidus, Uca* sp. and copepods). The copepods were homogenized and proceed in integral form by Polymerase Chain Reaction (PCR) probe, one hemolymph sample was taken from each of the different species to which was done the PCR getting the result of sequence in acrilamide, and having the corresponding marks. The samples for processing by *in situ* hybridization, were fixed in Davidson's solution for 48 hours and then in OH 70. The histological cuts were done 5 μm of thickness and were put on positively slides. By *in situ* hybridization, infected tissues of samples of *Callinectes sapidus* and *Litopenaeus vannamei* were easily distinguished. The presence of the blue precipitations it was present in branchial and connective tissues and gut subcuticular epithelium. In the other species of carcinological faun was negative by *in situ* hybridization probe.

FACTORS, RISKS AND SIGNIFICANCE OF EMERGENT NEOPLASIA DISEASES IN CULTURED AND WILD SOFT-SHELL CLAMS (*MYA ARENARIA*) IN ATLANTIC CANADA.

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In 1999, mortalities of soft-shell clams (*Mya arenaria*) caused by or associated with haemic neoplasia occurred at several sites around Prince Edward Island (PEI) and in Richibucto, New Brunswick (NB). Haemic neoplasia has been well documented in bivalves (clams, mussels and oysters) worldwide since the early 1970's. The cause(s) of haemic neoplasia is/are unknown. They have been linked to infectious triggers (neoplastic cells *per se* or a viral vector), anthropogenic carcinogens (e.g., polychlorinated biphenyls) and changing natural conditions (e.g., abnormally high water temperatures). The most urgent question, from an environmental and clam production perspective, is whether the neoplasia is infective or non-infective.

The objectives of this study are to: i) examine the transmissibility of this disease; ii) determine the geographic and seasonal distribution of haemic neoplasia in soft-shell clams from PEI (including a study to relate disease prevalence to five PEI clam populations); iii) examine common environmental variables between affected and unaffected sites on PEI, NB, and Nova Scotia (e.g., temperature, bottom-type, terrestrial run-off, human activities/input); iv) determine whether or not sediment exposure affects emergence of haemic neoplasia; and v) determine whether or not clams which have survived haemic neoplasia have developed a resistance to the disease which can be passed onto their offspring.

IDENTITY OF UNITED STATES MOLLUSK PRODUCTION DECLINES IN THE 1900S.

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In the 1900s, the historical declines in landings of estuarine mollusks along the east coast of the United States rarely were caused by overfishing, that led to permanent sharp declines in production of their larvae. Instead, habitat degration including adverse algal blooms, domestic pollution which led to closure of production beds, poor markets, and diseases have been the main causes. The oyster, *Crassostrea virginica*, landings, mainly, suffered from poor markets, and oyster habitats were despoiled by siltation and by dredgers removing shells while oysters were being harvested, and finally by diseases. Oysters companies sold their shells to the poultry industry and for hardening roads rather than spreading them on beds to produce more oysters. Northern quahog, *Mercenaria mercenaria*, landings declined due to the narrowing of bay openings, adverse algal blooms, and bed closures due to domestic pollution. Soft clam, *Mya arenaria*, landings declined due to destruction of beds, bed closures due to domestic pollution, diseases, and high temperatures. Bay scallop, *Argopecten irradians*, landings declined due to adverse algal blooms, changes in bay openings, and losses of eelgrass, *Zostera marina*. Attributing the declines to overfishing leads resource managers away from the actual causes and delays habitat restoration.

DEVELOPMENT OF A PCR-BASED ASSAY FOR DETECTION OF THE JOD-ASSOCIATED *ROSEOBACTER*.

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Juvenile Oyster Disease (JOD) has resulted in substantial losses of cultured Eastern oysters, *Crassostrea virginica*, in the northeastern United States. Despite management strategies utilizing selected lines and early deployment of hatchery-produced seed, JOD continues to occur annually in Maine's Damariscotta River. Further, during the past two years, mortalities have also been documented in three previously unaffected areas throughout Maine. In all years and locations, affected animals were extensively colonized by a novel species of marine α-proteobacteria (in the *Roseobacter* clade). We are currently evaluating a PCR-based diagnostic assay for detection of the JOD-associated *Roseobacter*. This assay uses specific primers to amplify the approximately 1300 base-pair internal transcribed spacer (ITS) region between the *Roseobacter* 16S and 23S rRNA genes. Direct amplifications from suspended cells are successful at concentrations as low as 1,000 cells per reaction. From sequence data and restriction length fragment polymorphism analyses, two genetic signatures are distinguishable. One is characteristic of the 1997–98 isolates, while the other is characteristic of the 2000–01 isolates. Thus PCR amplification followed by restriction enzyme digestion provides data regarding both the presence of, and specific genotype of, the JOD-associated *Roseobacter*. It is expected that this assay will be invaluable as a screening and diagnostic tool, and for regional management efforts to control the spread of JOD.

PRELIMINARY STUDY ABOUT FEEDING ECOLOGY OF THE ROCK LOBSTER, *PANULIRUS HOMARUS*, LINNAEUS, 1785, AT IRANIAN SEASHORES OF OMAN SEA.

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Preliminary aspects of feeding ecology of the rock lobster, *Panulirus homarus*, specimens collected by diving from January 1999 to November 2000 was considered. Stomach contents were recorded by using the point method. Fi and Pi of different prey items were measured. Monthly and seasonally frequencies of empty stomachs and different preys were compared using Chi-square tests. Monthly changes of macrobenthic communities sampled by diving were studied. Ivlev index used for comparing the importance of items between stomach contents and macrobenthics. Pearson correlation coefficient of different prey items against some hydrological factors was obtained. Spearman correlation coefficient was used for correlation between different preys against size groups, sexes and ovigerous females.

EVALUATION OF THE STRUCTURE AND FUNCTION OF A CREATED BLUE MUSSEL (*MYTILUS EDULIS*) REEF.

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In May 2001, blue mussels (*Mytilus edulis*) were collected from a donor site and transplanted into South Mill Pond, Portsmouth, NH, as part of a broad scale project to restore a degraded tidal salt pond. A total of six mussel reefs were established in two locations: three at the inner South Mill Pond (ISMP) and three at the outer South Mill Pond (OSMP). Two general parameters were used to evaluate the structure and function of the created reefs: (1) reef population dynamics (survivorship, size frequency distribution, density, and movement) assessed mussel response to transplanting and local pond conditions and (2) faunal utilization to evaluate the establishment of ecological functions for living marine resources (LMR). Mussel population dynamics were sampled once per month (June, July, August) using a random sample protocol. Mussels initially responded negatively to pond conditions (poor water quality; minimal tidal exchange), resulting in high mortality. Smaller mussels had a greater survival ratio as noted in a decreased average shell length. Survival and average shell length increased after water quality and tidal flow improved. Fish community dynamics were evaluated in reefs and reference areas through the summer (June, July, August). Reef areas had greater species diversity per sampling effort than reference areas under slow water conditions. *Apletes quadracus* and *Menidia menidia* were most common in reef areas. *Fundulus heteroclitus* and *M. menidia* were most common in reference areas. The constructed mussel reefs functioned as habitat for LMR's immediately, providing shelter and forage for small fish. Further assessment of utilization by other LMR's (invertebrates) is required to properly evaluate the functions and value of the created mussel reef.

GENETIC MONITORING OF OYSTER STOCK ENHANCEMENT IN THE CHOPTANK RIVER, CHESAPEAKE BAY.

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The spread of parasitic diseases (primarily MSX and Dermo), in conjunction with overharvesting, has led to the rapid decline of Eastern oyster (*Crassostrea virginica*) populations. Regional variation in disease resistance may be useful in restoration efforts. In collaboration with the University of Maryland Horn Point Laboratory, we have assessed the success of recent enhancement efforts within the Chesapeake Bay using genetic markers. *C. virginica* exhibits regionally diagnostic 16s mitochondrial DNA haplotype profiles (North Atlantic, South Atlantic, and Gulf Coast). In 1997 oysters from Louisiana broodstock were planted in the Choptank River, Maryland. The presence of newly settled spat with the Gulf Coast haplotype in the Choptank River confirms the survival and propagation of the Louisiana broodstock. An automated mini-sequencing technique (Pyrosequencing Inc.) was used to determine the mitochondrial haplotypes of oyster spat collected throughout the Choptank River. This rapid mass screening method revealed that 95% of spat collected were of the North Atlantic haplotype and approximately 5% were South Atlantic. Of 4538 spat screened, four (0.1%) possessed the Gulf Coast haplotype. The use of these genetic markers has enabled us to assess the survival, propagation, and dispersal of the Louisiana oyster stock within the Choptank River, Chesapeake Bay.

PROGRESS IN THE BIOLOGICAL CONTROL OF ZEBRA MUSSELS WITH MICROBIAL TOXIN.

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Recent progress in the development of bacterial strain CL0145A as a biocontrol agent of zebra mussels, *Dreissena* spp., is reviewed. Strain CL0145A is a North American isolate of *Pseudomonas fluorescens*, a ubiquitous, soil-water, Gram-negative bacterium, and a U.S. patent for its use for zebra mussel control has recently been issued. *Pseudomonas fluorescens* is not a parasitic species, and histological analysis indicates that mussels die from a biotoxin associated with strain CL0145A cells, not from infection. Therefore, future commercial products based on this microbe could contain only dead cells, further reducing environmental concerns. When zebra mussels ingest strain CL0145A cells, the biotoxin specifically destroys their digestive gland tissues. All zebra mussel sizes tested to date (ca. 1–30 mm long) are susceptible to kill by strain CL0145A, and high mussel mortality is achievable at all temperatures examined (range, 5–27°C). Static cultures currently produce cells of the highest toxicity, but shaking culture protocols are being developed in order to proceed to large-scale fermentation production. Very small-scale trials, designed to simulate flow-through conditions, have been conducted in temporarily-installed pipes within a hydropower facility, and they have confirmed that exposure to bacterial cells for 2 days at 23°C achieves high mussel kill. Evidence of nontarget safety has been demonstrated in laboratory and mesocosms trials. Current studies are focusing on biotoxin identification, fermentation scale-up, and identification of the key biotic and abiotic factors needed to maximize zebra mussel mortality.

CHARACTERIZATION OF A PARASITIC AMOEBA IN THE AMERICAN LOBSTER BY MOLECULAR SYSTEMATICS.

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Past mass mortality events of Long Island Sound (LIS) lobster (*Homarus americanus*) have been associated with a number of potential etiologies, one of which is neurologic infection by a parasitic amoeba. Histopathologic examinations of nerves and ganglia revealed tissue invasion by an amoeba, with and without attendant hemocytic infiltrates. This amoeba possessed a small, round, secondary nucleus differentially stained using the Feulgen technique. Transmission electron microscopic examination of nervous tissue confirmed the presence of this nucleus-like organelle, or Nebenkörper, a consistent feature of members belonging to the genus *Paramoeba* Schaudinn, 1896. Previous efforts to culture parasitic *Paramoeba* spp. and recent attempts to culture the lobster amoeba *in vitro* have been unsuccessful. Molecular data for the family Paramoebidae is absent, and the lack of such information contributes to the ambiguity associated with classification of these organisms. In this study we propose to characterize the rDNA of this parasitic *Paramoeba* by determining the molecular systematics of potentially related lobose amoeba. We have sequenced the small subunit (SSU) rRNA gene of twelve previously identified organisms that have morphologic (light microscopic and ultrastructural) similarity to the amoeba infecting lobster. This SSU rDNA has provided the data necessary to describe the current systematics of the Order Euamoebida using bioinformatic computer methods. Based on this rDNA sequence data, species of *Neoparamoeba and Korotnovella* occupy a separate clade between *Vannella* spp and the clade classically representing the Class Lobosa (*Acanthamoeba* spp and the leptomyxid amoebae). In addition, such rDNA nucleotide sequence data has allowed for identification of variable and conserved sequences that could be exploited for the purpose of amplifying SSU rDNA from the parasitic amoeba in lobster tissue. The rDNA sequence of this amoeba is expected to allow for molecular characterization using molecular evolution methods and provides the critical sequence elements necessary to develop primers and probes for future polymerase chain reaction (PCR) and in situ hybridization-based (ISH) diagnostic tests to detect the parasite in lobster tissue. These tests will be important to histopathologic and molecular diagnostics integral to future health surveillance programs.

SEASONAL CHANGES IN CELL PROLIFERATION OF OYSTER TISSUES.

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Identification of mitotically active tissues should assist development of cell lines from oysters and other bivalve molluscs. Except for evaluation of embryonic tissues, limited consideration has been given to identification of optimal tissue types and time of year to establish oyster cell lines. Thus, cell proliferation in tissues of the eastern oyster, *Crassostrea virginica*, was evaluated monthly for one year by an immunohistochemical assay for proliferating nuclear antigen (PCNA), an endogenous marker of cell proliferation that is evolutionarily conserved and present in all active phases of the cell cycle (G1, S, G2, M). A commercial monoclonal antibody to PCNA (PC10) was used to calculate a labeling index (percentage of labeled nuclei for 1000 nuclei counted) at 400× magnification for each tissue. We found highest proliferation in somatic tissues (labial palps > digestive diverticula and stomach epithelium > gills > mantle) in late fall to early spring (November-April in southern Louisiana). Cell proliferation ceased in these tissues in late Spring while proliferation in maturing gonads persisted. After spawning, proliferation resumed in somatic tissues for a month or two before gonad proliferation resumed in late summer. This agrees with earlier observations that cell proliferation in bivalves is highly variable throughout the year.

DEVELOPMENT OF A MEDIUM TO INDUCE HYPNOSPORE FORMATION AND ZOOSPORULATION OF *PERKINSUS MARINUS.*

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The difficulty in inducing zoosporulation of *P. marinus* hinders our ability to study zoospores. We recently developed a medium to induce hypnospore formation and zoosporulation of *P. marinus*. This medium was formulated in 3 steps by determining the effects of various solutions, individually and in combination, on the size, viability and zoosporulation of cultured parasites. The solutions tested included fluid thioglycollate medium (FTM) components (i.e., yeast extract, casein hydrolysate, dextrose, cystine, sodium thioglycollate, agar) in step 1; solutions of amino acids, carbohydrates, lipids, vitamins or nucleotides in step 2; and media supplements (lactalbumin hydrolysate, Hyprep 4601, pancreatin, Ex-cyte VLE, egg yolk, oyster lysate) in step 3. Solutions tested at each step which had significant positive effects on the enlargement, viability, and/or zoosporulation of cultured parasites were included in a medium for testing all solutions of subsequent steps. The size (35 ± 13 μm, N = 100), viability (95%, N = 400) and zoosporulation (35%, N = 400) of parasites incubated in our final medium for 6 days were significantly greater than the enlargement (11 ± 2 μm), viability (30%) and zoosporulation (0%) of parasites incubated in the FTM for 1 week. The initial mean parasite size was 4 ± 1 μm (N = 100). Interestingly, enlargement and viability of parasites incubated in our medium was similar to the enlargement (35 ± 9 μm) and viability (95%) of parasites incubated in oyster lysate. Zoosporulation (12%) of parasites incubated in oyster lysate was however significantly lower. The availability of *P. marinus* zoospores for study will enable investigations on the role of this cell stage in *P. marinus* life cycle.

THE EFFECTS OF *MARTEILIA SYDNEYI* ON THE HOST DEFENSE RESPONSES OF THE SYDNEY ROCK OYSTER, *SACCOSTREA GLOMERATA*. **Rodney Peters*** and **David Raftos**, Marine Biology Laboratory, Macquarie University, Sydney, Australia 2109.

Marteilia sydneyi (paramyxean parasite) is the causative agent of QX disease in *Saccostrea glomerata*, which has mortality rates of 90 to 98%. Outbreaks of QX disease have reduced oyster farming in some of the major oyster producing estuaries in New South Wales, Australia to critical levels. Once, farmers had a productive period of 2 years during which they could grow out, or "fatten," the oyster to a reasonable market size. Now this period lasts only from April/May to December of the same year in the QX contaminated rivers.

Invertebrates, according to all available evidence, lack antibodies and lymphocytes. However, they are still capable of mounting highly efficient cellular and humoral immune responses. Our premise is that these immune responses must be either evaded or overpowered by *M. sydneyi* to initiate QX disease. Specifically, our investigations have centered on proteolytic cascades, such as the prophenoloxidase (proPO) system, that in other species are closely associated with host defense. The prophenoloxidase cascade catalyzes the hydroxylation of tyrosine to dopa and the oxidation of dopa to dopaquinone, finally forming the pigment melanin. A number of intermediates in this pathway are cytotoxic or bacteriostatic, and melanin itself contributes to defense by encapsulating foreign material.

Data presented here describes an outbreak of QX disease on the Georges River, NSW during 2000/2001. A strict correlation was evident between infection intensity and prophenoloxidase activity. ProPO activities declined rapidly at precisely the time that infection was established. The data supports the contention that proPO activity is specifically suppressed by *M. sydneyi* in order to establish infection.

POPULATION BIOLOGY OF MELONGENID WHELKS IN THE INTERTIDAL ZONE IN WASSAW SOUND, GEORGIA. **Alan J. Power,*** **Mary Sweeney-Reeves**, **Todd C. Recicar**, **Dodie M. Thompson**, and **Randal L. Walker**, Marine Extension Service, Shellfish Research and Aquaculture Laboratory, University of Georgia, 20 Ocean Science Circle, Savannah, GA 31411-1011.

Four species of whelk (family Melongenidae) are found within the coastal waters of Georgia: the knobbed whelk, *Busycon carica;* the channeled whelk, *Busycotypus canaliculatus;* the lightning whelk, *Busycon cantrarium;* and the pear whelk, *Busycotypus spiratus*. These whelks are commercially harvested using trawls and are also taken intertidally by oystermen, clammers, and sport fishermen. To date the whelk fishery remains one of the most economically successful molluscan fisheries in the state. There is, however, a lack of fundamental biological information to allow for sound management decisions regarding the sustainability of the fishery. Consequently, the present study was initiated to examine the temporal variability in the abundance and population structure of each species in the intertidal zone in Wassaw Sound, Georgia. Whelks were collected at low tide from six locations, on a seasonal basis, over an annual period. Prior to releasing, all were tagged with an identifying label, measured (shell length, shell width, total wet weight), and sexed. Size frequency distributions, sex ratios, and sexual dimorphism in terms of body size were determined. Growth rates and seasonal migration patterns were examined by recapturing previously tagged specimens. The implications of these results for the whelk fishery in Georgia are discussed.

A RAPID METHOD FOR ASSESSING STRESS IN THE AMERICAN LOBSTER USING A HAND HELD GLUCOMETER. **Deanna L. Prince**, **Robert Bayer,*** **Christina Congleton**, **Shannon Colby**, **Danielle LaVine**, **Danielle Volmuth**, **Katrina Brooks**, **Margaret Berry**, and **William Congleton**, Department of Animal Sciences, and School of Marine Studies, University of Maine, Orono, ME 04469; **John Vetelino**, Department of Electrical and Computer Engineering, University of Maine, Orono, ME 04469.

A number of parameters were measured in lobsters exposed to temperature stress and anoxia from air exposure. Measurements included electrical resistance across the tail membrane, resistance across the body from mouth to anus, pH, and hemolymph calcium, magnesium, and glucose. Hemolymph glucose appeared to be the best indicator of stress as a result of an increase in hyperglycemic hormone. Based on these preliminary findings, a rapid technique was developed to measure hemolymph glucose levels using a commercial hand held glucometer available from any pharmacy. The technique involves centrifugation of the samples and placing the serum on a disposable strip, which is read by the glucometer. In a field sampling using this technique lobsters from areas that tended not to survive well in shipping and storage showed elevated blood glucose.

ROUTES OF *HEMATODINIUM* SP. TRANSMISSION INTO BLUE CRABS. Michael Sheppard,* Florian Rambow, Marc E. Frischer, and **Richard F. Lee,** Skidaway Institute of Oceanography, Savannah, GA 31411.

Hematodinium sp. is a parasitic dinoflagellate which causes mass mortality of blue crabs during infection peaks in late spring and fall in salt marsh estuaries of coastal Georgia. The life cycle of *Hematodinium* sp. in blue crabs involves several different stages including dinospores, prespores, trophonts and plasmodia [Shields, J.D., Ann. Rev. Fish. Dis. 4:241–271 (1994)]. A series of studies were carried out to determine the source and possible routes of transmission of *Hematodinium* sp. into blue crabs in the Wassaw Sound estuary system. Both histological and a recently developed molecular diagnostic techniques, including a quantitative real-time PCR method, were used to quantify *Hematodinium* sp. in crabs and in estuarine water samples. Three possible routes of transmission were investigated in these studies; consumption of infected tissues, injection of hemolymph containing *Hematodinium* sp., and exposure to estuarine water and sediments where infected crabs were found. Hematodinium disease was transmitted by all of these routes, but the most effective route was the transmission to healthy uninfected crabs after feeding on infected tissues from the diseased blue crabs. In several experiments there was a lack of transmission of *Hematodinium* sp. into crabs from water found to contain *Hematodinium*, which we speculate was due to lack of infectious forms in the water, e.g. dinospores. Partially supported by Georgia Sea Grant.

THE RELATIONSHIP BETWEEN BLACK SPOT DISEASE AND LIMB-LOSS IN *CANCER PAGURUS* FROM THE SHETLAND ISLANDS, SCOTLAND. Shelly M. L. Tallack, North Atlantic Fisheries College, Scalloway, Shetland Islands, ZE1 0TS, UK.

The edible crab, *Cancer pagurus*, is a heavily exploited crustacean resource throughout the UK, including the peripherally located Shetland Islands. Black spot disease is the primary infection recorded in the local population and is believed to be most prevalent in specimens with reduced immune systems, resulting from, for example, injury. The confrontational, minority fishing activity of removing only claws from this species in offshore Shetland waters has led to concerns regarding a possible increase in the number of severely injured crabs. Shetland's infection rates were compared with other UK studies.

Black spot disease rates were higher in Shetland than in studies on populations in Norfolk (Ayres & Edwards, 1982) and Wales (Davies, 1999), but lower than findings from Ireland (Vogan *et al.*, 1999). A positive relationship was shown between crab size and black spot disease. Greater infection severity was associated with later intermoult stages. Sex differences were evident with the proportion of infected individuals being higher in males (25.61%) than in females (12.78%). Finally, limb-loss and crab injury were positively correlated with infection.

It cannot be determined whether black spot disease rates in Shetland are linked to claw fishing activity rates. However, as vulnerability to black spot disease may be increased through injury, findings from the current study in addition to earlier research, imply the need for handling techniques which minimize severe limb-loss and injury in discarded crabs.

OYSTER GRAZING ON TOXIC AND NON-TOXIC *PSEUDO-NITZSCHIA* AND *THALASSISIORA WEISFLOGGII*, AND *DITYLUM BRIGHTWELLII*. Anne Thessen,*[1,2] Q. Dortch,[1] T. M. Soniat,[2] and **G. J. Doucette,[3]** [1]Louisiana Universities Marine Consortium, 8124 Hwy 56 Chauvin, LA 70344; [2]Biology Department, Nicholls State University, Thibodaux, LA 70310; [3]Marine Biotoxins Program, NOAA/NOS, Charleston LAB, 219 Fort Johnson Rd, Charleston, SC 29412.

Pseudo-nitzschia spp. are chain-forming diatoms that sometimes produce domoic acid, a potent neurotoxin that causes Amnesic Shellfish Poisoning (ASP). Despite high abundances of *Pseudo-nitzschia* over Louisiana oyster (*Crassostrea virginica*) beds, there have been no documented cases of ASP. Two possible explanations are that oysters cannot feed on long, pointed chains or they discriminate against toxic cells while grazing. Short-term (<2 hr) grazing experiments were conducted with non-toxic *P. pseudodelicatissima*, toxic *P. multiseries* (22–87 μm/cell, depending on species and clone; 4 cells/chain median chain length), *Thalassisiora weisfloggii* (15–23 μm), and *Ditylum brightwellii* (70–140 μm). Oysters (73 to 85 mm) were collected in the field, maintained on flowing ambient seawater, and then starved for 48 hours. Cultures were added at approximately 10^6 cells/liter to containers with individual oysters and to controls with no oysters or killed oysters. During experiments grazing was monitored by measuring decreases in *in vivo* fluorescence, but cell counts and volumes were also measured. Oysters grazed rapidly on all diatoms and cells appeared in feces within 1 hour. Grazing rates, based on fluorescence, were lower on both types of *Pseudo-nitzschia* than for the other diatoms, but those differences may not be sustained when grazing is based on cell volume/carbon.

HISTORIC EASTERN OYSTER *(CRASSOSTREA VIRGINICA)* DREDGE AND PATENT TONG SURVEYS IN THE MARYLAND PORTION OF THE CHESAPEAKE BAY.

Jessica M. Vanisko,* Mary Christman, and **Kennedy T. Paynter,** University of Maryland, College Park, MD 20742; **Stephen J. Jordan,** Maryland Department of Natural Resources (MD-DNR), Cooperative Oxford Laboratory, 904 S. Morris Street, Oxford, MD 21654.

The distribution of the Eastern oyster, *Crassostrea virginica,* in the Chesapeake Bay remains spatially variable. The densities often range from 0 to 250 oysters per m^2. The conversion of historic oyster surveys to spatial files (GIS) has provided the means to examine the potential relationships between oyster biomass and spat settlement, as well as past distributions of oysters in spatially complex ways. Extensive patent tong sampling was conducted by the Maryland Department of Natural Resources (MD-DNR) during two surveys from 1975 to 1979, and again from 1989 to 1995. Data were compared to determine if significant changes in the density of spat, small, and market oysters occurred during the time period between the two surveys. Changes in oyster density in Maryland can be attributed to environmental disturbances and the impacts of disease. Oyster biomass, as calculated from the MD-DNR dredge survey, was paired with patent tong samples in an effort to test for, among other things, a relationship between oyster biomass and spat settlement on several spatial scales. A significant relationship between biomass and spat settlement could not be established with the data available, however data from current sampling regimes are being analyzed for relationships between oyster density, disease incidence, and/or spat settlement.

HEMATODINIUM INFECTION IN BLUE CRABS, SPIDER CRABS AND STONE CRABS.

Anna Walker,* Department of Pathology, Mercer University School of Medine, Macon, GA 31207; **Michael Sheppard, Richard F. Lee,** and **Marc Frischer,** Skidaway Institute of Oceanography, Savannah, GA 31411.

Hematodinium sp. is a histoinvasive parasitic dinoflagellate. We report heavy infections of *Hematodinium* sp. in blue crabs (*Callinectes sapidus*) and spider crabs (*Libinia emarginata*) collected during late fall in a Georgia estuary. The prevalence of *Hematodinium* sp. in blue crabs and spider crabs ranged from 20 to 80% at different sampling stations. Prevalence of *Hematodinium* sp. in stone crabs (*Menippe mercenaria*) from this estuary was only 5% (n = 20). Both hemolymph and tissues were examined microscopically and by molecular techniques. Most infected crabs had a high intensity of infection (15 to 90% of the hemolymph cells were parasite cells). A sequence analysis from the 18S rRNA gene (1682 bp) of *Hematodinium* sp. from spider crabs and stone crabs showed 99.6 and 99.5% sequence similarity, respectively, to *Hematodinium* sp. from blue crabs. Infected crabs had interstitial infiltrates of parasites in all tissues, but most notably in gills, hepatopancreas, cardiac and striated muscle. Focal muscle necrosis was present in heavy infections. Following injection of infected hemolymph, tissues of healthy crabs demonstrated an influx of granular hemocytes with encapsulations.

SHELL MOVEMENT AND JUVENILE SURVIVAL OF THE OYSTER *CRASSOSTREA VIRGINICA* ON INTERTIDAL REEFS ADJACENT TO WATERS WITH INTENSE BOATING ACTIVITY IN THE INDIAN RIVER LAGOON, FLORIDA.

Linda Walters,* Kevin Johnson, Lisa M. Wall, and **Neysa Martinez,** Department of Biology, University of Central Florida, 4000 Central Florida Blvd., Orlando, FL 32816; **Ray Grizzle,** Jackson Estuarine Laboratory, University of New Hampshire, Durham, NH 03824.

Resulting from anthropogenic influences such as boat wakes or natural disturbance events, reefs of the eastern oyster *Crassostrea virginica* in the Indian River Lagoon have been declining in size in recent years. Additionally, dead margins (i.e. mounds of tightly packed, disarticulated shells extending above the high tide line) are commonly found adjacent to boating channels. To determine the impact of boating activity on: 1) shell movements, and 2) juvenile oyster survival, experiments were run on eight oyster reefs in Mosquito Lagoon, the northernmost region of the Indian River Lagoon, Florida. Four reefs had large dead margins, while the other four monitored reefs had no dead margins. To measure shell movement, 25 pre-weighed, oyster shells were deployed within $0.25m^2$ quadrants on the exposed, middle and protected regions of each reef and dispersal of individuals was assessed weekly for 8 weeks. Boat activity adjacent to the eight reefs was also recorded during the eight-week trials. There was a positive correlation between shell dispersal in the exposed regions of reefs and the average number of boats per hour that passed by the study reefs. Additionally, shells that weighed less than 50 grams and those which exceeded 100 grams experienced the least amount of overall movement, and the protected sides of the reefs experienced the least amount of shell movement, even for those reefs with dead zones. To determine the impact of shell movement on juvenile oyster survival, 1-cm diameter clay mimics were attached to 20 shells at the exposed, middle and protected regions on all eight reefs. At weekly intervals for the first 4 weeks of the 8-week trial, damage to the clay was recorded and used as a proxy to estimate damage to *C. virginica* at this vulnerable stage in its life-history.

DEVELOPMENT AND TESTING OF A SIMPLE FIELD SYSTEM FOR MONITORING MUSSEL SHELL GAPE SIZE.

Win Watson, Steve Jury, Jennifer Wishinski, Dan O'Grady, Walter Golet, Darren Scopel, Heidi Pye, and **Chris Rillahan,** Zoology Department and Center for Marine Biology, University of New Hampshire, Durham, NH 03824.

Mussels and other bivalves are known to vary their shell gape size in response to changes in various environmental conditions such as salinity, current or the presence of contaminants. In fact, several research groups have developed biomonitoring systems based on measuring the shell status of freshwater bivalves. The purpose of our project was to develop and test a simple and relatively inexpensive system for measuring the shell gape size of estuarine and saltwater mussels (*Mytilus edulis*) in their natural habitat. Battery-powered Hall-Effect sensors were used to detect the gape size of mussels and their output, in volts, was logged at 10 sec intervals onto a HOBO datalogger. The datalogger, batteries and customized electronics were placed in a waterproof case inside a buoy that communicated with mussels on the bottom via a waterproof cable. Monitoring systems were deployed at 6 different locations in the Great Bay Estuary, NH and along the N.H. coastline. Data obtained was examined to determine if changes in gape size were correlated with changing tides, light levels and/or ambient concentrations of heavy metal contaminants. Laboratory studies, under more controlled conditions, were used to determine their response thresholds to these same stimuli. While the system developed proved very sensitive and reliable, the complex factors influencing the gape size of mussels made it difficult to definitively explain all the variations in gape size recorded from mussels in their natural habitat. This study was supported by CICEET and Gulf Watch grants to W.H.W.

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Erratum 299
Abstracts of technical papers presented at the 5th International Conference on Shellfish Restoration. Nanaimo B.C., Canada, September 18–20, 2001 301
Abstracts of technical papers presented at the 55th Annual Meeting of the Pacific Coast Oyster Growers Association & National Shellfisheries Association, Silverdale, Washington, September 20–22, 2001 321
Abstracts of technical papers presented at the 22nd Annual Meeting of the Milford Aquaculture Seminar, Milford, Connecticut, February 25–27, 2002 329
Abstracts of technical papers presented at the 94th Annual Meeting of the National Shellfisheries Association, Mystic, Connecticut, April 14–18, 2002 357

COVER PHOTO: Carpet shell clam, *Tapes decussatus* (Linnaeus, 1758). The species is of Atlantic-Mediterranean origin and is distributed along the Euro-African coast from England to Senegal and throughout the Mediterranean with penetration in the Red Sea. While the introduced short-necked clam or striped venus (*Tapes philippinarum*) has almost superseded the carpet clam in Italian waters, *T. decussatus* is grown commercially in Sardinia. Photo courtesy of Consorzio Promozione Prodotti Ittici, Italy.

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Huayong Que and Standish K. Allen, Jr.
Hybridization of tetraploid and diploid *Crassostrea gigas* (Thunberg) with diploid *C. ariakensis* (Fujita) 137

Pedro Saucedo, Carmen Rodríguez-Jaramillo, and Mario Monteforte
Microscopic anatomy of gonadal tissue and specialized storage cells associated with oogenesis and spermatogenesis in the calafia mother-of-pearl oyster, *Pinctada mazatlanica* (Bivalvia: Pteriidae).............. 145

Alan J. Power and Randal L. Walker
Growth and gametogenic cycle of the blood ark, *Anadara ovalis* (Bruguiére, 1789) in coastal Georgia 157

J. A. Rodríguez-Valencia, and F. Caballero-Alegría
Temporal fluctuations (1989–1999) in the populations of *Haliotis fulgens* and *H. corrugata* (Gastropoda: Haliotidae), at Islas San Benito (Baja California, Mexico) 163

M. Kruatrachue, P. Laimek, C. Wanichanon, V. Linthong, P. Sretarugsa, E. S. Upatham, and P. Sobhon
Development of the nerve ganglia of abalone, *Haliotis asinina* Linnaeus.............. 173

Ingrid Bahamondes-Rojas and Marta Bretos
Induction of spawning and early development in *Fissurella picta* (Mollusca: Archaeogastropoda) from southern Chile. 185

Ludwig C. A. Naegel and Chris J. Cooksey
Tyrian purple from marine muricids, especially from *Plicopurpura pansa* (Gould, 1853) 193

Dominique Sud, Jean-Marc Poncet, Armelle Saïhi, Jean-Marc Lebel, Dominique Doumenc, and Eve Boucaud-Camou
A cytological study of the mantle edge of *Haliotis tuberculata* L. (Mollusca, Gastropoda) in relation to shell structure. 201

Getian Wu, Kangsen Mai, Beiping Tan, and Wei Zhu
Dietary biotin requirement of juvenile abalone, *Haliotis discus hannai* Ino.............. 211

Nicholas G. Elliott, Jason Bartlett, Brad Evans, and Neville A. Sweijd
Identification of southern hemisphere abalone (*Haliotis*) species by PCR-RFLP analysis of mitochondrial DNA........ 219

Alberto de Jesús Navarrete
Distribution and abundance of *Strombus costatus* (Gmelin, 1791) larvae and adults at the biosphere reserve: Banco Chinchorro, Quintana Roo, Mexico.............. 227

O. R. Chaparro, A. E. Soto, and C. E. Bertran
Velar characteristics and feeding capacity of encapsulated and pelagic larvae of *Crepidula fecunda* Gallardo, 1979 (Gastropods, Calyptraeidae).............. 233

Oscar Efraín Holguin Quiñones and Jesús Emilio Michel-Morfín
Distribution, density, and length-weight relationship of *Chiton articulatus* Sowerby, 1832 (Mollusca-Polyplacophora) on Isla Socorro, Revillagigedo Archipelago, Mexico 239

Beatriz Novoa, Carolina Tafalla, Angel Guerra, and Antonio Figueras
Cellular immunological parameters of the octopus, *Octopus vulgaris*.............. 243

H.-Jörg Urban and José M. Riascos V.
Estimating gonado-somatic indices in bivalves with fused gonads 249

Arnold G. Eversole and Yavuz Mazlum
Comparative fecundity of three *Procambarus* species.............. 255

D. J. Macintosh, J. L. Overton, and H. V. T. Thu
Confirmation of two common mud crab species (genus *Scylla*) in the mangrove ecosystem of the Mekong Delta, Vietnam 259

Biplob Das, Yusuf Sharif Ahmed Khan, Khan Towhid Osman, Pranab Das, and Md. Nurul Amin
Physico-chemical changes in acid sulfate soil during semi intensive culture of *Penaeus monodon* fabricius, in cleared mangrove areas of The Chakaria Sundarbans, Bangladesh 267

Craig S. Lawrence, Noel M. Morrissy, Philip E. Vercoe, Ian H. Williams, and Yuk W. Cheng
Physical, chemical, and biological variation among freshwater crayfish (*Cherax albidus* Clark, 1936) research ponds .. 273

Fernando Negrete-Soto, Enrique Lozano-Álvarez, and Patricia Briones-Fourzán
Population dynamics of the spiny lobster *Panulirus guttatus* (Latreille) in a coral reef on the Mexican Caribbean 279

Roy Melville-Smith and Yuk Wing Cheng
Responses of migrating western rock lobsters *Panulirus cygnus* (George, 1962) to two different tagging release procedures.............. 289

A. Battison, R. Cawthorn, B. Horney, and A. Mackenzie
Mushroom tyrosinase as a control material for phenoloxidase assays used in the assessment of crustacean "health" 295

The Journal of Shellfish Research is indexed in the following: Science Citation Index®, Sci Search®, Research Alert®, Current Contents®/Agriculture, Biology and Environmental Sciences, Biological Abstracts, Chemical Abstracts, Nutrition Abstracts, Current Advances in Ecological Sciences, Deep Sea Research and Oceanographic Literature Review, Environmental Periodicals Bibliography, Aquatic Sciences and Fisheries Abstracts, and Oceanic Abstracts.

CONTENTS CONTINUED ON PREVIOUS PAGE

JOURNAL OF SHELLFISH RESEARCH

Vol. 21, No. 1 JUNE 2002

CONTENTS

Kenneth Chew
Honored Life Member: John B. Glude 1

Craig L. Appleyard and Joseph T. DeAlteris
Growth of the northern quahog, *Mercenaria mercenaria,* in an experimental-scale upweller 3

Bassem Allam, Kathryn A. Ashton-Alcox, and Susan E. Ford
Flow cytometric measurement of hemocyte viability and phagocytic activity in the clam, *Ruditapes philippinarum* 13

M. J. Stewart and R. G. Creese
Transplants of intertidal shellfish for enhancement of depleted populations: preliminary trials with the New Zealand little neck clam 21

Edward Fahy, Maria Lyons Alcantara, Mark Norman, Ronan Browne, Vincent Roantree, and Nick Pfeiffer
Mortalities in *Ensis arcuatus* (Jeffreys) (Solenacea) in western Ireland 29

Michael L. Zettler
Ecological and morphological features of the bivalve *Astarte borealis* (Schumacher, 1817) in the Baltic Sea near its geographical range 33

R. W. Penney and M. J. Hart
Is survival genotype-dependent in North American populations of farmed blue mussels, *Mytilus* spp? 41

Qin-Feng Gao, Zheng-Lin Wang, Wai-Hing Wong, and Siu-Gin Cheung
Effects of food quality and quantity on feeding and absorption in black-ribbed mussels, *Septifer virgatus* (Wiegmann) (Bivalvia: Mytilidae) dominating wave-exposed habitats in Hong Kong 51

K. R. Freeman, E. Kenchington, and S. P. MacQuarrie
Comparative settlement depths of *Mytilus edulis* C. Linnaeus, 1758 and *M. trossulus* Gould, 1850: I. A. mesocosm study 59

E. Kenchington, K. R. Freeman, B. Vercaemer, and B. MacDonald
Comparative settlement depths of *Mytilus edulis* C. Linnaeus, 1758 and *M. trossulus* Gould, 1850: II. Field observations 67

Anthony S. Anderson, A. Lelania Bilodeau, Matthew R. Gilg, and Thomas J. Hilbish
Routes of introduction of the Mediterranean mussel (*Mytilus galloprovincialis*) to Puget Sound and Hood Canal 75

Julie A. Maguire, Maurice O'Donoghue, Stuart Jenkins, Andrew Brand, and Gavin M. Burnell
Temporal and spatial variability in dredging induced stress in the great scallop *Pecten maximus* (L.) 81

Marcel Fréchette and Gaétan Daigle
Reduced growth of Iceland scallops *Chlamys islandica* (O.F. Müller) cultured near the bottom: a modelling study of alternative hypotheses 87

Rosalío Maldonado-Amparo and Ana M. Ibarra
Ultrastructural characteristics of spermatogenesis in diploid and triploid catarina scallop (*Argopecten ventricosus* Sowerby II, 1842) 93

Marcial Villalejo-Fuerte, Marcial Arellano-Martínez, Bertha P. Ceballos-Vázquez, and Federico García-Domínguez
Reproductive cycle of *Spondylus calcifer* carpenter, 1857 (Bivalvia: Spondylidae) in the "Bahía de Loreto" National Park, Gulf of California, Mexico 103

Young Jin Chang, Min-Do Huh, Myung-Joo Oh, and Yoshio Sugawara
Baculovirus-like particles in epithelial cell of digestive diverticula of the scallop, *Patinopecten yessoensis* 109

I. Sunila, N. A. Stokes, R. Smolowitz, R. C. Karney, and E. M. Burreson
Haplosporidium costale (seaside organism), a parasite of the eastern oyster, is present in Long Island Sound 113

Kwang-Sik Choi, Kyung-Il Park, Ki-Wan Lee, and Kazumi Matsuoka
Infection intensity, prevalence, and histopathology of *Perkinsus* sp. in the Manila clam, *Ruditapes philippinarum,* in Isahaya Bay, Japan 119

Pedro Saucedo, Ilie Racotta, Humberto Villarreal, and Mario Monteforte
Seasonal changes in the histological and biochemical profile of the gonad, digestive gland, and muscle of the calafia mother-of-pearl oyster, *Pinctada mazatlanica* (Hanley, 1856) associated with gametogenesis 127

CONTENTS CONTINUED ON INSIDE BACK COVER

JOURNAL OF SHELLFISH RESEARCH

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IN MEMORIUM
HAROLD HALEY HASKIN
1915–2002

Hal Haskin died on June 23, 2002 at the place he loved best—his Cape Shore cottage on Delaware Bay. Most of his 87 years–since he spent the summer after his junior year at Rutgers working on oyster drills—had been devoted to teaching and research on the marine environment, and Delaware Bay was its focus. Hal's "Honored Life Member Biography" appeared in 1999 in Volume 18(2) of the Journal of Shellfish Research. Here we reflect more on his character and recount memories of the man.

Hal was born in 1915 at Niagara Fall, NY to George and Laura Haley, the second of three children. Three years later, the children were orphaned when both parents and a grandmother died within a week of each other in the 1918 flu pandemic. A family friend, Frederick Haskin, adopted Hal—an unusual arrangement, particularly for the time, because Fred Haskin was a bachelor. Haskin was a pipefitter, a job that forced him to travel around the country, so he lodged young Hal with a retired farm family living in southern New Jersey, near the DuPont chemical plant where he sometimes worked. The daughter of the family, whom Hal knew as Aunt Jenny, supervised the home and became his *de facto* mother. Later, Aunt Jenny and Fred Haskin married so that Jenny officially became the mother that she had unofficially been throughout most of his childhood. It wasn't until many years later that Hal became reacquainted with his many Haley relatives still living around Niagara Falls and for the first time was called "Uncle Hal".

Always a good student and with an unusually strong work ethic, Hal graduated from Rutgers College in 1936, the first student to do so with a perfect grade score. It was at Rutgers that he came under the tutelage of Thurlow Nelson and began his life-long fascination with oysters. During summers spent investigating the predatory oyster drills, Hal observed that the snails preferred young oysters to older ones. Curious about mechanisms of chemoattraction, he entered Harvard to work with John Welsh on this phenomenon, but he spent his first summer as a graduate student at the Bermuda Biological Station studying lobster neurohormones. He grew a beard, which came in red, was introduced to sailing, and acquired a taste for his research subjects.

Hal switched to algal physiology for his PhD dissertation, which he carried out under the supervision of the oceanographer Alfred Redfield. His work included the development of a method for estimating chlorophyll concentrations using spectrophotometry—a precursor to the present-day Strickland and Parson's method. At Harvard, Hal supported himself as a dorm proctor and a teaching assistant. He was a natural teacher who excelled at hands-on instruction in both the laboratory and the field.

Upon receiving his PhD in 1941, Hal entered the US Army and spent the next 5 years training troops and supervising units guarding the coast from Long Island to the Virginia Capes. He returned to Rutgers as an assistant professor in the Department of Zoology in 1946 and began developing a research program devoted to marine bivalves. His early work involved culturing and rearing of hard clams on a grant from Campbell's Soup Company. In 1950, however, he succeeded his mentor Thurlow Nelson as head of the Oyster Investigation Laboratory in the New Jersey Agricultural Experiment Station, and for many years thereafter devoted most of his research efforts to protecting and enhancing the oyster resource of Delaware Bay. Although he later became deeply involved in acquiring data on, and developing a management plan for, offshore surf clams, his heart and mind never strayed far from oysters and Delaware Bay. From his early efforts to institute harvest limits on the depleted natural seed oyster beds, through the devastation brought by the MSX parasite, to providing management data when populations later rebounded, Hal considered the Bay's oysters to be his personal responsibility. He brought to this and other tasks a unique combination of intelligence, scientific integrity, and the ability to work with people from all sides of a problem. He never shirked from controversy, but he was always armed with reams of data, which he carried in whiskey boxes in the back of his car, to backup his point of view. Hal took pains to make the data understandable to both the regulators and the fishermen. He spoke his mind, albeit diplomatically, and he rarely lost a battle.

One ongoing stuggle, which he inherited from Thurlow Nelson, was maintaining freshwater flow into the Delaware estuary. In this battle against diversions and dams, he had the enthusiastic participation of his wife, Peg, whom he had met when she was a recent graduate of Smith College and he was a graduate student at Harvard. The two of them eventually became a formidable team fighting for rational water management in New Jersey, particularly concerning those policies affecting Delaware Bay and its oyster populations.

Although he ran a large research laboratory, Hal devoted much of his time to teaching. As a young assistant professor, he taught mainly undergraduate courses in general biology, limnology, animal physiology, and invertebrate zoology. Somewhat later, he developed graduate courses in coastal oceanography, estuarine ecology, and malacology. Most of the graduate students in the Rutgers Zoology Department took at least one of these courses at some point in their academic careers—and never forgot them. Hal did not confine his teaching to the classroom, nor did he limit it to official students. He delighted in showing anyone the anatomy of a shucked oyster or talking about the intricacies of an oyster community. His audience might be a businessman or an oyster grower—it didn't matter—his enthusiasm captivated them all. Anyone who was associated with Hal in any capacity always wound up considering himself or herself a student. And Hal, himself, never stopped being a student.

Hal retired in 1984 but he remained active long thereafter. During his nearly 50-year career, he touched many people—students, colleagues, university and government officials, and shellfish producers. More than 200 of them gathered at the Haskin Shellfish Laboratory on September 28, 2002 to remember him. People came from all along the eastern seaboard and from as far west as Colorado to tell stories, share memories, renew acquaintances, look at old photographs, and eat good food—including freshly shucked oysters farmed at the Cape Shore near the Haskin cottage.

His first and last graduate students were there, as were a host of students in between. They recalled how taking one of his courses had been the stimulus to go into marine science or shellfish biology. Several remarked on how tough a questioner he was during thesis defenses and how his criticisms were right on the mark, sometimes requiring a return to the bench to do a critical experiment. Although Hal was remembered most for applied research that directly benefited the shellfish industry, his intellectual curiosity was profound. He insisted that applied research conform to the standards of basic research. Further, how could one provide sound scientific advice for management without understanding fundamental biological and ecological principals? Woe to any student or colleague who failed to convince Hal that he or she knew the basic scientific principles underlying a concept.

Students remembered his Saturday courses and that he worked well into the night on Friday, and often into the early morning hours of Saturday, preparing. The classes officially ran from 8 in the morning to 5 in the evening, but leaving at 5 was viewed with a highly disapproving eye. Several students remarked on his indifference to physical discomfort on the field trips—and that he was equally indifferent to discomfort of the students. The discomfort usually took the form of "cold and wet" or "seasick", but on one occasion it was much more serious. On an oceanography class field trip, Hal lacerated his hand severely on the pulley wheel of an outboard motor that he was attempting to start. The deep cut ran along the base of his fingers and down the outside of his palm. Flapping fingers indicated severe damage to his hand, but he merely wrapped it in a handkerchief and was fully prepared to continue the trip. At the overwhelming insistence of his students, however, he reluctantly canceled the excursion in favor of a trip to the hospital and several hours of microsurgery to rejoin the tendons leading into two fingers.

Neighbors, who grew up with the Haskin children at the Cape Shore, and whose fathers were businessmen and auto mechanics, couldn't figure out what Hal did for a living. He was like a kid in an adult body who actually got paid for mucking about on the tide flats playing with oysters. But he could answer all the questions they had about the organisms they found in the bay and along the shore.

Hal was an advocate of simplicity. He was not interested in "building an empire" because he felt that he would then have to spend all his time supporting and defending it—certainly much less stimulating to him than the hands-on field and lab activities that he relished. Colleagues and students remembered him as a quiet, low-keyed person who was a good listener. He was thoughtful and one knew that he had truly reflected upon a question or an issue before he gave advice.

One of the fondest memories, shared by nearly all the students, was of the biannual "tray moves". These events took place each spring when trays of selectively bred oysters had to be moved from winter quarters, where they were protected from ice damage, onto the tidal flats in front of the Cape Shore Laboratory where they were exposed to disease pressure and could be easily tended. Each fall, the reverse move took place. All able hands—students, faculty, technicians, friends—were marshaled for the event because the oysters in each tray—and there were often more than 100 trays—had to be counted, samples removed, and the oysters had to be placed in freshly prepared trays. The weather was usually cold and miserable, and ones fingers quickly became numb. Tying lines on the trays so that they could be hung in the marina that served as winter quarters was especially challenging under these conditions. Hal would check every single knot (four per tray, half-hitches, not bowlines) and retie most, while being silently cursed by the small group of students standing around on the dock—cold, wet, tired, and anticipating the reward that awaited them at the Haskin cottage where Peg was preparing the traditional turkey dinner and a fire was blazing in the wood stove. Fifteen, twenty, occasionally up to thirty famished people would arrive. The first order of business was the preparation and distribution of gin and tonics, and if Hal made them, they contained plenty of gin. People pitched in to set the table, make the salad, and cook the peas, but certain tasks were Hal's own province: making the gravy, mashing the potatoes, and carving the turkey. These jobs had to be performed in a very specific fashion, with Hal describing to anyone within earshot his way to successful gravy or mashed potatoes. The evening ended with homemade (by Peg) pie—cherry, pumpkin, blueberry, and pecan—along with ice cream, coffee and tea, and very muted memories of the earlier discomforts. The tray moves were planned to end on Saturday night when the turkey dinner was held. Sunday morning one could sleep late, but not so late as to miss the "flapper" breakfast—again at the Haskin cottage with Hal busy making blueberry pancakes. The tray moves eventually became very popular events that attracted ex-students and often, their friends, back to the Cape Shore for a weekend of work and good fun.

Hal's publication record was modest and belies his research accomplishments. He was totally disinterested in maintaining a CV. In fact, it was difficult to locate citations, among his own files, to list in this remembrance. His exacting standards applied to writing—both style and content—and to his own as well as that of others. He had difficulty with the concept of publishing a paper that didn't tell a complete story, and he usually felt that he needed more data than he had. This was particularly true for field studies, even though his data sets extended for years, and, in some cases, for decades. Although Hal always analyzed the results and used them in meetings or to advise management agencies, formal reporting was less interesting than starting a new project or going on a field expedition. Students recalled late night sessions in which everyone pitched in to help copy and assemble reports at the very last minute—typically well after the deadline. Nevertheless, Hal had little difficulty obtaining funds to run his laboratory because grant managers knew the quality of the data would be high—when they eventually received it.

When he wasn't working, which often seemed like never, Hal had three great recreational passions: woodcutting, sailing, and growing lilacs. For years, Hal and Peg heated their house in southern New Jersey and their Cape Shore cottage with wood stoves. Their house in Piscataway had multiple fireplaces. Hal always carried a chain saw and splitting wedges in the trunk of his car in case he had an hour or two free to cut wood. Friends recalled that he cut down only dead or dying trees and knew exactly when they should be cut to maximize dryness and minimize decay.

The principal Haskin sailboat was a 17 ft Thistle that was anchored on the tidal flats in front of the summer cottage, and sailed as often as possible at high tide with Hal at the helm and a boat full of family, friends, and students from the Cape Shore Laboratory, just down the beach. Annual beach parties were a chance for sailboat races between the Thistle and any other sailboat that could be pressed into service.

More than 100 lilac bushes representing 70 different varieties, formed a fragrant hedge around the Piscataway house. Hal knew all the varietal names and delighted in escorting visitors around the hedge, "introducing" them to each plant and pointing out its unique characteristics. Each May when the lilacs bloomed, he would bring buckets full of blossoms into the lab to distribute among the staff and faculty. He stored them in the cold room, which temporarily at least, smelled delightfully of lilacs rather than aged oysters.

The memorial service included tributes from representatives of numerous agencies, organizations, and institutions that Hal served, including the National Shellfisheries Association. He joined NSA in the late 1940s and rarely missed a meeting for almost 50 years. He was Vice President in 1966–67, and President during 1967–69. He became an Honored Life Member in 1979 and, most precious to him, was given the David Wallace Award in 1984.

In addition to Peg, Hal is survived by five children, Kathleen Haskin of New York City, Jean Haley of North Kingstown, RI, Elizabeth Haskin of Cape May Court House, NJ, Frederick James Haskin of Piscataway, NJ, and Mary D. Haskin of Washington, DC; and four grandchildren, Harold Haskin II, a student at Rowan University, Allegra and Elijah Penny, of Washington, DC, and William Schroer of Cape May Court House, NJ.

Hal, alone and with Peg, received numerous awards over the years (see Kraeuter and Ford, 1999. J. Shellfish Res. 18: 337–339), but one of the finest was presented at the memorial service: The New Jersey Nature Conservancy named a portion of its Delaware Bayshores Tract near the Cape Shore cottage "The Harold and Margaret Haskin Nature Preserve." It was an entirely fitting honor for a lifetime of dedicated service to environmental teaching and preservation.

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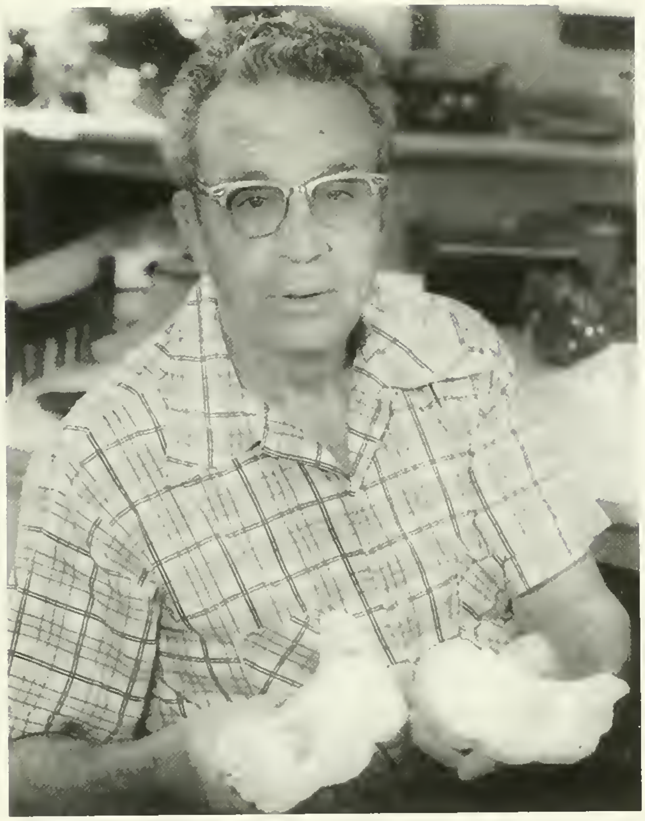

Jay Donald Andrews
Honored Life Member

Jay Andrews, affectionately known as "Andy" by his many friends and colleagues, is widely respected for his fundamental research on the ecology of the major oyster pathogens in the Chesapeake Bay, *Haplosporidium nelsoni* and *Perkinsus marinus*. Less well known, but equally important, are his 22-y dataset on oyster spatfall patterns in the Chesapeake Bay and the disease management strategies he developed for industry.

Andy was born on September 9, 1916 in Bloom, Kansas. He grew up on a wheat and cattle farm in western Kansas south of Dodge City during the Depression and drought of the 1930s. Andy attended high school in Bloom and was the top student in a class of 10. Studies were apparently easy for him and he spent much time reading Zane Gray wild-west stories and following baseball scores and players. When Andy graduated from high school in 1934, the nation was in full depression, and the western plains were in a severe ten-year drought that was making farming very difficult, if not impossible. Lack of moisture prevented raising wheat or fodder for cattle. Over time, Andy remembers, pastures of the family farm were filled with annual weeds, prickly pear cactus, and an explosion of jackrabbits. After graduation, Andy's father took him to a bank in Dodge City, borrowed $100 and sent him to Kansas State College in Manhattan. College opened a new world for Andy, and he worked hard. In addition to classes, he worked 40 hours each month in the state 4-H Club for 25 cents an hour and participated in the ROTC, which paid $15 a month. He was designated the top agricultural student and obtained a degree in agriculture in 1938, but farming in western Kansas was not an attractive proposition for Andy. Instead, he began graduate study at the University of Wisconsin in Madison, where he obtained a teaching assistantship in biology. He earned a salary of $600 a year and had money for extracurricular activities for the first time. His love of opera developed during these years. Andy earned an M.S. in 1940 and continued on for his Ph.D.

Andy's graduate studies were interrupted by World War II; he was called up in January 1941 and spent four years in the infantry. He saw action in the South Pacific at the battle of Leyte Gulf and the battle of Bataan and survived multiple kamikaze attacks on his troop ship. In 1946, he returned to Madison to finish his degree, which he earned in 1947. In the spring of 1946, Andy and fellow student, Willard Van Engel, attended a fisheries meeting in St. Louis, where they met the Director of the Virginia Fisheries Laboratory in Yorktown, VA (the precursor of VIMS), who offered them jobs that they both accepted. Van Engel was requested to work on blue crabs, and Andy was asked to concentrate on oysters. A few months later, he was advised by a visiting professor from Yale not to work on oysters, because there was already a large literature and "everything had been done already." Thankfully, Andy ignored the advice. When Andy and "Van" arrived in Virginia in the fall of 1947, they learned that the three scientists who had been at the laboratory had left that summer for positions at Texas A&M. They had been working with mussels on some compound important to the war effort. The three scientists were John Mackin, Sewell Hopkins, and Winston Menzel.

Shortly after he arrived in Virginia, Andy began monitoring oyster spatfall patterns in the tributaries. After years of monitoring, it became clear that setting patterns could be easily separated into two distinctive types. The large tributaries and the Chesapeake Bay proper require very large stocks of brood oysters to acquire regular spatfall because of the large tidal exchange and flushing. Only the upper James River estuary met this requirement and that was because of its special circulation patterns and the large beds of transplanted oysters in the lower James being grown by private industry. Such other large rivers as the York, Rappahannock, and Potomac never had enough broodstock to produce regular spatfall, but occasional large sets did occur. The small tributaries, with low runoff, exhibit a completely different setting pattern than the large tributaries. These coastal plain subestuaries have moderate annual setting patterns, even though oyster populations are low because of high larval retention caused by the winding channels and shallow flats. Uncharacteristically, Andy never published these data on spatfall patterns. Beginning in 1950, Andy tried to persuade managers in Virginia to use the small estuaries to produce seed oysters for transplantation to larger tributaries, but this advice was ignored until long after he retired. When the reef restoration strategy was initiated in Virginia in 1996, the first few reconstructed oyster reefs were placed in these small tributaries because of the larval retention patterns that Andy had documented.

Andy's research on oyster diseases began when John Mackin visited VIMS from Texas to determine if *Perkinsus marinus* (Dermo disease) was present in Chesapeake Bay oysters. The discovery of this pathogen in an area lacking oil drilling confirmed that oyster mortality in the Gulf of Mexico was the result of *P. marinus*, not the oil industry. Andy produced fundamental papers on the epizootiology of Dermo disease during the 1950s, and his 1988 review on the pathogen is a classic paper still widely read and cited. When *Haplosporidium nelsoni* (MSX disease) appeared in the Chesapeake Bay in 1959, Andy immediately began conducting research on this organism and produced many seminal papers on the ecology of this pathogen as well as on *Haplosporidium costale*. One of Andy's most important traits was that he published his results in a timely manner. In 1960, Andy initiated a monitoring program for *H. nelsoni* at VIMS using imported susceptible oysters. This program continues to the present day and provides a 42-year database on *H. nelsoni* prevalence and intensity that has proved invaluable for examining climate effects on pathogen abundance. Because of his broad training and experience and his inquisitive nature, Andy was very insightful. For example, he was the first to hypothesize, in 1980, that *H. nelsoni* was an introduced pathogen. It wasn't until 2000 that molecular data were obtained that support his supposition.

Andy worked hard and expected hard work of others. You arrived on time, and you didn't leave early. Andy loved the rigors of fieldwork and scoffed at suggestions for an easier way to do things. He hauled oyster trays by hand and cleaned them by throwing countless buckets of water that he dipped by hand. After such trips he returned to the campus at VIMS wet, covered with mud, shirtless, in shorts, and barefoot. He surely startled more than one unsuspecting graduate student.

Andy is internationally recognized for his fundamental research on oyster diseases, but he was also a long-time educator. During his career at VIMS he taught Ichthyology, Field Biology, Taxonomy and Systematics, Marine Ecology and Biologic Oceanography.

Andy was a capable and energetic volleyball player. In the "interesting" lunch time volleyball games at VFL/VIMS he played with gusto, spiking near net-balls down the throats of opposing players with great vigor and glee. Road trips with Andy were always an experience. Once, while driving north for a meeting, Andy spied a walnut tree along the road. He pulled over and took an old pair of coveralls out of the trunk. He tied off the end of each leg, walked over to the tree and began filling the legs with walnuts that had fallen to the ground, commenting that he hoped we would not be shot by the owner.

Andy is an avid vegetable gardener and has a large plot near VIMS. He can still be seen hauling countless old milk jugs full of water in his old truck during drought periods and undoubtedly still cursing the varmints that take half his crop.

Andy is a long-time member of the National Shellfisheries Association and served as President and Editor. He was elected Honored Life Member in 1983. In 1998, he received the David Wallace Award in recognition of his career-long efforts to use science for the betterment of shellfish management and a sustainable industry.

Eugene Burreson
Gloucester Point, Virginia

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Neil F. Bourne
Honored Life Member

Neil was born August 11, 1929 in London, Ontario, Canada. He began his elementary school education there, and it was in London, mainly through the influence of his father, that he became interested in natural history and particularly bird watching, a hobby and passion that have continued to the present. It was this interest in natural history that eventually led him to become a biologist. The family moved to Hamilton, Ontario in 1939, where he completed his elementary and high school education. By the time he moved to Hamilton, he had over 150 bird species on his life list! He entered McMaster University in Hamilton in 1948 and graduated with a BSc in biology in 1952. In 1953, he completed a MSc degree at McMaster under an Ontario Research Scholarship and in 1953–54 spent a year in Germany, where he undertook studies in limnology at the Freshwater Institute in Ploen and marine biology at the University of Kiel. He returned to Canada in 1954, entered the University of Toronto, and graduated from there with a PhD in 1959. His thesis was entitled, "Determination of carbon transfer from *Chlorella vulgaris* Beyerinck to *Daphnia magna* Strauss using radioactive carbon (C14) as a tracer." Three years of his studies were supported by National Research Council of Canada scholarships.

On completion of his PhD in March 1959, Neil joined the staff of the Fisheries Research Board of Canada at the Biologic Station in St. Andrews, New Brunswick working under the direction of the late Dr. J. C. Medcof, a former Honorary Member of NSA. At St. Andrews, he was in charge of the sea scallop investigation that included investigations of the basic biology and ecology of sea scallops, dynamics of sea scallop populations on Georges Bank and in the Bay of Fundy, scallop gear efficiency trials, and exploratory work to locate sea scallop resources in other areas along Canada's Atlantic coast. While at St. Andrews, Neil spent two weeks at Dr. V. Loosanoff's laboratory in Milford, Connecticut studying bivalve breeding technology. Thus began a life-long commitment to shellfish aquaculture. He used knowledge gained at the Milford laboratory to begin initial attempts to culture sea scallops and was successful in conditioning and spawning adults and raising larvae to the mature stage.

In 1965, Neil transferred to the Pacific Biologic Station in Nanaimo, British Columbia, first working for the Fisheries Research Board of Canada and later with the Department of Fisheries and Oceans. Initially, he worked with the late Dr. Dan B. Quayle, another former Honored Member of NSA, and remained at the Pacific Biologic Station until his retirement. During his career at the Pacific Biologic Station, Neil undertook research studies on a wide range of molluscan subjects, including the basic biology of several Pacific coast molluscan species, population studies of commercially important clams, harvesting gear efficiency, and studies to develop techniques to culture several species. He developed techniques to predict Pacific oyster spatfall accurately for the industry, a service he continues to provide. He undertook extensive surveys of bivalve resources in B.C. and, in particular, followed the dispersal of Manila clams northward along the coast. As a result of these studies, a fishery for Manila clams became established in the central coast of B.C.

As a result of previous work and the influence of his stay at the Milford laboratory, Neil remained intensely interested in bivalve culture and continued studies in this field. Initial studies were undertaken to investigate the potential of butter and littleneck clams and abalone. He undertook studies with Manila clam culture that were important in establishing the Manila clam culture industry that exists in B.C. today.

From 1981 to 1991 Neil led a program to investigate the feasibility of scallop culture in B.C. Several species were studied, but the decision was made to work with the Japanese scallop, *Mizuhopecten yessoensis*, and methods were developed to produce juveniles in a hatchery and raise them to commercial size within a period of two years. Much of the information culminated in the publication, *A Manual for Scallop Culture in British Columbia*, which continues to be used by many to the present day. Results of the work led to a private company building a scallop hatchery and beginning commercial scallop culture in B.C. He continues to serve as an advisor to this industry.

Neil has been active in foreign aid work, assisting at Universities and has been involved with several scientific societies. He worked for the Canadian International Development Agency for 1 1/2 years in Fiji, where he served as Director of a Fisheries Training Program at the University of the South Pacific in Suva. He undertook an assessment of the Cuban oyster industry for the Department of Fisheries and Oceans and advised on a joint Canada–Cuba program to expand oyster culture in Cuba. He worked for the United Nations Food and Agriculture Organization in the Peoples' Republic of China and continues to serve as a volunteer advisor to the Canadian Executive Service Organization. He served on the Aquatic Resources Subcommittee of the Science Council of British Columbia from 1987–2002 and was chairman from 1991 to 2002.

He is an affiliate professor at the University of Washington, School of Fisheries and has been a committee member for over 20 graduate students, mostly at the University of Washington, but also at the University of Victoria, the University of British Columbia, and Simon Fraser University.

Neil served on the executive boards of the Canadian Society of Zoologists, the World Aquaculture Society, the Aquaculture Association of Canada, serving as President of the latter Society from 1987–1988. He joined the National Shellfisheries Association (NSA) in 1961 and has been active in the affairs of the society since then. He served on the Executive Committee of the Association from 1974–1983, including tenure as President in 1981–1982; he was the first Canadian to serve as President of NSA. He has been a member of the editorial board of the *Journal of Shellfish Research* for many years. He was made an Honored Life Member of the Association in 1990 for his exemplary service to NSA and the profession. In 1991, he received the David H. Wallace award for promoting understanding, knowledge, and cooperation among industry members, the academic community, and all levels of government and for his outstanding success in bringing together shellfish scientists and industry officials for the benefit of shellfisheries.

Neil received an Award of Merit from the Department of Fisheries and Oceans in 1993 for an exceptional and distinguished contribution to the effectiveness and efficiency of the Public Service and the Department, particularly for contributions to the aquaculture industry of British Columbia. Neil retired from the Department in March 1994.

In May 2000, Neil was presented with an Honorary Lifetime Achievement Award from the Aquaculture Association of Canada. The selection board cited long-time service to the Association, the research community, industry, and young scientists as exemplary examples of the goals of that organization. He was the first recipient of this award.

Neil lives in Nanaimo and continues to work at the Pacific Biologic Station as a volunteer Scientist Emeritus, publishing results of past and current work and encouraging young scientists in their careers. He still has time to enjoy his grandchildren, hike, work around the house, and undertake other activities. He is keenly interested in golf and is a rabid bird watcher with the distinction of having birded on all seven continents. One activity in his retirement is to compile his bird life list, he believes he has seen about 2,500 species of birds! Those who have golfed with him have noted that he never lets golf interfere with birding although the opposite has occurred on occasion.

Kenneth Chew
University of Washington
Seattle, Washington

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Victor G. Burrell
Honored Life Member

Dr. Victor (Vic) G. Burrell, Jr. has been a member of the National Shellfisheries Association (NSA) for over 30 years and served as its president from 1982–1983. He was born in Wilmington, NC in 1925 and served in the U.S. Navy during WW II from 1943–1946. He received a B.S. degree in English from the College of Charleston in 1949. In 1965, after 15 years in his family's meat-packing business, he went on to earn both Master's Degree and a Ph.D. Degree in Marine Science from the College of William and Mary in 1968 and 1972, respectively. In large part, this interest may have been kindled through his experiences operating a charter boat in South Carolina and working part time in the commercial oyster industry. While a student at Virginia Institute of Marine Science (VIMS), he served as the liaison to the fishing industry, especially with oystermen. At that time, he developed a method of denaturing the red algal-derived seasonal color of oysters using the blower system.

In 1972, he left the staff of the VIMS and returned to the Carolinas as an Associate Marine Scientist with the then just established Division of Marine Resources in Charleston. He spent from 1972 to 1991 at the Marine Resources Research Institute (MRRI) of the Division of Marine Resources, South Carolina Department of Natural Resources (then the Wildlife and Marine Resources Department). He served as the Institute's Director from 1974 to 1991, "retiring" in 1991. Since then, he has continued to take an active role in South Carolina's marine resources, including co-authoring numerous scientific and lay review publications for NOAA and the DNR as an emeritus director and scientist at Fort Johnson. In total, he has published over 65 scientific papers on zooplankton ecology, shellfish biology, and oyster culture. These include a series for NOAA in 1996–1997 entitled "The History, Present Condition, and Future of the Molluscan Fisheries of North and Central America and Europe, Volumes 1–3, with Clyde Mackenzie and others. In 2000, he authored a DNR educational report entitled "The Recreational Fishery in South Carolina: the Little River Story," reviewing the first organized offshore recreational fishing industry in South Carolina. He is also currently writing a history of South Carolina's oyster industry for publication.

During his leadership at MRRI, Dr. Burrell saw the staff double to about fifty professional individuals, including eleven Ph.D-level marine scientists. In addition to his active participation in the NSA, Dr. Burrell served also as president of the Southeastern Estuarine Research Society (SEERS) and was named an honorary life member of SEERS. He served as president of the College of Charleston Alumni Association and was honored as the college's Alumnus of the Year in 1963. He also saw the development and construction of the SC Aquarium serving on the executive board of the aquarium as a founding member.

He is listed in Community Leaders of America, Personalities of the South, American Men and Women of Science, International Directory of Distinguished Leadership, Who's Who in the South and Southwest, Who's Who in America, and Men of Achievement. He recently moved to new digs and we will miss him walking along the road tipping his hat and waving his hand as he traveled daily to MRRI from his James Island house. However, we still expect to see him almost daily in his office (when he is not taking a vacation cruise) chatting with everyone and being the gentleman that he is. He is an aficionado of big band swing and jazz, particularly that from the Depression era through post-war periods. We remember his enjoyment of Ken Burn's "Jazz" and discussed with him the pros and cons of Burn's choice of influential "characters," such as Louis Armstrong. He's knowledgeable enough about the music and musicians of that era to do a creditable history. He's been an active member of St. James Episcopal on James Island for many years, serving as Junior and Senior Warden on several occasions.

Vic and his wife Katherine have four children, eight grandchildren, and four great-grandchildren.

Loren D. Coen
M. Yvonne Bobo
MRRI
SCDNR
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Original works of art surround the artist and NSA Honored Life Member, Herb Hidu, at his home in Alna, Maine.

Herbert Hidu
Honored Life Memeber

Herb has traveled an unusual and convoluted path to distinction in American shellfisheries science. He was born in 1931, the son of a German immigrant and a 2nd generation Hungarian farmer and estate manager. The family never had the slightest inclination for academia. He spent his youth hunting, fishing, and observing the pleasantries of living on a Connecticut rich man's estate as a cottage inhabitant. He joined the army in 1951 and for 2 years was a paratrooper with the 82nd airborne division, which gave him his mindset for later years. After his hitch, his drinking buddy made him aware of Korean G.I. Bill eligibility. Entering the University of Connecticut, he majored in fisheries management and forestry (B.S., 1958). Along the way he had the opportunity to resume his parachuting paranoia, working a summer as a smokejumper at the United States Forestry Service base in Winthrop, Washington, during which time he made four fire jumps.

At UConn, Dr. Slater of the Entomology Department convinced him to pursue a graduate degree in the biological sciences. He received a MS degree in Zoology in 1960 from Penn State University, working under the tutelage of Dr. Edwin L. Cooper on population structure of largemouth bass in a farm fish pond.

Then, for some unexplained reason, Herb accepted a job in the alien science of shellfisheries biology, working for Dr. Victor Loosanoff at the U.S. Bureau of Commercial Fisheries shellfish laboratory in Milford, Connecticut. His position entailed conducting bioassays with shellfish larvae. The 3-year experience taught him the intricacies of shellfish hatchery culture and, perhaps more importantly, perseverance. Indeed, having endured the extremely confining environment created by the very intense Russian scientist Loosanoff, the world was now Herb's oyster and the oyster was now Herb's world.

The Mid-Atlantic MSX oyster mortalities of the mid-1960s provided Herb an excellent avenue for advancement. Rutgers University and Dr. Harold Haskin were in great need of workers (students) who were versed in hatchery techniques. Efforts were concentrated on the production of experimental disease-resistant stocks. This became Hidu's "bread and butter" as a graduate student, where for over 3 years he begat many of Rutgers' experimental oysters stocks at the Cape May laboratory. He used the abundant larval stocks to conduct his study on laboratory behavior and field recruitment of Delaware Bay oysters. His 1967 Ph.D thesis is cited to this day as the definitive study on Delaware Bay oyster recruitment.

After Rutgers, Hidu became a shellfisheries research biologist at the Chesapeake Biological Laboratory (CBL) in Solomons, Maryland. It was hoped that he would fill the shoes and carry on the traditions of the late Francis Beavin, the "dean" of Chesapeake Bay oyster science. With four older veteran shellfisheries biologists, he pursued, for 3 years, field shellfish surveys of Chincoteague Bay and the effects of power plant effluent on the early life history stages of Chesapeake Bay oysters. He developed the natural feeding method for shellfish hatcheries in Maryland and stimulated an early commercial hatchery (Frank Wilde, Shadyside, MD). However, he was miscast in the position at CBL, which he viewed as a mixture of research and personnel management. As an advocate of hatchery production of shellfish to augment recruitment, he locked horns with the Maryland public oyster fishery. Furthermore, his lack of contact with students at the University of Maryland's College Park campus, 100 miles distant inland, created for Herb an untenable situation and he decided it was time to move on. His lifetime break came in 1970 when the University of Maine's Darling Marine Center entered the Federal Sea Grant Program with a "Coherent Areas" grant entitled "Culture of Resources in a Cold Water Marine Environment". Hidu was offered and accepted a position that required shellfish hatchery expertise and, more importantly to Herb, allowed access to excellent students who would pursue M.S. and Ph.D degrees in Oceanography while accomplishing the Sea Grant aquaculture research.

The 15+ graduate students Hidu sponsored at the University of Maine won a record 5 NSA Thurlow Nelson Awards and contributed significantly to the shellfisheries literature: blue mussel biology, R. Lutz, L. Incze, G. Podniesinski; triploid shellfish, S. Allen, M. Lee; intertidal shellfish capacity adaptations, R. Gillmor; hatchery pathology and techniques, L. Plunket, M. Logue–Keller, R. Clime, and others. In the 1970s Hidu developed the nation's first mariculture course. That field course and the associated aquaculture extension effort helped stimulate a significant new Maine industry in oyster grow-out and hatchery production. For this, in 1990, he received the

University of Maine's Presidential Public Service Award and, in the eyes of those of us who know Herb well, he took his place as Maine's "father of aquaculture".

His own research resulted in over 75 papers, with topics ranging from hatchery techniques to field recruitment of oysters, gregarious setting, biological fouling control, triploid shellfish, air winter storage of shellfish seed, blue mussel field recruitment, effects of pollutants on shellfish larvae, and behavior of shellfish larvae. He ran the gamut of offices in the National Shellfisheries Association (NSA), becoming President in 1980. He won the NSA David Wallace award in 1991 and became an NSA Honored Life Member in 1994. Herb retired in 1992 and is now Professor Emeritus of Animal and Veterinary Science at the University of Maine.

In retirement, Herb has gravitated toward his old roots with a move from aquaculture to horticulture, maintaining a small farm in Alna, Maine at which he raises commercial Hosta plants. In fact, his farm bears a remarkable resemblance to a certain Connecticut rich man's estate, with extensive trimmed pine hedges and espaliered trees. In 2001 he was a retired smokejumper volunteer in the Bob Marshall Wilderness Area of Western Montana working trails and helping build a wilderness bridge. He is active in golf, watercolor art, and woodlot management. He has been married 47 years to Judith and has 3 children and 4 grandchildren, all of whom live in Maine. He is the man to whom the author of this biography owes his career—thanks, Herb.

Richard A. Lutz
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Sandra E. Shumway
Honored Life Member

Sandra "Sandy" Shumway was born March 29, 1952 in Taunton, Massachusetts. She attended Mulcahey Grammar School before going on to Taunton High School, where she graduated as Salutatorian in 1970. A tomboy as a youngster, she was more interested in cowboys and Indians, football and baseball than dolls. She began piano in the second grade, but it was not her instrument. In fourth grade, she took up the clarinet and later bass clarinet and played first chair in the All-State Band twice. While continuing to play throughout college, music was for fun and science would win out. From the time that school let out until Labor Day, summers were spent at the family summer home in Portsmouth, Rhode Island. She dug her first clam by the age of three, and as she got older she fished, sailed, and tended a few lobster pots. She spent countless hours collecting marine life and cataloging it (unlike other normal children). She began a small science project by the 5th grade and was involved in science fairs thereafter. She regularly won local and regional fairs and took first place at the Massachusetts State Science Fair at MIT during her junior year. In her senior year, she was named a Ford Future Scientist of America and to the NASA Youth Science Congress. She credits her parents and several tolerant and progressive teachers for nurturing her scientific interests.

Thus, her love of the sea and its creatures has carried over into her adulthood, and it is not surprising that she majored in this field in college. Sandy graduated *Summa Cum Laude* in 1974 with a B.S. in Marine Science/Biology from Southampton College, Long Island University. From there, she went on to the University College of North Wales in Bangor, Gwynedd, Wales as a Marshall Scholar, where she received her Ph.D. in 1976 and later a D.Sc. in 1992. As a post-graduate she continued at the Marine Science Laboratories in Menai Bridge, Gwynedd, North Wales, then at the University of Otago in Dunedin, New Zealand and with the Department of Ecology and Evolution, State University of New York at Stony Brook.

In addition to her investigations in Wales and New Zealand, Sandy has also conducted research in laboratories in Brazil and numerous states including Georgia, Maine, Washington, and North Carolina. Her formal teaching experiences have been limited to several levels at Southampton College, but she teaches constantly wherever she goes, and she has served on thesis committees at the University of Maine, the College of William and Mary, University of Washington, College of Charleston, North Carolina State University, University of South Florida, University of Maine, University of Connecticut, and Rutgers University. She has served as an External Examiner for Ph.D. candidates at various universities in Australia, Canada, England, India, New Zealand, and South Africa.

Her travels have taken her far and wide. After returning from abroad, where she spent 1974–1980 in Wales, New Zealand and Brazil, Sandy spent two years at Stony Brook and then moved north to the Maine Department of Marine Resources as a Research Scientist from 1983 to 1993 and an adjunct scientist at the Bigelow Laboratory for Ocean Sciences. After this, she moved back to Southampton College and spent the next seven years as a Professor of Marine Science and Biology. In 2001, she moved to the Department of Marine Science at the University of Connecticut in Groton, where she is presently an Adjunct Professor in Residence.

Sandy's research covers more than 27 years in shellfish biology, filter feeding, and physiological ecology of marine invertebrates. She pioneered the study of impacts of harmful algae on shellfish and introduced the use of flow cytometry to determine particle selection in filter-feeding invertebrates. A primary focus has been on problems associated with the shellfish industry: for example, distribution of toxins in shellfish tissues, detoxification rates, and timing and extent of toxicity between shellfish species with the goal of establishing species-specific closures and helping the industry to prosper in the presence of harmful algal blooms. She has recently worked with scientists at North Carolina State University to determine the impacts of *Pfiesteria* spp. on shellfish and public health. Two of these collaborators, Drs. JoAnn Burkholder and Howard Glasgow, recently named a new species of dinoflagellate, *Pfiesteria shumwayae*, in her honor. Sandy has a permanent illustration of it with her at all times.

Sandy has authored 120 publications, edited two books, and co-authored a shellfish cookbook. She has served as editor of the National Shellfisheries Association's *Journal of Shellfish Research* since 1986 and also edits the *Journal of Experimental Marine Biology and*

Ecology. In addition, she serves on the editorial boards of several other journals. Although this takes an incredible amount of time, she finds it satisfying to see the results in print and says it also gives her a fantastic overview of the fields of shellfish biology and experimental marine biology. The number of manuscript submissions to JSR from foreign countries has increased dramatically in recent years, and Sandy enjoys helping scientists from underdeveloped countries get their work published. She recently launched a new journal with Elsevier, *Harmful Algae*, co-edited by Ted Smayda.

Sandy has been responsible for much of the recent growth of the National Shellfisheries Association. When she became Editor of the *Journal of Shellfish Research*, the publication was often behind schedule and barely 100 pages a year. Since then, the JSR has averaged 600–700 pages annually and reached a new level with the publication of five issues and more than 1,700 pages in 1998. She was the first woman president of NSA, serving in 1991–1992, and was re-elected in 2002 to serve again in 2003–2004. She has worked to increase membership in the association and has played an important role in the annual meetings of NSA with her organizational skills, her management of students for registration, sales, and as AV operators, and for the student auctions, which she initiated in 1993. These auctions raise money for the Student Endowment Fund, which helps defray the costs of students who attend the annual meetings. She has good-humoredly dressed for these occasions in various costumes, including a court jester, a blue quahog, a geoduck, and a lobster. On another occasion, she made her own bikini top, with the help of a hotel employee, from a pair of sea scallop shells, and has actually sold the shirt(s) off her back. In her never-ending effort to nurture students, in 1992, Sandy began the Recruits, the organization of student members of NSA. The Recruits now have their own forum on the NSA website, a nonvoting representative on the EXCOM and write articles for the Newsletter.

Sandy has also held several offices in the World Aquaculture Society and was the push that NSA needed to become a sponsor of the Aquaculture Triennial Meetings, first as an affiliate sponsor of Aquaculture '86 and then as a full sponsor of Aquaculture '89 in Los Angeles. These meetings have allowed NSA members to meet with members of the World Aquaculture Society, the American Fisheries Society Fish Culture Section, and many other smaller societies. Since that first meeting, NSA has enjoyed the intellectual and financial advantages that these larger meetings provide, and Sandy has been a major part of the organization and production of each of these including San Diego, Las Vegas, two in Orlando and the upcoming AQ '04 in Hawaii.

Despite her seemingly endless efforts with students, journals, scientific meetings, and the shellfish industry, Sandy still has time to spend with her miniatures which she began building in 1982. She is a nationally recognized miniaturist, yet many of the people who know her are unaware of her love of the small. Students are particularly surprised to find out that she devotes time to things other than science. Her buildings have been featured in several magazines including *Miniature Gazette, Nutshell News, International Dollhouse*, and *Miniature Collector*. "Sandra Shumway's Sea-Born Treasures" by Mary Kaliski in the April 1998 issue of *Miniature Collector* is a wonderful example. At one time, she ambitiously wanted to build an entire village in miniature (1 inch = 1 foot), but she has had to settle for less because of lack of time and space. Thus far, however, she has created a general store, two fishing shacks, a bordello, a bakery, a drug and candy store, a barber shop, a school house, and a funeral parlor (all complete with people).

Sandy is also an avid pool player. She began when she was in college, but became more serious about it when she moved to Maine. She not only loves the game for what it is, but for the opportunity it provides to interact with fishermen. She organized local tournaments for years and has a number of trophies to show for her efforts. Often after a day of paper sessions and a dinner at the annual NSA meeting, she will find a local pool hall to unwind. Of all the honors and awards she has received over the years, one of her most prized recognitions came from a well-seasoned, cynical fisherman in a local bar who, when asked "Who's the broad at the pool table?" replied "She's a scientist, but she's okay."

Sandy was awarded NSA's highest honorary citation, the Honored Life Member award at Aquaculture '01 in Orlando, Florida on January 18, 2001 following the eloquent remarks of Dr. Melborne R. Carriker, who stated that "it is her warm outgoing personality, genuine interest in people, and readiness to extend an unselfish helping hand to those in need that has endeared her most to so many of us."

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EVALUATION OF MIST ALERT™ RAPID TEST KITS FOR THE DETECTION OF PARALYTIC AND AMNESIC SHELLFISH POISONING TOXINS IN SHELLFISH

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ABSTRACT Rapid test kits (MIST Alert™) for the detection of paralytic shellfish poisoning (PSP) and amnesic shellfish poisoning (ASP) toxins in shellfish have recently been developed. In this study, these kits have been evaluated for their potential use in shellfish toxin-monitoring programs and by the shellfish industry. These antibody-based tests were used to assess the presence of shellfish toxins qualitatively in a variety of shellfish species (mussels, scallops, oysters, cockles, razor fish) while routine methods of detection were simultaneously used to quantify any toxin present. All shellfish extracts found to contain PSP toxins at the regulatory limit of 80 μg saxitoxin equivalents (STX eq) 100 g^{-1} shellfish flesh using the mouse bioassay (MBA) were confirmed as positive by MIST Alert for PSP. Shellfish farmers and other professionals in the industry also used these test kits successfully, with all positive samples being correctly identified, clearly demonstrating its potential application in shellfish harvest management and end product testing. MIST Alert for ASP also detected toxin in all monitoring samples containing the regulatory limit for amnesic shellfish poisoning (ASP) toxins, 20 μg g^{-1} shellfish flesh, as determined by high-performance liquid chromatography (HPLC). In addition, among samples in which HPLC did not detect toxin, the kit agreed in 99% of tests. Overall, these results suggest that MIST Alert™ for PSP and ASP could be used as part of routine monitoring programs.

KEY WORDS: paralytic shellfish poisoning toxins, amnesic shellfish poisoning toxins, MIST Alert, shellfish monitoring, mouse bioassay, high-performance liquid chromatography

INTRODUCTION

Recently we have reported on the use of a commercial antibody-based rapid test kit (MIST Alert™ for PSP) for the qualitative (yes/no) detection of paralytic shellfish poisoning (PSP) toxins in shellfish (Mackintosh et al. 2002). These potent neurotoxins are produced by certain algal species and can accumulate in filter-feeding shellfish. Current European legislation requires that shellfish are monitored for these toxins, and if they are detected in shellfish flesh above the regulatory limit of 80 μg saxitoxin equivalents (STX eq) 100 g^{-1} shellfish flesh, restrictions on shellfish harvesting are imposed (Gallacher et al. 1998). The initial study (Mackintosh et al. 2002) highlighted that the MIST Alert for PSP kit could be used in monitoring programs as a prescreen for toxin-negative samples and in end-product testing and thus warranted further investigation.

Since our preliminary assessment of MIST Alert for PSP a further antibody-based qualitative test for amnesic shellfish poisoning (ASP) toxins has also been developed (MIST Alert™ for ASP). This kit utilizes sheep antibodies developed by Garthwaite et al. (1998) for the detection of neurotoxic domoic acid (DA), the principal compound of ASP toxins, produced by several species of the diatom *Pseudo-nitzschia*. MIST Alert for ASP utilizes the same working principals of immunoflow chromatography used in MIST Alert for PSP (Fig. 1).

The first recorded outbreak of human ASP, following consumption of contaminated mussels, was recorded in 1987 (Wright et al. 1989). Subsequently, many countries implemented monitoring programs for the detection of ASP toxins in shellfish with a regulatory level of 20 μg DA g^{-1} shellfish flesh adopted. In the UK, the Scottish shellfishery has been particularly adversely affected by ASP toxin contamination of king scallops which was responsible for widespread harvesting closures during 1999 and 2000 (Campbell et al. 2001, Gallacher et al. 2001).

To date, the main tools for PSP and ASP toxin-monitoring programs have been the mouse bioassay (MBA) and the high-performance liquid chromatography (HPLC) method of Quilliam et al. (1995) with UV diode array detection, respectively. The use of mice for the detection of PSP toxins is considered ethically questionable, and the assay is known to be susceptible to matrix interference (Schantz et al. 1958, Park et al.1986). Chemical analysis for ASP toxins is expensive and requires skilled operators. Hence, the requirement for simple, cheap, quick, and accurate detection methods for PSP and ASP toxins in shellfish have been widely discussed (Gallacher et al. 1998, Garthwaite 2000, Llewellyn et al. 2001).

The aim of this study was to evaluate further the use of MIST Alert for PSP in regulatory monitoring, as a harvest management tool, and end-product test by the shellfish growing and processing industries and to assess the potential of MIST Alert for ASP in routine shellfish monitoring.

MATERIALS AND METHODS

Assessment of MIST Alert for PSP During Routine Shellfish Monitoring

Throughout 2001, the MBA was used for the quantitative assessment of PSP toxicity (μg STX eq 100 g^{-1} of shellfish flesh) in acidic (0.1 M HCl) shellfish extracts (n = 547) as part of the Scottish shellfish-monitoring program. Simultaneously, MIST Alert for PSP test kits (Jellett Biotek Ltd., Nova Scotia, Canada) were used for the yes/no detection of PSP toxins and results compared with those from the MBA in four categories: not detected, <40; 40 – <80; and ≥80, μg STX eq 100g^{-1}· as previously described (Mackintosh et al. 2002).

Investigation into the Use of MIST Alert for PSP in Shellfish Harvest Management and End-Product Testing

MIST Alert for PSP test kits were supplied from Fisheries Research Services (FRS) to eight participants to evaluate its po-

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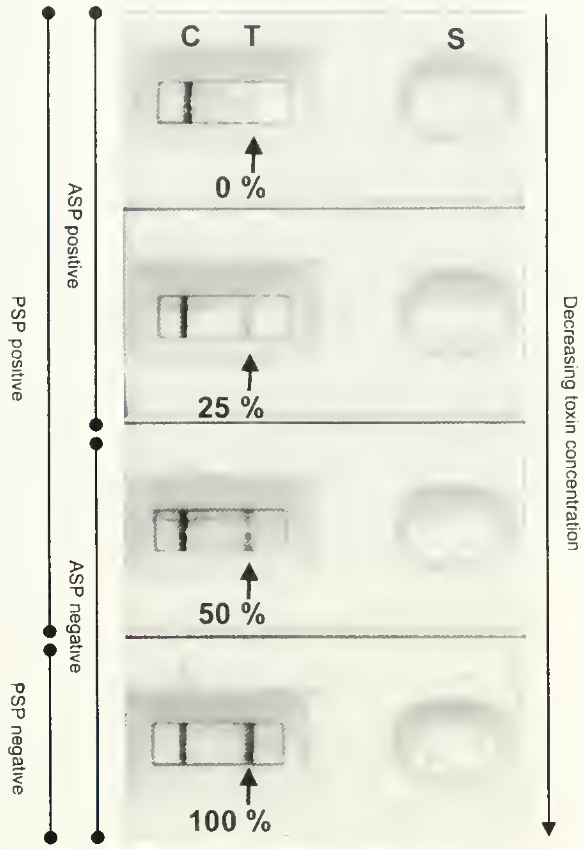

Figure 1. The MIST Alert™ test kit. Test kit strips consist of an absorption pad (S), a membrane striped with a mixture of toxin analogs (the "T" line), and an antibody detection reagent (the "C" line), a sample pad, and a conjugate pad containing the antibodies. A visible "T" line indicates absence of toxin in the sample and no line indicates the presence of toxin. The "C" line indicates that the sample fluid has sufficiently resuspended and mobilized the antibody color complex. As the level of toxin increases, the "T" line progressively disappears. Remnant "T" lines are scored as having 50, 25, or 0% of color intensity relative to the "C" line. MIST Alert for PSP™—samples are considered positive if the 'T" line is 50% or less the intensity of the "C" line. MIST Alert for ASP™—samples are considered positive if the "T" line forms only a faint shadow (i.e., less than 25%) or is completely absent.

tential for field use. Participants comprised shellfish farmers, processors, and environmental health officers (EHOs). Each participant was supplied with either 50 (farmers) or 100 (processors and EHOs) test kits and the equipment/chemicals necessary to perform acidic shellfish extraction as described by the manufacturer. Before the study, each participant received training in sample extraction and kit use. Subsequently, each participant assessed the presence/absence of PSP toxins in shellfish extracts using the test kits. An aliquot of each shellfish extract analyzed, the test kit, and the participant's interpretation of the result were returned to FRS. The kit result was reinterpreted by laboratory personnel and extracts reanalyzed by MIST Alert for PSP by an experienced user within the laboratory. Positive samples were further analyzed by the MBA. If this was not possible (e.g., if the sample volume supplied from the participants was too small), samples were diluted 1:1 in a negative shellfish extract and retested using MIST Alert for PSP.

Assessment of MIST Alert for ASP During Routine Monitoring

Shellfish samples (500) from the 2001 Scottish shellfish-monitoring program were extracted in aqueous methanol, [50:50 (v/v) water:methanol] according to the extraction procedure of Quilliam et al. (1995) and as detailed in Hess et al. (2001). Extracts were concurrently analyzed by MIST Alert for ASP (Jellett Biotek Ltd., Nova Scotia, Canada) and HPLC (Quilliam et al. 1995). MIST Alert for ASP is performed as for MIST Alert for PSP, but samples are only considered positive if the "T" line is completely absent or appears only as a faint shadow (i.e., less than 25%) (Fig. 1). The qualitative presence of ASP toxins by the test kits was compared with the quantitative analysis of these toxins (DA + epi DA = total amnesic shellfish toxins: AST) by HPLC categorized into five groups: not detected, the limit of detection (lod) that is about 2.5 μg AST g^{-1} shellfish flesh, >lod – <6, 6 – <20, and ≥20 μg AST g^{-1} shellfish flesh.

Reproducibility of MIST Alert for ASP

Fifty aqueous methanol extracts obtained from a variety of different shellfish species (mussels, *Mytilus edulis*; cockles, *Cerastoderma edule*; queen scallops, *Aquipecten opercularis*; Pacific oyster, *Crassostrea gigas*; king scallop, *Pecten maximus*—whole animals, gonad, adductor, residual tissue—gut, hepatopancreas, gills) were tested in triplicate using MIST Alert for ASP to investigate the reproducibility of the test kits. Extracts contained varying concentrations of AST, as determined by HPLC, and were chosen to contain levels within the five categories above.

RESULTS

Use of MIST Alert for PSP in Routine Shellfish Monitoring

Results from 547 shellfish extracts tested by MIST Alert for PSP were compared with results obtained using the MBA (Table 1, Fig. 2). All extracts ($n = 77$) determined to contain toxin concentrations above the regulatory limit (80 μg STX eq 100 g^{-1}) by the MBA were also found to be positive for PSP toxins by MIST Alert for PSP. In addition, test kits gave a positive result for 97% (131/135) of samples in which the MBA detected toxin below the regulatory limit (grouped as 40 – <80 and <40 μg STX eq 100g^{-1} in Fig. 2). The kit did not detect toxin in two mussel extracts (*Mytilus edulis*), a king scallop gonad (*Pecten maximus*), and a European oyster (*Ostrea edulis*) extract, the mussel extracts both being in the <40 μg STX eq 100 g^{-1} category.

The MBA did not detect toxin in 335 shellfish extracts. Using MIST Alert for PSP, 97 of these samples gave a positive result (29%) giving an overall "false-positive" rate of 18%. Of these samples, king scallop extracts (both gonad and whole animal) gave a higher proportion of MIST Alert PSP positive results (35 and 53%, respectively) than mussel extracts (25%), the most commonly tested species in the PSP toxin monitoring program.

Field Trials of MIST Alert for PSP

Results from 259 shellfish extracts from eight field trial participants are shown in Table 2. In general, participants and FRS agreed in the positive or negative interpretation of the test kit results. In one case, Participant 8 recorded a negative result, but FRS considered it positive. A positive result was confirmed when this extract was re-analyzed by MIST Alert for PSP at FRS. However, when further tested by the MBA, toxin was not detected. All but one Participant recorded positive results when using the kit.

TABLE 1.

Comparison of MIST Alert for the detection of PSP in shellfish extracts with the MBA.

Tissue Type	No. Tested	MBA ≥80[a]	MIST +	MIST −	MBA 40 – <80	MIST +	MIST −	MBA <40	MIST +	MIST −	MBA ND[b]	MIST +	MIST −
Pecten maximus (G)[c]	115	14	14	0	22	21	1	19	19	0	60	21	39
Pecten maximus (W)[d]	126	37	37	0	39	39	0	12	12	0	38	20	18
Mytilus edulis	239	25	25	0	20	20	0	15	13	2	179	44	135
Ostrea edulis	6	0			1	0	1	0			5	0	5
Crassostrea gigas	27	0			0			0			27	1	26
Aquipecten opercularis	16	0			1	1	0	2	2	0	13	6	7
Cerastoderma edule	9	1	1	0	1	1	0	0			7	2	5
Ensis. sp.	9	0			0			3	3	0	6	3	3
Total	547	77	77	0	84	82	2	51	49	2	335	97	238
% compliance		100%			97.6%			96.1%			71.0%		

[a] MBA (μg STX eq 100 g^{-1}).

[b] ND, not detected.

[c] G, gonad.

[d] W, whole animal.

Only one sample was found to contain levels over the regulatory limit when analyzed by the MBA.

Interpretation of "T" Line Intensity

The majority of disagreement in results between participants and FRS were attributable to differences in "T" line intensity rather than whether a sample was considered positive or negative (Table 2). Participant 2 seemed to misinterpret kit instructions and in some cases, read the "C" line as a percentage of the "T" line. Despite this, positive and negative samples were correctly identified. Some trial participants attempted to read the kit too precisely; for example, rather than scoring the "T" line as 0, 25, 50, or 100% (see Fig. 1), results were reported as 5, 15, 30%, or so forth. Much of the difference in interpretation between FRS and Participants 6 and 7 was attributable to this "overinterpretation," but results were still correctly read as positive or negative.

Reanalysis of Shellfish Extracts

When shellfish extracts were retested at FRS, there was over 90% agreement with the positive or negative result previously obtained by Participants (Table 2). On four occasions when FRS reanalyzed extracts (from Participants 3, 6, and 8) by MIST Alert for PSP, the result changed from negative (on-site) to positive (at FRS). In one extract from Participant 3, (a king scallop gonad) a low level of toxicity (31 μg STX eq 100 g^{-1}) was detected by the MBA. In the extract from Participant 8, the MBA gave a negative result, while insufficient extract volume was available for further MBA testing from Participant 6, but when diluted and tested by MIST Alert for PSP, the result was considered negative. Two extracts from Participant 7 changed from a positive to negative score when reanalyzed by the kit, but because the "T" line intensity was close to 50% (see Fig. 1), the response was considered difficult to interpret.

Previous studies using HPLC showed that PSP toxin profiles of stored Association of Official Analytical Chemists (AOAC) acidic shellfish extracts do not change significantly over a five-day period (data not shown). It was, therefore, considered that during transportation of shellfish extracts from participants to FRS toxin levels remained unchanged.

Use of MIST Alert for ASP in Routine Shellfish Monitoring

MIST Alert for ASP was used to test 500 shellfish extracts, and the results were compared with the HPLC analysis routinely used in the ASP toxin shellfish-monitoring program (Table 3 and Fig. 3). All samples over the regulatory limit (20 μg AST g^{-1}), as determined by HPLC, were scored positive by MIST Alert for ASP (n = 162). In samples (n = 114) that were below the regulatory limit but above the HPLC lod (2.5 μg AST g^{-1}), MIST Alert for ASP detected toxin in 93 (81.5%). The kit also detected toxin in 10.6% (13/123) of samples at the HPLC lod and a further sample (king scallop gonad) that was negative by HPLC. Of 39 samples falling into the range >lod – <6 μg AST g^{-1} 24 yielded a positive result by the kit (61.5%). Of the commonly tested species, mussels yielded the fewest positive results at the lod.

Reproducibility of MIST Alert for ASP

Overall, 90% of samples tested gave three identical "T" line responses (Table 4). In samples wherein toxin was not detected, at

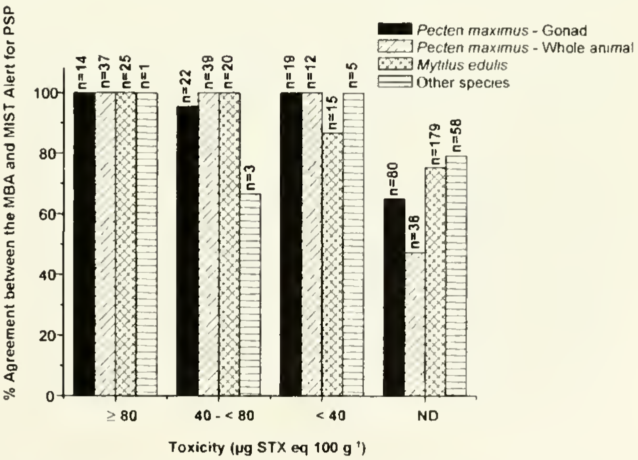

Figure 2. Percentage agreement between the MBA and MIST Alert for PSP results for shellfish as determined by the MBA.

TABLE 2.

Summary of results obtained using MIST Alert for the detection of PSP toxins in shellfish by field trial participants and FRS.

Participant	No. Kits Used	No. Samples Showing Disagreement in +ve/-ve Interpretation Between FRS and Participants	No. Samples Showing Disagreement In "T" Line Interpretation Between FRS and Participants	No. Samples Showing Disagreement in +ve/-ve Interpretation Between FRS and Participants when Reanalyzed by MIST at FRS	No. Samples Showing Disagreement in "T" Line Interpretation Between FRS and Participants When Reanalyzed by MIST at FRS	No. Positive Samples	Tissue Type of Positive Samples[a]
1	13	0	0	0	5	7	*Pecten maximus* (W, G, R)
2	15	0	14	0	2	3	*Pecten maximus* (G, W)
3	74	0	2	2	4	9	*Pecten maximus* (W, G)
4	45	0	0	0	0	0	NA[b]
5	20	0	1	0	0	1	*Pecten maximus* (W)
6	34	0	9	1	6	10	*Pecten maximus* (W, G, A)
7	50	0	16	2	6	14	*Mytilus edulis*, *Ensis* spp., *Aquipecten opercularis*,
8	8	1	0	1	2	5	*Mytilus edulis*

[a] W, whole animal; G, gonad; R, residual tissue; A, adductor muscle.

[b] NA, Not applicable.

the lod or ≥20 μg AST g^{-1} by HPLC, the kit gave three identical responses in agreement with HPLC results. Eight extracts in the range >lod – <6 μg AST g^{-1} gave three identical responses—two negative and six positive. The remaining two samples each gave two identical responses—one, two negatives, and a positive; and the other two positives and a negative. Ten samples in the 6 – <20 μg AST g^{-1} category all gave three positive results, but only seven samples gave three identical results (Table 4).

DISCUSSION

MIST Alert for PSP in Routine Monitoring

Using MIST Alert for PSP, a positive result was recorded for all shellfish extracts, from a wide range of tissue types, known to contain ≥80 μg STX eq 100 g^{-1} as determined by the MBA, indicating that shellfish considered a threat to public health would be detected by this method during routine PSP toxin monitoring in Scotland (Table 1, Fig. 2). In addition, approximately 97% of samples containing toxins below the regulatory limit (by the MBA) were also found to be positive by MIST Alert for PSP, suggesting the kit has a similar sensitivity to the MBA (about 33 μg STX eq 100 g^{-1}), an important factor when considering its potential use. MIST Alert for PSP failed to detect toxin in four samples in which the MBA detected toxin below the regulatory limit (33, 38, 47, and 60 μg STX eq 100g^{-1}). MIST Alert for PSP detects the amount of toxin based on the ability of the antibody mixture on the test kits to detect different STX analogs. It has been reported that the test kit is less sensitive to neo-STX derivatives and requires levels close to the regulatory limit to give a positive result (Laycock et al. 2001).

Differing sensitivities of MIST Alert for PSP to different toxin profiles may also account for the observed "false- positive" results (i.e., MIST Alert recorded a positive result; whereas, the MBA indicated a negative result) observed in this study. For example,

TABLE 3.

Comparison of MIST Alert for the detection of ASP in shellfish extracts with HPLC.

Tissue Type	No. Tested	HPLC 20	MIST		HPLC 6 – <20	MIST		HPLC >lod – <6	MIST		HPLC lod	MIST		HPLC ND[a]	MIST	
			+	−		+	−		+	−		+	−		+	−
Pecten maximus (G)[b]	121	18	18	0	55	50	5	18	12	6	23	6	17	7	1	6
Pecten maximus (R)[c]	64	54	54	0	5	5	0	2	2	0	3	0	3	0		
Pecten maximus (W)[d]	101	86	86	0	13	12	1	1	1	0	1	0	1	0		
Pecten maximus (A)[e]	36	1	1	0	1	1	0	11	8	3	18	2	16	5	0	5
Mytilus edulis	128	3	3	0	1	1	0	5	0	5	54	2	52	65	0	65
Aquipecten opercularis	17	0			0			1	1	0	12	3	9	4	0	4
Ostrea edulis	12	0			0			0			5	0	5	7	0	7
Crassostrea gigas	14	0			0			0			4	0	4	10	0	10
Cerastoderma edule	7	0			0			1	0	1	3	0	3	3	0	3
Total	500	162	162	0	75	69	6	39	24	15	123	13	110	101	1	100
% compliance			100%		92%			61.5%			10.6%			99.0%		

[a] ND, Not detected; [b]G, gonad; [c]R, residual tissue; [d]W, Whole animals; [e]A, adductor muscle.

TABLE 4.

Summary of the reproducibility of triplicate analyses of shellfish extracts using MIST for ASP.

HPLC (µg AST g^{-1} Shellfish Flesh)	Percentage of Samples Giving Three Identical Responses	Number of Samples Giving Three Identical Responses	Percentage of Samples Giving Two Identical Responses
ND[a]	100	10/10	
lod[b]	100	10/10	
>log – <6	80	8/10 (6 positive, 2 negative)	20
6 – <20	70	7/10	30
320	100	10/10	
Total	90	45/50	10

[a] ND, Not detected.

[b] lod, limit of detection (about 2.5 µg AST g^{-1}).

extracts containing high levels of low-toxicity toxins (e.g., some C toxins) (Oshima 1995) may yield a negative result by the MBA but a positive result by MIST Alert for PSP (Laycock et al. 2001). This may be considered an advantage of the kit, because these toxins can be readily converted to other more potent PSP toxins during shellfish consumption. Shortcomings of the MBA must be taken into account when considering inconsistent results. The MBA is known to underestimate toxicity with an associated error of 20% (60% near the detection limit) (Park et al. 1986).

Use of MIST Alert for PSP in the Field

Results from data obtained by shellfish farmers, processors, and EHOs show very little difference in the interpretation of positive or negative results obtained by these lay persons with less experience of the test kits than staff at the routine monitoring laboratory (FRS). Similarly, in-house (FRS) and on-site (Participants) results from shellfish extracts were, for the most part, comparable (Table 2). In this study, variability in the interpretation of the "T" line response was observed. Previous trials (Mackintosh et al. 2002) also demonstrated variation in interpreting the "T" line response of MIST Alert for PSP by novice users. This trial suggests that this may not be a serious problem, because positive and negative samples were still correctly identified, and the manufacturer advises that if doubt exists because of "T" line intensity, a positive result should be recorded. It is also considered that this variable will be reduced with more extensive use of the kit.

Importantly, an extract determined to contain over 80 µg STX eq 100 g^{-1} by the MBA was correctly identified by a participant. A further positive extract that was not retested by the MBA (attributable to insufficient sample volume) was diluted 1:1 in a negative shellfish extract and retested using MIST Alert at FRS. This extract produced a negative response, suggesting toxicity in this sample was <80 µg STX eq 100 g^{-1}, although this would have been dependent on the toxin profile of the extract.

Overall, even when considering misinterpretation of instructions (e.g., recording of the "T" line intensity), participants correctly identified positive and negative samples. Previously, we have shown that novice users can make errors in both use and interpretation (Mackintosh et al. 2002), emphasizing that careful attention to the instructions and interpretation of the results is essential.

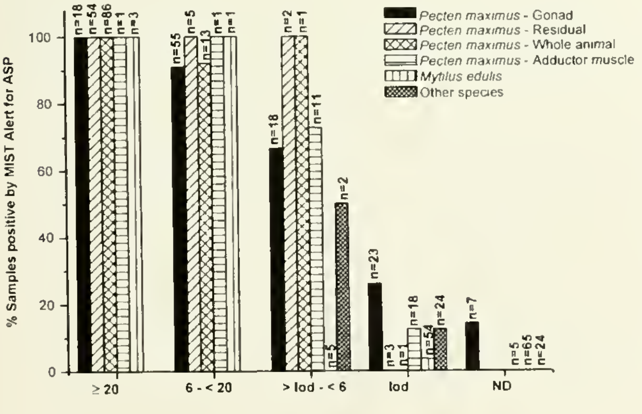

Figure 3. Percentage of samples found to be positive by MIST Alert for ASP when compared with HPLC results.

MIST Alert for ASP in Routine Monitoring

MIST Alert for ASP detected toxin in all samples containing the regulatory limit of ASP toxin (20 µg AST g^{-1}) and above, with good reproducibility observed between test kits (Table 3, Table 4, Fig. 3). Toxin was also detected in a high percentage (82%) of extracts containing between the regulatory limit and the lod of the HPLC method used. The manufacturer of MIST Alert for ASP reports a detection limit of between 8 and 12 µg AST g^{-1} (Jellett et al. in press); our results show that the kit will detect toxin at lower levels, particularly in king scallops. In Scotland, the contamination of king scallops with ASP toxins has adversely affected the offshore scallop fishery (Campbell et al. 2001, Gallacher et al. 2001); whereas, other shellfish species remain relatively unaffected. Results from routine monitoring of Scottish king scallops demonstrate that the proportion of scallops that currently do not contain ASP toxin as determined by HPLC is about 30% (J. Petrie pers. comm.). This implies that the majority of scallop extracts tested by MIST Alert for ASP will give a positive result, regardless of whether the scallops contain toxin below the regulatory limit, and would still be marketable.

Overall, results from this study indicate that MIST Alert for ASP is currently too sensitive to screen out low- toxicity king scallop samples in routine monitoring programs, but it may be suitable for other shellfish species (e.g., mussels). However, the requirement for an alternative method to the current HPLC technique for ASP toxin detection in shellfish is not considered paramount, because the ethical concerns surrounding the MBA do not need to be considered. It has been reported that MIST Alert for ASP and PSP can be performed using the same extract; that is, AOAC extract (sample homogenate extracted in 0.1N HCl). Before this study, AOAC acidic extracts were examined by MIST Alert for ASP and PSP (data not shown). It was found that, unless these extracts were used immediately, they were unsuitable for ASP testing because of the instability of DA in HCl, suggesting a single extraction protocol would not be feasible. In addition, acidic extracts are unsuitable for HPLC analysis if further investigation were required.

CONCLUSION

In conclusion, the MIST Alert for PSP has been successfully used during two monitoring seasons (Mackintosh et al. 2002) to determine the presence/absence of PSP toxins in Scottish shellfish. The test kits confirmed the presence of PSP toxins in samples considered to contain toxin at and above the regulatory limit (80 µg STX eq 100 g^{-1}). MIST Alert for PSP could be used in monitoring programs as a screen to eliminate negative and low PSP toxicity samples before use of the MBA for quantitative analysis of positive samples. For example, if MIST Alert for PSP had been used for this purpose during the study period in 2001, there would have been a reduction of 43% of the MBAs. Furthermore, field trial results indicate that the PSP kit has the potential to be used by shellfish farmers as a shellfish harvest management tool and by EHOs and processors in end product testing.

Results suggest that MIST Alert for ASP is too sensitive to use as a screen in the Scottish ASP toxin-monitoring program and in end-product testing for king scallops. A higher detection limit would be desirable to reduce the high number of MIST Alert for ASP positives observed at toxin levels <6 µg AST g^{-1} by HPLC. In contrast, it is possible that the kit may be used as a screen for ASP toxins in other shellfish species and this should be studied further.

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DIARRHETIC SHELLFISH POISONING ASSOCIATED WITH *PROROCENTRUM LIMA* (DINOPHYCEAE) IN PATAGONIAN GULFS (ARGENTINA)

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ABSTRACT A serious diarrhetic shellfish poisoning (DSP) intoxication caused by the consumption of mussels harvested in the Gulfs of San José and Nuevo, Patagonia, Argentina occurred in March (autumn) 1999. This was the first observation of DSP toxins along the Argentine coast. Cells of the dinoflagellate *Prorocentrum lima* (Ehrenberg) Dodge were found in water samples, as were epiphytes upon macroalgae and in the stomach contents of the mussels *Aulacomya atra* (Molina) and *Mytilus edulis platensis* (d'Orbigny). Extracts from both mussel species were positive for DSP-like activity using the fluorimetric phosphatase inhibition assay. When the extracts were analyzed using liquid chromatography and tandem mass spectrometry, peaks of okadaic acid or dinophysistoxin 1 (DTX-1) were not observed in the nonhydrolized samples. DTX-1 was detected in hydrolized extracts of *Mytilus* and *Aulacomya*. The *Mytilus* extract contained 21.2 ng DTX-1 per gram of the whole tissue; the *Aulacomya* extract contained 94.0 ng DTX-1 per gram.

KEY WORDS: Argentina; diarrhetic shellfish poisoning; okadaic acid; *Prorocentrum lima*, toxic dinoflagellate

INTRODUCTION

Harmful algal blooms have increased in geographic distribution, magnitude, and frequency during the last decades (Anderson 1989; Smayda 1990; Hallegraeff 1993). Diarrhetic shellfish poisoning (DSP), a severe gastrointestinal disturbance following the consumption of shellfish, is also an increasing problem. DSP results from the accumulation, in shellfish tissues, of polyether toxins produced by dinoflagellates belonging to the genera *Dinophysis* and *Prorocentrum* (Wright & Cembella 1998 and references therein). In his 1993 review, Hallegraeff recognized only the coast of Chile as being affected by DSP in South America. Ferrari et al. (1993) and Méndez and Ferrari (1994) later reported DSP incidents occurring in Uruguay. Proença and Rörig (1995) and Proença et al. (1998) documented DSP occurrences in southern Brazil. To date, only species of *Dinophysis* have been the causative agents of DSP cases reported from South America (Lembeye et al. 1993, Lembeye et al. 1996, Proença et al. 1998, Uribe et al. 2001).

Since 1995, oceanographic research along the North-Patagonian coast of Argentina has included phytoplankton investigation, microscopical observations of stomach content of mussels, and analysis of physical and meteorological data. Because of recurrent blooms of another toxic dinoflagellate, Alexandrium tamarense, in the area (Esteves et al. 1992, Carreto et al. 1998, Gayoso 2001), monitoring programs are conducted only for paralytic shellfish poisoning toxins in shellfish (mouse bioassay) by the local (Chubut) government, who is responsible for imposing bans on shellfish harvest. Although the known DSP producers, *Dinophysis acuminata* and *Prorocentrum lima*, are present (Esteves et al. 1992, Santinelli et al. 1994, Gayoso 2001), monitoring programs for DSP are not currently conducted in Argentina.

On March 20, 1999, shellfish harvested in the Gulfs of San José and Nuevo (Chubut) were served at a social event despite voiced concerns from one of us (A.M.G.) that toxic dinoflagellates other than *Alexandrium tamarense* were potentially present in the water column and in the shellfish digestive glands. More than 40 people suffered from gastrointestinal disorders after consuming the shellfish. Physicians from the Dr. Andrés Isola Public Hospital reported symptoms of diarrhea, nausea, and abdominal pain, consistent with the DSP syndrome. After the incident, samples were collected from the two north-patagonian gulfs for phytoplankton and epiphytic dinoflagellate identification, chemical analysis of mussel tissues, and microscopic examination of the stomach content of mussels was undertaken to document the causative toxin(s) and the responsible organisms. This work reports on the first recognized episode of diarrhetic shellfish poisoning on the Argentine coast.

MATERIALS AND METHODS

Water samples at two depths (surface and bottom) with a 2.5 L Van Dorn bottle and vertical and oblique hauls with a 25 μm mesh plankton net were taken at two stations: Puerto Madryn, situated on the west coast of Golfo Nuevo (42°46′S, 65°02′W) and San Román, located on the north coast of Golfo San josé (42°15′S, 64°15′W). Dominant macroalgae and mussels (*Aulacomya atra* and *Mytilus edulis platensis*) were manually collected from the bottom (ca. 18 m depth) at each station by scuba divers. The samples were collected twice a month from March to June 1999.

The identification of species was made using the net samples, a light microscope provided by Nomarsky illumination, and a scanning electron microscope. For quantitative analysis, subsamples (from the bottle samples) were settled in a chamber from a cylinder of 50 mL and counted with an inverted microscope. The species of macroalgae were analyzed separately, subsamples (2–6 g wet weight) were placed in a tube containing a solution of formaldehide-filtered seawater, the suspended epiphytic dinoflagellate populations were examined and their abundance, expressed as cells per gram wet weight of macroalga, was estimated from counts using a 1 mL Sedwick Rafter chamber. The mussel stomach contents were analized within 4 h of collection, the stomach content of at least six mussels was extracted with a Pasteur pipette, the contents were pooled, diluted with filtered seawater, and observed on a slide using a compound light microscope. Microalgae present were identified and their relative abundance was estimated.

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Toxin Analysis

Toxin analysis was performed on the following two mussel samples: (1) *Aulacomya atra*, which were collected in Golfo Nuevo on March 18, 1999; and (2) *Mytilus edulis platensis*, which were collected in Golfo San José on March 25, 1999. Both were frozen whole until the time of analysis.

Sample Extraction

For each species, 5 g wet weight of whole mussels were homogenized in 80% methanol for 2 min using a Polytron. The resulting extract was filtered through a GHB glass fiber filter and the filtrate brought to a volume of 5 mL. To convert all metabolites to the parent okadaic acid (OA) or dinophysistoxin-1 (DTX-1), 2.5 mL of each sample were hydrolyzed by heating at 76°C for 40 min after addition of 250 μL of 2.5 N NaOH (Mountfort et al. 1999). Therefore, for each species two separate samples were analyzed for DSP-toxins.

Protein Phosphatase Inhibition Assay

The protein phosphatase inhibition assay was performed in a 96 well format using the procedure of Vieytes et al. (1997). The assay tests the ability of OA standard or unknown sample to inhibit activity of purified protein phosphatase 2A against a fluorimetric substrate, 6,8-difluro-4-methylum belliferyl phosphate (DiFMUP). All samples were diluted in reaction buffer (50 mM Tris HCl pH 7.0, 0.1 mM $CaCl_2$) at least 4-fold to reduce the methanol concentration to ≤5% in the assay. This step was to eliminate inhibition of the enzyme by methanol. For the assay, 10 μL of a 1:1 dilution of 40 mM $NiCl_2$ and 1 mL/mL bovine serum albumin, 17 μL of reaction buffer, 33 μL samples and standards (0.03–10 nM OA; NRC, Ottawa Canada) and 200 μL of purified PP2A enzyme (Upstate Biotechnology, Lake Placid, NY) were added to duplicate wells of a 96 well plate (Costar, Corning, NY). To start the reaction, 120 m DiFMUP (10 mM; Sigma, St. Louis, MO) was then added to each well and the reaction allowed to proceed for 1.5 h at 37°C, followed by 30 min at 4°C. Protein phosphatase activity was determined by fluorescence (360 nm excitation; 460 nm emission) in the wells using a fluorimetric plate reader (Fluostar, BMG Laboratory Technologies, Durham, NC). OA-like activity in the sample was quantified relative to the standard curve. The detection limit of the fluorimetric phosphatase inhibition assay was approximately 1×10^{-10} M okadaic acid equivalents.

Liquid Chromatography and Tandem Mass Spectrometry (LC-MS/MS)

Samples that displayed protein phosphatase inhibition activity were analyzed by LC-MS/MS using a Finnigan LCQ mass spectrometer. The methanolic extracts were injected on a C18 column (Zorbax 2.1 × 150 mm) and eluted with a gradient of 50 to 95% methanol/water containing 0.1% TFA at a flow rate of 0.2 mL/min. A splitter device was used to direct 10% of the column effluent to the electrospray source. The mass spectrometer was operated in positive ion mode. Toxins were analyzed by trapping [M+Na]+ species for each toxin and conducting selected ion monitoring experiments for distinctive fragment ions from the collisionally activated dissociation of the trapped parent ions. Chromatographic traces were acquired for the detection of the fragment ions and undissociated parent ions. The limits of OA and DTX-1 detection were approximately 1×10^{-7} and 1×10^{-8} M, respectively, as measured by LC-MS/MS.

RESULTS

P. lima (Fig. 1) was unequivocally identified in net samples from the gulfs of San José and Nuevo. The two main thecal plates (valves) of the specimens found were obovate (length 33–38 μm, width 22–24.2 μm, $n = 10$) and cellular morphologic features agreed with the descriptions given by Faust (1991) and McLachlan et al. (1997, as *Exuviaella lima*). A row of conspicuous marginal pores, scattered valve pores, and valve center free of pores were observed. The species was registered in water samples (cell densities less than 100 cells L^{-1}) on March 18, April 13 and June 29, 1999 (16.5–18°C). Its occurrence coincided with the autumn phytoplankton peak in which *Rhizosolenia setigera* Brightwell, *Chaetoceros curvisetus* Cleve, *C. diadema* (Ehrenberg) Gran, *C. didymus* Ehrenberg, and *Pseudo-nitzschia pungens* (Grunow ex Cleve) Hasle were most abundant, and *A. tamarense* (Lebour) Balech was also present. Dominant macroalgae in the gulfs San José and Nuevo, *Dictyota dichotoma* (Hudson) Lamouroux (Phaeophyceae), *Anotrichium furcelatum* (J. Agardh) Baldock, *Ceramium rubrum* (Hudson) C. Agardh (Rhodophyceae), and *Ulva rigida* (C. Agardh) Thuret (Chlorophyceae) showed an epiphytic assemblage of diatoms and dinoflagellates that included *P. lima*. Cell densities of *P. lima* varied from approximately 750 cells per g wet weight of macroalga at station San Román to 1782 cells per g wet weight at station Puerto Madryn.

Intact cells (with protoplasm) of *P. lima* were observed in the stomach contents of the mussels on March 18, May 18, and June 29, 1999. During the study period, the diet of *A. atra* and *M. edulis platensis* consisted mainly of diatoms, with benthic forms outnumbering pelagic species. *Prorocentrum micans*, benthic pennate diatoms, *Paralia sulcata*, and *Thalassiosira* spp. were the most abundant organisms found in the mussel stomachs.

Extracts of both mussel species were positive for DSP-like activity using the fluorimetric phosphatase inhibition assay. The *Mytilus* sample contained 33.5 ng of OA-equivalent activity per gram of whole tissue, whereas the *Aulacomya* sample contained 150 ng/g. The difference between hydrolized and nonhydrolized samples using this assay were not significant.

Extracts of the nonhydrolized samples analyzed using LC-MS/MS did not exhibit peaks corresponding to okadaic acid or dino-

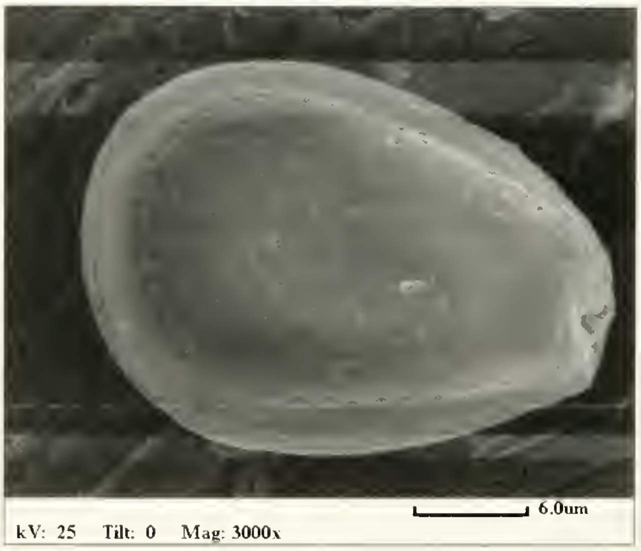

Figure 1.

physistoxin-1. However, dinophysistoxin-1 was detected in the *Mytilus* and *Aulacomya* extracts that had been hydrolized, yielding 21.2 ng DTX-1/g whole tissue and 94.0 ng/g, respectively.

DISCUSSION

Dinophysis acuminata is a frequent component of the phytoplankton in the area; its abundance can reach up to 1.9×10^3 cells L^{-1} (Gayoso 2001). However, no known gastrointestinal disorders have accompanied its presence. The episode of diarrhea following shellfish consumption observed in March 1999 appears to be related to the presence of *P. lima*. It is an important component of the epiphytic community associated with the dominant macroalgae in the gulfs. The species was also present in the mussel stomach contents and in the water samples.

Not only was *P. lima* present in mussel digestive glands, but DSP toxin activity was also detected in *M. edulis platensis* and *A. atra*, strengthening the causal relationship between *P. lima* and the gastrointestinal disorders of March 1999. If toxin regulations such as those implemented in the European Union had been in effect, the shellfish harvest would have been halted; most EU countries tolerate no DSP toxins above detection levels (Shumway et al. 1995).

LC-MS, a highly selective and sensitive method for detection of DSP toxins (Quilliam & Wright 1995), showed a peak corresponding to DTX-1 in hydrolyzed samples of *M. edulis platensis* and *A. atra*, whereas neither OA nor dinophysistoxin was observed in nonhydrolyzed extracts. This finding is indicative of the esterified nature of DTX-1 within the mussels; esterified DSP toxins (also called DTX-3 complex) are acyl derivatives that also possess toxic activity (Quilliam & Wright 1995). Upon sample hydrolysis, the esterified toxin converts to the active parent compound. Esterified DTX-1 is found to be the main DSP toxin in two other shellfish, the scallop *Patinopecten yessoensis* and the mussel *Mytilus galloprovincialis* (Suzuki & Mitsuya 2001). A significant difference was found in the toxin concentration of the two hydrolyzed samples: 21.2 ng DTX-1/g of whole *Mytilus* tissues versus 94.0 ng/g for *Aulacomya*. This finding may be explained by the time of collection (1 week apart for the two species), different specific feeding and digestion behavior, and location of the two populations.

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REDUCING NEUROTOXIC SHELLFISH POISON (NSP) IN PACIFIC OYSTERS (*CRASSOSTREA GIGAS*) TO LEVELS BELOW 20 MOUSE UNITS · 100 G^{-1}

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ABSTRACT Pacific oysters previously exposed to the algae *Karenia breve*, which produces neurotoxic shellfish poisoning (NSP), were held in experimental depuration tanks for up to 5 days prior to determination of their NSP levels by mouse bioassay. Although depuration reduced NSP levels in the oysters to around the regulatory limit (20 mouse units · 100 g^{-1}), levels below the regulatory limit could not be assured at temperatures of 15°C and 20°C and salinities of 24‰ and 34‰ with the standard bioassay used. Detoxification appeared less effective at 12°C. The use of an in-line filter to remove free algae or ozone to eliminate algae and toxins did not enhance detoxification. Use of a biological filter at 12°C in the tanks or feeding the oysters with *Isochrysis galbana* also did not assure NSP levels below the regulatory limit. Examination of NSP distribution in the shellfish tissues revealed that during depuration, the percentage of this lipid soluble toxin associated with the organs of the digestive system (gills and digestive viscera) decreased while levels increased in high lipid tissues, such as the gonads. We postulate that NSP is difficult to eliminate from oysters because it is incorporated into the lipid stores of the animal.

KEY WORDS: neurotoxic shellfish poison, Pacific oyster, *Crassostrea gigas*, detoxification

INTRODUCTION

Although the uptake of algal toxins by bivalve shellfish is a problem worldwide for shellfish producers, to date there have been very few successful strategies to mitigate the effects of toxic algal blooms. Current controls rely on growers monitoring toxin levels in shellfish and ceasing harvesting when levels exceed prescribed limits. This is in contrast to the situation when shellfish are exposed to bacterial pathogens. In this case growers may have the options of either relaying (moving shellfish to unaffected areas and harvesting again when the shellfish have naturally purified themselves after a period of weeks) or processing the shellfish in land-based depuration tanks where they will be cleared from bacterial pathogens within a few days (Roderick 1994). Similar strategies for the detoxification of shellfish from algal toxins are not commercially available.

The uptake and elimination of three of the four main algal toxin groups affecting shellfish have been investigated in laboratory studies: paralytic shellfish poison (PSP) (Bricelj & Shumway 1998), diarrhetic shellfish poison (DSP) (Cembella et al. 1998) and amnesic shellfish poison (ASP) (Wohlgeschaffen et al. 1992, Whyte et al. 1995). These studies suggest that land-based detoxification only appears to be feasible for ASP. Relaying to eliminate toxins could be developed but further research is required and there would be risks of introducing toxic algae to unaffected areas. Little work has been reported on detoxification of shellfish affected by the fourth toxin group, neurotoxic shellfish poisoning (NSP). Despite promising work that showed that NSP toxins were susceptible to oxidation in ozonated seawater (Schneider & Rodrick 1995) little work on the toxins within shellfish has been published (Fletcher et al. 1998). NSP occurs when people eat shellfish that have been subjected to dinoflagellate blooms composed of *Karenia* (syn. *Gymnodinium*) species. These blooms commonly occur in the Gulf of Mexico where the causative alga is *Karenia breve* (syn. *Gymnodinium breve, Ptychodiscus brevis*). One outbreak, caused by a *K. breve*-like species, has been recorded in New Zealand (Chang 1995). We have previously reported the results of six experiments designed to determine whether Pacific oysters (*Crassostrea gigas*) might be successfully detoxified of NSP toxins in a depuration system (Fletcher et al. 1998). We found that, although mean NSP levels could be reduced to levels below the regulatory limit of 20 mouse units · 100 g^{-1}, levels below this limit could not be assured in individual tests using the regulatory method (APHA 1970). This was regardless of whether ozone was used in the seawater. We now report on four experiments designed to confirm the previous results, investigate why levels below 20 mouse units were not consistently achieved and to evaluate other detoxification methods that may result in additional decreases in NSP levels.

MATERIALS AND METHODS

K. breve cells were grown in a medium derived from the GP medium of Loeblich and Smith (1968). The original GP medium contained 85% seawater, but because a higher percentage of seawater gave better growth rates for *K. breve* (unpublished data) we modified the GP medium in this study to the following composition: seawater (1 L), KNO_3 (200 mg in 2 mL H_2O), vitamin solution (1 mL), trace element solution (5 mL), and soil solution (5 mL). This medium was autoclaved (121°C, 15 min), and then K_2HPO_4 (8.7 mg in 4 mL autoclaved) was added aseptically. The vitamin solution contained thiamin HCl (100 mg), vitamin B_{12} (0.1 mg), biotin (0.2 mg) and H_2O (100 mL). The trace element solution contained Na_2EDTA (6 g), $FeCl_3.6H_2O$ (0.29 g), H_3BO_3 (6.85 g), $MnCl_2.4H_2O$ (0.86 g), $ZnCl_2$ (0.06 g), $CoCl_2.6H_2O$ (0.026 g), and H_2O (1 L) adjusted to pH 7.9 with NaOH. The soil solution was the supernatant after filtering (Whatman No. 2 filter) a mixture of soil (1 kg) and H_2O (2 L) that had been autoclaved (121°C, 60 min).

Oysters were supplied with *K. breve* cells at rates of 10 to 25 × 10^6 cells.oyster^{-1} (Table 1) and left for 24 h to ingest the cells and take up the NSP toxin, as described previously (Fletcher et al. 1998). This feeding regime gave initial toxin levels from 29.6 to 70.7 mouse units · 100 g^{-1} (Table 2).

Four detoxification experiments (designated Experiments 1 to 4) were carried out in the experimental tanks described previously (Fletcher et al. 1998). Briefly the tanks consisted of two closed systems: one of four 50 L tanks plumbed in parallel to a tempera-

TABLE 1.

Experimental conditions and NSP levels for uptake and detoxification of NSP from Pacific oysters.

Experiment Number	Oysters Weight[1] (S.E.)[2]	Oysters Lipid[3] (S.E.)	Detoxification Start Date	Treatment Number	Uptake *K. breve* Supplied	Detoxification Conditions Water Treatment	Temp.	Salinity	Filter
1	12.14 (0.22)	1.79 (0.08)	3/4/97	1a	25	U.V.	15	24	
				1b	10	U.V.	15	24	
				1c	10	U.V.	15	24	
2	15.91 (0.56)	2.18 (0.19)	4/10/97	2	10	Ozone	20	34	5
3	16.29 (0.74)	3.94 (0.11)	8/27/97	3a	18	U.V.	20	34	5
				3b	18	Biofilter	12	34	Bio
4	10.81 (0.14)	3.17 (0.09)	10/20/97	4a	23	U.V.	12	34	
				4b	23	U.V.[4]	12	34	
				4c	23	Biofilter	12	34	Bio
				4d	0	U.V.	12	34	

[1] Mean drained wet weight of soft tissues for all oysters in the experiment.

[2] (S.E.)—Figures in parentheses are the standard errors of the means.

[3] Mean lipid levels for all oysters in the experiment expressed as a percentage of the drained wet weight of soft tissue.

[4] Fed *Isochrysus* during the experiment (days 2 and 3).

ture controlled sump and the other of a single 50 L tank maintained at the same temperature. Both systems had in-line 5 μm cartridge filters (FilterPure 5PW10, Contamination Control, Auckland) and ultraviolet lamps (Steriflo 369P, Contamination Control, Auckland) available. Ozone could be supplied to tanks 1-4 by a corona discharge generator (CD1000, Novozone, Auckland) using oxygen as the source gas. When applied, water was ozonated using an electronic controller to provide a reading of 350 ± 20 mv (redox electrode MC241Pt, Radiometer) in the shellfish tanks, which converts to an Eh of 549 mv using the correction factor of +199 mv applicable to the Ag/AgCl, KCl reference electrode (Vogel 1961). Additionally, in Experiments 3b and 4c, oysters were placed in a 1000-L biofilter tank (12 ± 0.5°C) where recirculating water was purified by passing it through a sand and shell-based biologically active filter.

Ozone and ammonia levels in the seawater were measured using Palintest Photometer 5000 methods (Anon. 1994) and salinity was determined using a refractometer (Atago). NSP levels in the oysters were measured using the APHA method (APHA 1970). Each NSP test was carried out on at least 100 g drained meats from 10–12 oysters. One or two such pooled samples were tested from each treatment on each testing occasion with the two samples being taken from separate tanks when the treatment was spread over more than one tank (details of timing and numbers of samples taken are recorded in Fig. 1). The NSP method can detect toxin levels down to about 10 mouse units · 100 g^{-1}, depending on the size of mouse used (18–22 g). Where toxicity was observed but the relevant mice did not die, the toxin level was scored at half the minimum detectable level. Crude lipid levels were determined from the weight of lipid extracted in the ether extraction during the NSP extraction procedure.

The experimental conditions under which the oysters were made toxic and maintained during depuration are presented in Table 1. The depuration conditions in Experiments 1 and 2 were designed to complement the half factorial design used in previous work (Fletcher et al. 1998), increasing the replication for each

TABLE 2.

Fitted exponential curves.

Treatment	NSP Levels (m.u. · 100 g^{-1}) Start (S.E.)[2]	NSP Levels (m.u. · 100 g^{-1}) End (S.E.)[3]	Parameters (standard errors)[1] *a*	Parameters (standard errors)[1] *b*	Parameters (standard errors)[1] *r*	Regression Coefficient (R^2)
1a	60.3 (21.0)	15.0 (3.2)				
1b	62.5 (11.9)	23.7 (7.1)				
1c	70.7 (3.5)	24.5 (3.9)				
1a,b,c			22.97 (3.49)	41.52 (6.04)	0.116 (0.226)	0.715
2	54.4 (1.2)	12.8 (1.4)	13.36 (1.20)	41.04 (2.45)	0.065 (0.080)	0.976
3a	29.6 (5.5)	12.0 (0.5)	11.77 (4.89)	17.78 (6.24)	0.329 (2.408)	0.859
3b	29.6 (5.5)	15.2 (3.2)	0.00 (157.14)	29.73 (156.45)	0.880 (0.813)	0.710
4a	51.9 (0.02)	24.7 (6.3)	26.13 (6.73)	25.19 (10.62)	0.343 (0.414)	0.426
4b	51.9 (0.02)	23.2 (4.4)	0.00 (58.22)	48.57 (55.71)	0.823 (0.299)	0.640
4c	51.9 (0.02)	31.6 (2.0)	31.95 (2.54)	19.95 (4.76)	0.000 (0.312)	0.787

[1] Parameters fitted to equation 1: $Y = a + b.r^x$ with the standard error of the parameter in parentheses.

[2] Mean NSP level of the 1–2 samples taken at the start of the experiment.

[3] Mean NSP levels of all samples taken from Days 3, 4, and 5.

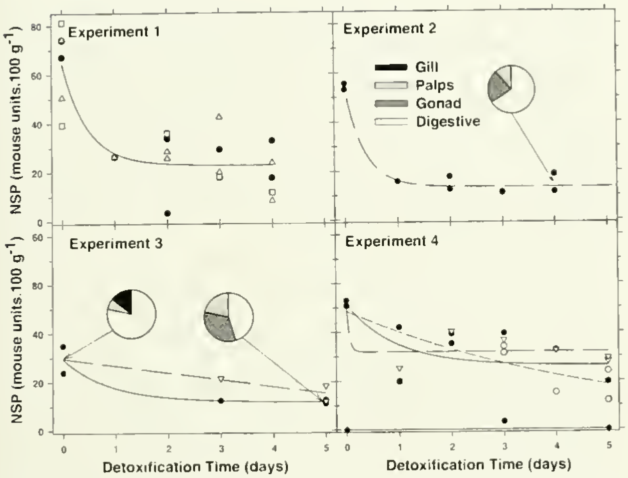

Figure 1. Detoxification progress. Each point represents one NSP analysis of a pooled sample of 10 to 12 oysters. Curves represent the best fit to Eq. 1: $Y = a + b.r^X$. Pie diagrams represent the distribution of NSP in various tissues (as in Table 3) at the indicated times. .... dotted line = regulatory limit (20 mouse units · g^{-1}).
Experiment 1: ——— Oysters in experimental detoxification tanks with 15°C seawater at 24% salinity disinfected with U.V. light. (a) ☐ toxin level 1, (b) △ toxin level 2, (c) ● toxin level 3.
Experiment 2: — — — ● Oysters in experimental detoxification tanks with 20°C seawater at 34% salinity disinfected by ozone.
Experiment 3: Detoxification with U.V. (20°C) or Biofilter (12°C). ——— ● (a) Oysters in experimental detoxification tanks with 20°C seawater of 34% salinity disinfected with U.V. light. — — — ▽ (b) Oysters in biofilter tanks with 12°C seawater at 34% salinity.
Experiment 4: Detoxification with U.V., (12°C), feeding with *Isochrysus* or not, or biofilter (12°C). ——— ● (a) Oysters in experimental detoxification tanks with 12°C seawater of 34% disinfected with U.V. light. - - - ○ (b) Oysters in experimental detoxification tanks with 12°C seawater of 34% disinfected with U.V. light fed with *Isochrysus galbana*. — — — ▽ (c) Oysters in biofilter tanks with 12°C seawater at 34% salinity. ——— ◆ (d) Oysters with no initial toxin.

factor (ozone or ultraviolet light sterilization, in-line filtration to 5 μm or no filtration, water temperatures of 15°C or 20°C and salinities of 24‰ or 33‰). Also, in Experiment 1, three batches of oysters were separately fed different levels of *K. breve* (Treatments 1a, 1b, and 1c), and then subjected to identical detoxification conditions. Experiment 3 compared UV sterilization at 20°C (Treatment 3a) with the use of the biofilter at 12°C (Treatment 3b). Experiment 4 also compared UV sterilization (Treatment 4a) with the use of the biofilter (Treatment 4c) but all oysters were held at 12°C. Additionally, (Treatment 4b) oysters were held in the same conditions as Treatment 4a except that they were fed with 10 million cells *Isochrysus galbana* per oyster on days 2 and 3. Treatment 4d was a control where oysters that were not toxic were placed in the same tanks as Treatment 4a oysters and tested at the end of the experiment (after 5 days) to see whether detectable levels of toxin had been accumulated during the trial.

Nonlinear regression analysis was used to fit exponentially declining detoxification curves of the form:

$$Y = a + b.r^X \tag{1}$$

where Y was the mean NSP level for a treatment on a given day and X was the number of days since the beginning of the experiment. The parameter a is the asymptote of the curve (the level to which NSP approaches after some large number of days); $a + b$ is the initial NSP level; and r is related to the speed of approach to the asymptote. The model was constrained with $r < 1$ and $a \geq 0$. Accumulated analysis of variance was used to compare the fitted curves. Analysis of variance (ANOVA) was applied to the mean NSP levels after day 2 (exclusive) and on parameter a in Equation 1 to determine whether any of the experimental factors had statistically significant ($P < 0.05$) effects on the outcome of detoxification for 6 experiments providing a balanced set of factors (Runs 1 to 4 of previously published work (Fletcher et al. 1998), with Experiments 1 and 2 of the current work). Residual Maximum Likelihood (REML) statistics were similarly applied to compare the effect of treatments in all experiments.

At the end of Experiment 2 (day 4) and at the beginning and end (days 0 and 5) of Experiment 3a, the distribution of toxin in various tissues was determined as well as the NSP levels in two pooled samples of 10 oysters. To determine toxin distribution, 100 oysters were dissected [see Quayle (1969) for a description of *C. gigas* anatomy] into mantle (up to the junction between the mantle and gill, adductor muscle, gill, labial palps, gonad (i.e., the white colored tissue surrounding the digestive organs) and digestive tissues (remaining visceral mass). The tissues from the 100 oysters were pooled, tested for NSP, and the results were related to the total NSP level found in the pooled samples of the 10 whole oysters.

RESULTS

The NSP results for the 4 detoxification experiments are presented in Figure 1 and Table 2. Initial toxin levels ranged from 29.6 (Experiment 3) to 70.7 (Experiment 1 Treatment c) mouse units g^{-1} with considerable variation in levels recorded from identical conditions of uptake (Table 1). As observed previously (Fletcher et al. 1998), there was a period of rapid detoxification followed by a period of no significant changes in toxin levels (from Days 3 to 5 inclusive). The results of fitting these data to Eq. 1 are shown in Table 2. As separating the 3 batches of shellfish in Experiment 1 did not improve the goodness of fit the NSP results were analyzed as a single treatment. Statistical analyses of Experiments 3 and 4 did not demonstrate significant differences in NSP levels between treatments, with or without an in-line biofilter or with or without feeding with *I. galbana*. Treatments with an in-line biofilter did not result in increased ammonia levels in the water with time while treatments in the smaller tanks did, as reported previously (Fletcher et al. 1998). Adding the extra replication of factors provided in Experiments 1 and 2 to the previously reported (Fletcher et al. 1998) half factorial experiment (Runs 1–4) effectively reduced the confounding effect of shellfish weight and initial NSP level from the analysis and allowed valid ANOVA. However, this still showed no significant differences between the four factors evaluated: temperature (15°C, 20°C), salinity (24‰, 34‰), in-line filter (5 μm, absent), disinfection agent (UV, ozone). Further, REML analysis of all the experiments also showed no significant effects ($P > 0.05$), although detoxification from treatments at 12°C was possibly less effective than from those at 15°C ($P < 0.10$).

Results from the analyses of different tissues in Experiment 2 and Experiment 3 Treatment a are presented in Table 3. Immediately after toxification (Day 0, Experiment 3), the toxin was concentrated in organs associated with ingestion and digestion (gills, palps, digestive tissues) while at the end of the detoxification

TABLE 3.

Distribution of toxin in oyster tissues.

Tissue	Weight	Lipid	NSP	NSP
Experiment 3a, Day 0				
mantle	20.9	3.74	<10.0	0
adductor	13.1	0.38	<10.0	0
gill	18.3	1.66	17.9	14
palps	11.2	5.27	14.6	7
gonad	22.8	4.98	<10.0	0
digestive	13.7	5	131	78
Experiment 3a, Day 5				
mantle	30.3	3.3	<10.0	0
adductor	12.3	0.39	<10.0	0
gill	16.3	2.04	<10.0	0
palps	10.3	5.55	13.4	22
gonad	17.8	5.6	11.9	33
digestive	13.1	5.57	22.1	45
Experiment 2, Day 4				
mantle	30	2.75	<10.0	0
adductor	14	0.34	<10.0	0
gill	19.4	1.28	<10.0	0
palps	8.9	4.67	12.4	12
gonad	17	3.88	12.4	22
digestive	10.7	4.38	57.9	66

[1] Weighted contribution of the tissue to total amount of toxin for all tissues.

experiments (Day 4 of Experiment 2 and Day 5 of Experiment 3) the remaining toxin was located in the palps, digestive tissues and gonads, all tissues with high levels of lipid.

DISCUSSION

The fit of data to Eq. 1 (as indicated by the R^2 statistic) ranged from a very good fit for Experiment 2 to poor for Experiment 4 Treatment a. However, the poor fits can be explained by the large variability between results from pooled samples of identically treated oysters on a given day rather than the inappropriateness of the exponential model. Similar results were found when fitting the previously published data (Fletcher et al. 1998) from Runs 1 to 6 with the R^2 statistic ranging from 0.565 to 0.999 and the a parameter from 5.21 to 18.29. In New Zealand's only confirmed NSP event, NSP stopped being recorded in shellfish (including Pacific oysters) shortly after the causative organism disappeared from the water column (Chang et al. 1995). In an open system where fresh seawater was continually supplied to the oysters it could be expected that parameter a in Eq. 1 would be zero (i.e., given sufficient time the oysters would completely purify themselves of the toxin as happened in the wild). In fitting the curves this only proved true for two treatments: Experiment 3 Treatment b where the oysters were in large tanks filtered through a biofilter and Experiment 4 Treatment b where oysters were fed with algae during the detoxification experiment. The levels of NSP after 3–5 days for these two treatments were no lower than other experiments, so detoxification was not more rapid in these treatments, but the value of 0 for parameter a suggests that complete detoxification would occur with time. The overall results suggest that the closed tank system is fundamentally limited in its ability to totally detoxify oysters of NSP.

Practical requirements for bacterial depuration of shellfish allow that salinity can vary from ambient by up to 20% without affecting performance (e.g., Interstate Shellfish Sanitation Conference 1997). In our work, reducing salinity from an ambient of 34 to 24‰ (30% reduction) did not have a significant effect on toxin loss, so a similar parameter could be applied for NSP detoxification. Previous work showed that bacterial depuration of Pacific oysters was more effective at 15°C–20°C than at temperatures below 14°C (Fletcher et al. 1991). The current work confirms that a similar pattern applies for NSP detoxification in this species with some evidence of reduced detoxification at 12°C, but no differences in detoxification effectiveness at 15°C and 20°C. Inclusion of an in-line 5 μm filter did not improve the effectiveness of detoxification, suggesting that uptake of NSP associated with particles in the water (e.g., whole algal cells) is not important in this system. As the products of ozonating seawater break down NSP toxins (Schneider & Rodrick 1995) it was expected that NSP detoxification would be more effective for oysters in ozonated water. However, the results did not show any such effect, probably due to the relatively low levels of ozone we were able to add to the water, levels that produced a change in redox potential in seawater from the ambient of around 450 to 550 mv. This level was used because, in preliminary trials, Pacific oysters were observed to close their shells and cease pumping at redox potentials of over 600 mv. Thus, ozonation did not appear to be beneficial in reducing NSP toxin levels within oysters.

In the initial work, we observed that the levels of ammonia in the water increased substantially during the detoxification experiments. We postulated that these levels of ammonia and other metabolic byproducts might have inhibited oyster function, thereby preventing detoxification after the first 2 days of the experiments. Holding oysters in the larger tank where water was filtered by the biological filter in Experiments 3 and 4 was designed to test this. Although there was no build-up of ammonia in this tank, there was still no significant detoxification after the first 2 days (Fig. 1) and rates and levels of detoxification were not significantly different to those oysters in the experimental tanks without the biological filter. Similarly, feeding the oysters did not result in any marked improvement in detoxification. Although these treatments may have slightly improved detoxification, the differences were not sufficient to assure the production of oysters below the regulatory level within the 5-day period.

The location of toxins in the different oyster tissues goes some way towards explaining the failure to achieve complete detoxification. The locations of the NSP toxin at the start of Experiment 3 support the hypothesis that the toxin is associated with algal particles that are in the process of being ingested or digested. However, during the detoxification period the toxin was eliminated from the gills and appeared in the gonad tissues. After detoxification, although the total levels of NSP were reduced, the remaining toxin only appeared in high lipid tissues at the end of Experiments 2 and 3. This suggests that while some of the algal particles may have been passed out in feces others have been digested and some of the lipid-soluble toxin has been transferred to the animals' lipid stores. Subsequent detoxification, therefore, will be a much slower process, reliant on the oysters' need to metabolize their lipid stores.

The considerable variability in toxin levels in oysters held under identical conditions is of concern. The method we used is the one accepted by regulatory authorities and the laboratory is approved under FDA protocols for this test. Although considerable variability is expected when testing individual shellfish (e.g., Scotti et al. 1983, White et al. 1993), pooling 10 to 12 oysters should limit the effects of oyster-to-oyster variability on the results. In some experiments (e.g., Experiment 2) the results of replicate samples were acceptably close, but in others using the same test procedures (e.g., Experiment 1) wide variances were observed. The control in Experiment 4 Treatment d was designed to test whether some of this variability might be due to the uptake of toxins excreted by other oysters. However, this did not appear to be the case, as the non-toxic oysters in this treatment did not accumulate toxin to detectable levels. The observed variability is critical in a regulatory environment where the test is usually carried out on a single pooled sample of 10 to 12 oysters from a given area. For example, in Experiment 1 Treatment c on Day 4 one sample was below the regulatory limit while another was well above (Fig. 1a). Due to the variability in results, an argument could be made that it would be better to base the regulatory limit on the mean of a number of samples rather than a single pooled sample. In this case many of our experiments would have met the regulatory limit. The extra testing required to obtain mean values would place an extra cost on the industry when the current regulatory limit has been effective in preventing NSP illness. There is little knowledge of the human dose-response curve for NSP but a 100-g sample is a reasonable reflection of an average serving for a consumer.

CONCLUSIONS

The levels of NSP in Pacific oysters were reduced to levels near the regulatory limit of 20 mouse units · 100 g^{-1} in 2–3 days when held at temperatures between 15°C and 20°C and salinities between 24‰ and 34‰. However, no treatment consistently produced levels below the regulatory limit within the 5-day trials. This failure can be explained by the hypothesis that toxins migrate from the readily accessible surfaces of digestive tissues and are incorporated into the animals' lipid stores. The level of variability encountered with the standard APHA test procedures suggests that they need to be reviewed to confirm their suitability for assuring safe levels of NSP toxins in shellfish.

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RECIRCULATION OF DINOFLAGELLATE CYSTS BY THE MUSSEL, *MYTILUS EDULIS* L., AT AN AQUACULTURE SITE CONTAMINATED BY *ALEXANDRIUM FUNDYENSE* (LEBOUR) BALECH

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ABSTRACT Holding suspension-feeding bivalves at an aquaculture site may facilitate the maintenance of toxic dinoflagellate populations by concentrating transient vegetative cells or resuspended cysts. To examine the role of the mussel, *Mytilus edulis*, in recirculating cysts within an aquaculture site contaminated with the dinoflagellate *Alexandrium fundyense*, sediment cores and fecal samples were collected in September and October 1996. In the interim period, a bloom of *A. fundyense* vegetative cells began. Mussels egested similar concentrations of dinoflagellate cysts (*Scrippsiella* sp., *A. fundyense*, and an unknown Grey species) regardless of the location of the mussel sock in the site, or the position of the mussel in the water column. In September, more putative *A. ostenfeldii* cysts were egested in feces collected from the bottom of two socks than in those from the top. One sock was located at greater depths near a barrier island and the other in a shallow northeastern cove. Within each dinoflagellate species, there were no significant differences between cyst concentrations in sediment throughout the site, the exception being the high concentrations in September of putative *A. ostenfeldii* beneath the sock located near a barrier island (182 cysts·cm^{-3}). Post-bloom, there were significantly fewer *A. fundyense* cysts in the sediment underlying the sock near a barrier island. In contrast, there were significantly more putative *A. ostenfeldii* cysts in the sediment in the shallow northeastern cove (580 cysts·cm^{-3}). The daily replenishment rate of *A. ostenfeldii* cysts in bottom sediments by mussel fecal deposition was estimated as 2×10^5 cysts m^{-2} d^{-1}, or about 8%. This may be a considerable contribution to the maintenance of this dinoflagellate species in a mussel aquaculture site, but further studies are required to compare other inputs and outputs of cysts to establish the relative importance of bivalve aquaculture.

KEY WORDS: dinoflagellate, *Alexandrium*, cysts, mussel, aquaculture, PSP

INTRODUCTION

Accumulations of resting cysts (or hypnozygotes) of benthic dinoflagellates have been observed in various marine environments: offshore trenches and depressions, fjords, and the shallow coastal embayments often selected for shellfish aquaculture operations (Dale 1976, Dale et al. 1978, Anderson & Morel 1979, White & Lewis 1982). Certain locations that accumulate cysts as a result of selective deposition due to hydrographic and sedimentary processes can act as "seed beds", producing motile cells that then initiate blooms (Steidinger 1975, Anderson & Morel 1979, Tyler et al. 1982). More than 80 species of marine dinoflagellates are known to produce cysts (Matsuoka et al. 1989), including species of the toxic dinoflagellate *Alexandrium* (Halim) Balech (formerly classified as *Gonyaulax* spp. or the *Protogonyaulax catenella/tamarensis* species complex). In many parts of the world, *A. tamarense* (Lebour) Balech has been responsible for incidents of paralytic shellfish poisoning (PSP; see review by Hallegraeff 1993).

PSP is a serious public health risk that threatens the commercial harvest of both wild and cultured bivalve populations. Potent neurotoxins can accumulate in bivalves through ingestion of vegetative cells of toxic dinoflagellates during suspension feeding (Shumway et al. 1987, Shumway 1990). Cysts formed at the end of *Alexandrium* blooms have also been implicated as vectors of PSP toxin transfer to bivalves (Dale et al. 1978, White & Lewis 1982). There has been some debate concerning the toxicity of the cysts relative to the motile vegetative cell (Dale et al. 1978, Yentsch & Mague 1979, White & Lewis 1982, Cembella et al. 1990), although there is a consensus that *A. tamarense* cysts contain PSP toxins. The abundance of *A. fundyense* (Lebour) Balech cysts in sediments has been positively correlated with maximum-recorded levels of PSP toxins in the blue mussel, *Mytilus edulis* (Schwinghamer et al. 1994). Mussels ingest *A. tamarense* cysts and pass them in fecal pellets (Anderson 1984), but whether bivalves can extract the toxins from cysts and assimilate them remains to be established.

Bivalves inhabiting areas with bottom sediments contaminated with toxic cysts cannot avoid encountering these cells during resuspension events. Dense concentrations of suspension-feeding bivalves in areas such as aquaculture sites may facilitate the maintenance of a seed bed of toxic dinoflagellate hypnozygotes by concentrating transient vegetative cells or resuspended cysts and depositing these cells onto the underlying sediment in the form of pseudofeces or feces.

Mussel aquaculture is an expanding industry in Newfoundland: production has increased from 320 tonnes in 1991 to over 1700 tonnes in 1999 (Statistics Canada 1999). Surveys of coastal sediments around the Province have revealed contamination by *A. fundyense* cysts in many areas (McKenzie 1993, McKenzie 1994, Schwinghamer et al. 1994). One former mussel farm, located in Barred Island Cove, Notre Dame Bay, is permanently closed to aquaculture due to persistent PSP contamination, and has been the focus of ongoing research (Fig. 1). Sediment analysis at this site, a cove with two barrier islands and a shallow sill, has revealed *A. fundyense* cyst concentrations ranging from 20 to >1,000 cysts·cm^{-3} (McKenzie 1993, McKenzie 1994, Schwinghamer et al. 1994). This site experiences frequent sediment resuspension as the result of strong winds.

The objective of this study was to examine the role of the mussel, *M. edulis*, in recirculating dinoflagellate cysts and maintaining *Alexandrium* populations within an aquaculture site. The dinoflagellate cyst composition of feces collected from mussels from the tops and bottoms of socks was determined to test the hypothesis that mussels suspended further away from the contaminated sediment egest fewer cysts. To facilitate comparisons be-

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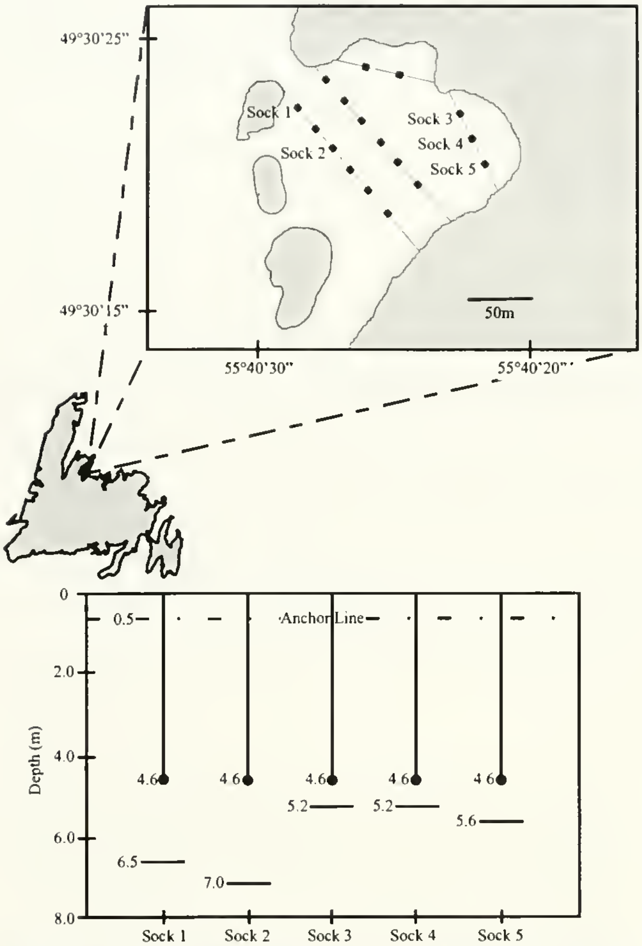

Figure 2. Location of mussel long-lines (—) and socks (◆) at the study site, Barred Island Cove, in Notre Dame Bay, Newfoundland. Lengths of socks and sediment sampling depths are depicted for specified socks (insert).

tween different sampling locations in the site, the concentrations of cysts in the sediment beneath the mussel socks were determined. The biodeposition rate of dinoflagellate cysts in fecal pellets was then calculated to estimate the rate at which cultured mussels returned resuspended cysts to the sediment surface.

MATERIALS AND METHODS

Collection and Examination of Mussel Fecal pellets

Mussels were sampled in September and October 1996 from the tops and bottoms of mussel socks previously established at the study site, Barred Island Cove, Notre Dame Bay, Newfoundland (see Fig. 1). Wind speed and tide stage, factors that could induce heavier sediment resuspension, were similar for the two sampling periods. Sediment traps deployed in September and retrieved in late October contained *Alexandrium* cells in various stages of encystment, suggesting a mid- to late-October bloom (McKenzie et al. 1998). Socks (4.6-m length) were selected based on results of earlier studies conducted at this site, which demonstrated that the highest cyst concentrations occurred in the shallow sediment at the northeastern edge of the cove (McKenzie 1994, Schwinghamer et al. 1994). Socks 1 and 2 were located near the barrier islands; socks 3–5 were situated in the northeastern cove (see Fig. 1).

On September 24, 1996, five mussels (*Mytilus edulis*, mean shell length 63.5 mm ± 10.2 SD) were collected from the top and five from the bottom of each of socks 1, 2, and 4. Two species of mussels, *M. edulis* and *M. trossulus*, co-exist in Newfoundland; those used in this study were identified as *M. edulis* based on size and morphologic characteristics (Freeman et al. 1992). Furthermore, over 95% of mussels in this part of the coast of Newfoundland are *M. edulis* according to genetic markers used by Innes and Thompson (unpublished data).

Mussels were transported on ice (8 h) to the Ocean Sciences Center, Logy Bay; no mortalities occurred. On arrival, mussels were scrubbed free of epibionts and placed in individual 1-L containers with 700 mL UV-sterilized, filtered (1.0 μm) seawater (FSW) at 15°C. Mussels were removed after a 15 h incubation period and the fecal pellets carefully collected and stored in the dark at 4°C. No preservative was necessary, as low storage temperature (4°C) and darkness do not stimulate germination of cysts (Perez et al. 1998). Preliminary trials indicated that the gut retention time of dinoflagellate cysts was approximately 9 h, therefore 15 h was considered adequate time for complete digestion and/or egestion; Scarratt et al. (1993) proposed that 12 h would be sufficient time to purge cysts from mussels. Since the mussels remained closed during transport, there was no egestion of feces. Prolonged retention of the material within the digestive tract during transport did not appear to affect the egestion of intact dinoflagellate resting cysts, although some cysts may have been digested so that the values for cysts egested may be underestimates.

On October 29, four mussels (mean shell length 43.6 mm ± 6.7 SD) were sampled from the top and bottom of each of socks 1, 2, 3, and 5. Fecal pellets were collected on-site: after being scrubbed free of epibionts, individual mussels were placed in plexiglass cylinders (diameter = 70 mm; height = 75 mm) filled with surface water. Cylinders were sealed with a rubber stopper, leaving an air pocket, and submerged for 19 h at 7°C. Fecal material was then collected and transported to the laboratory for processing. No pseudofeces production was ever observed.

Fecal contents were disaggregated by repeated aspiration through a micropipette tip, followed by gentle sonication for thirty minutes in an ice-water ultrasonic bath (Cole Parmer 8851). In preliminary testing of methods for disaggregating fecal pellets, gentle sonication in the bath did not destroy any cysts and resulted in a homogeneous suspension of fecal material (Harper 1997). Sonication by microprobe and by ultrasonic bath has no significant negative effects on germination of *A. tamarense* cysts collected from sediment (Perez et al. 1998). Cysts in each sample were counted (triplicate samples) with an Utermöhl settling chamber. Dinoflagellate cysts were identified and enumerated by direct cell counting using phase and epifluorescence microscopy. Although this approach is laborious and time-consuming, it yields the most accurate information about the composition of the phytoplankton and the condition of the cells (Sakshaug 1990). An electronic particle counter could not be used for enumeration of the cells in the fecal material as the cysts of each species were approximately the same size (40 μm diameter) and shape, and thus cysts of any given species could not be distinguished from those of other species.

To facilitate comparisons between mussels from different locations and of different sizes, cyst abundance values were expressed per mg total dry weight (DW) and per mg ash-free dry weight (AFDW) of feces. Duplicate samples of 0.7–1.2 mL homogeneous fecal suspension were retained on preweighed, precombusted (450°C) Whatman GF/C filters (25 mm) under gentle vacuum, then rinsed with 10 mL 3% ammonium formate to remove salts. Filters were oven-dried to constant dry weight at 60°C, then combusted at 450°C for 12 h, cooled in a desiccator and reweighed to determine the AFDW of feces. Weights were determined within 1 μg with a Mettler UM3 microbalance.

Collection, Concentration, and Examination of Cysts from Sediment

On each of the sampling dates, four sediment cores were collected by hand by SCUBA divers within a 1-m^2 area beneath mussel socks 1–4 in September and socks 1–5 in October. The sediment was composed of small rocks, fine gravel and silt. The top 6–8 cm of sediment and 2–3 cm of overlying water was collected using a 50-mL polycarbonate centrifuge tube (cross-sectional area 5.92 cm^2) with the conical tip removed (McKenzie 1993, McKenzie 1994; McKenzie & Schwinghamer 1994). Cores were transported on ice in the dark to the laboratory where the top 3 cm of sediment and the overlying water were transferred to clean 50-mL tubes, then stored in the dark, unpreserved, at 4°C. Dinoflagellate cysts were enumerated in the top 3 cm of sediment rather than just in the flocculent surface layer (Anderson & Wall 1978, Dale et al. 1978, Lewis et al. 1979). As many *Alexandrium tamarense* (*Gonyaulax excavata*) cysts can be present in the top 3–4 cm of sediment as are found at the surface (White & Lewis 1982).

To ensure adequate dispersal of detritus, sediment samples were sonicated with two bursts (each 45 sec., power level 4) of a Branson 250 Probe Sonifier. Samples were then fractionated to separate and concentrate dinoflagellate cysts following the density-gradient technique of Schwinghamer et al. (1991). In brief, after sonication the sediment was sieved through 80- and 20-μm Nitex nylon sieves, retaining the material on the 20-μm sieve. A step gradient was then formed by slowly injecting "light" and "dense" solutions of a non-toxic, aqueous colloidal silica suspension (Nalco 1060), made isosmotic with seawater (salinity 32‰) using sucrose, beneath the sieved cyst suspension in a 50-mL centrifuge tube. The tubes were then centrifuged at 1,500 g for 30 min at 4°C and the concentrated layer of dinoflagellate cysts at the interface between the light and dense layers removed for examination.

The dinoflagellate cyst composition of each sediment core was determined in triplicate by examining the concentrated cyst layer with phase and epifluorescence microscopy. Each count was performed on 100–200 μL of the homogenous cyst slurry, the volume settled for each core being adjusted to yield a total of 100–300 cysts per slide (Venrick 1978, with reference to Lund et al. 1958). Results were expressed as the number of cysts·cm^{-3} sediment.

Dinoflagellate Cysts in Feces and Sediment

The cysts of four dinoflagellate species were enumerated in fecal and sediment samples: *Scrippsiella* sp. (Stein) Loeblich, *Alexandrium fundyense*, and two unknown species. Cysts were identified using morphologic characteristics: the shape of the cyst body and its ornamentation, cyst size and diameter, wall structure, and the presence of food reserves and pigmented bodies (Matsuoka & Fukuyo 1995). The first unidentified form resembled the resting stage of *A. ostenfeldii* (Paulsen) Balech et Tangen, a toxic dinoflagellate that has been observed in the Gulf of St. Lawrence (Levasseur et al. 1996), and in Nova Scotian coastal embayments (Cembella et al. 1998, Cembella et al. 2000). These cysts matched the description of the *A. ostenfeldii* cyst provided by MacKenzie et al. (1996): a spherical cell (~40 μm diameter) containing one or two red-brown pigmented granules and areas of condensed, golden-brown pigmentation radiating from the center of the cell towards the periphery. Identification of the vegetative cells that emerged from these cysts was not possible, as repeated attempts at establishing cultures were unsuccessful, but these vegetative cells had the typical gonyaulax form (Hallegraeff 1995), although they did not survive more than 48 h. These cysts are referred to here as putative *A. ostenfeldii* pending further germination studies.

The second unidentified cyst is referred to as the Grey cyst because of its appearance in phase microscopy. It is possible that these cysts were partially digested *A. fundyense* cysts as they were approximately the same dimensions and clearly contained a red pigmented body, but all were tapered at one end, in contrast with the two rounded ends of cylindrical *A. fundyense* cysts. Grey cysts did not germinate under culture conditions and currently remain unidentified.

Statistical Analyses

The concentrations of the dinoflagellate cysts in feces from mussels collected from the top and bottom of each sock were analyzed by two-way analysis of variance with the sock and the position of mussel on the sock as fixed factors and the individual mussel as the unit of analysis. Separate analyses were performed for the abundance of each dinoflagellate species present in mussel feces, expressed per mg DW and per mg AFDW feces. September and October data were treated separately as only socks 1 and 2 were sampled in both months.

The concentrations of the four species of dinoflagellate cysts in the sediment beneath the socks were analyzed by one-way analysis of variance with the sock as the fixed factor. Analyses were performed separately for September and October data, and for each cyst type, expressed as the total number present·cm^{-3} sediment.

A two-way analysis of variance was performed to compare pre- and post-bloom concentrations of the cysts in the sediment beneath socks 1–4. Month and sock were fixed factors in the analysis; the individual cores were the experimental units.

Where the F value exceeded the critical value at $P = 0.05$, planned comparisons were made using Fisher's LSD method (Milliken & Johnson 1984). The criterion for statistical significance in all analyses was $P \leq 0.05$.

RESULTS

Concentrations of Dinoflagellate Cysts in Mussel Feces

The concentrations of dinoflagellate cysts egested were highly variable between individual mussels (Fig. 2, Fig. 3). In September (see Fig. 2), the concentrations of *Scrippsiella* cysts egested were low, ranging from 0–142 cysts·mg^{-1} DW and 0–98 cysts·mg^{-1} AFDW feces. Few *Alexandrium fundyense* cysts were egested, from 0–28 cysts·mg^{-1} DW and 0–846 cysts·mg^{-1} AFDW feces. Concentrations of the unknown grey morphotype were also low, ranging from 0–71 cysts·mg^{-1} DW and 0–474 cysts·mg^{-1} AFDW feces. There were no significant differences in the concentrations of *Scrippsiella* sp., *Alexandrium fundyense*, and Grey cysts between mussel feces collected from the tops and bottoms of socks.

More putative *Alexandrium ostenfeldii* cysts were found in the mussel feces collected in September (see Fig. 2) than any of the

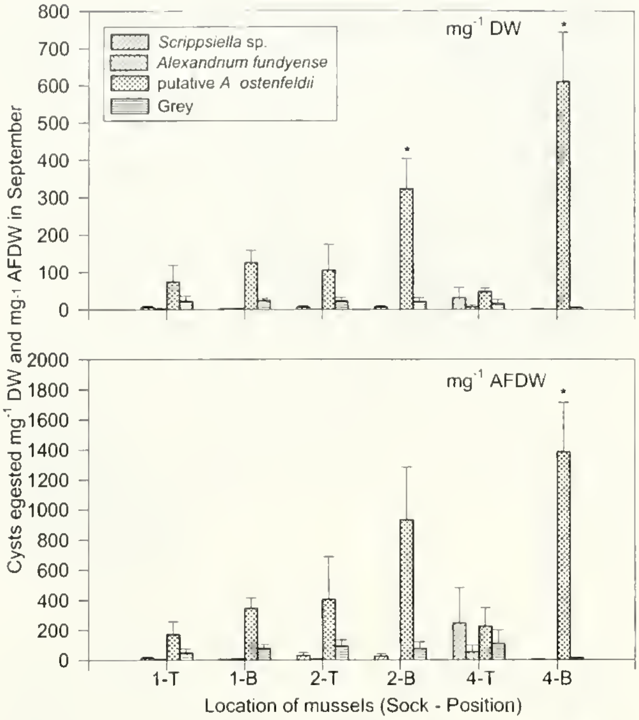

Figure 2. Concentrations of dinoflagellate cysts egested in mussel feces collected from the tops (T) and bottoms (B) of socks in September. Values are standardized per mg dry weight of feces and per mg ash-free dry weight. Each bar is the mean ± standard error ($n = 5$). The asterisk (*) indicates significant differences (P 9 0.05) between the concentrations of a dinoflagellate species in feces collected from the top and bottom of a specific sock.

other dinoflagellate cyst types quantified. Putative *A. ostenfeldii* concentrations varied from 10 to 846 cysts·mg^{-1} DW and 18 to 2,190 cysts·mg^{-1} AFDW feces. Significantly more of these cysts were found in mussel feces collected from the bottom of sock 4 than from the top ($P = 0.0001$ for values expressed in terms of DW; $P = 0.004$ for AFDW) and from the bottom of sock 2 than from the top ($P = 0.039$ for DW). Sock 4 was located in the northeastern cove; the bottom of the sock was only 0.6 m above the sediment. Socks 1 and 2 were located near the barrier islands and were further from the bottom, 1.9 and 2.4 m from the sediment respectively (see Fig. 1).

In October (see Fig. 3), there were no significant differences in the concentrations of any of the dinoflagellate species between fecal samples from mussels collected from the tops and bottoms of the socks. The concentrations of *Scrippsiella* sp. egested were similar to those found in September, ranging from 0–90 cysts·mg^{-1} DW and 0–216 cysts·mg^{-1} AFDW feces (see Fig. 3). *Alexandrium fundyense* cysts concentrations in the mussel feces remained low, varying from 0–170 cysts·mg^{-1} DW and 0 to 181 cysts·mg^{-1} AFDW feces. Concentrations of putative *A. ostenfeldii* cysts egested ranged from 0–130 cysts·mg^{-1} DW and 0 to 325 cysts·mg^{-1} AFDW feces. Grey cyst concentrations were also low; consistent with amounts egested in September, ranging from 0 to 130 cysts·mg^{-1} DW and 0 to 325 cysts·mg^{-1} AFDW feces.

The error in counting cysts in the fecal suspension was estimated from five replicate counts of a single sample containing 494 cysts (putative *A. ostenfeldii*). The coefficient of variation (CV) was 18% mg^{-1} DW and mg^{-1} AFDW feces. The error in this method is comparable to the 20% CV reported by Ishikawa and Taniguchi (1994) for enumeration of cysts of *Scrippsiella* sp. in sediment. The counting error (CV) for the sediment cores was 14%, estimated by five replicate counts of putative *A. ostenfeldii* cysts in one sediment core that contained 156 cysts·cm^{-3}.

Concentrations of Dinoflagellate Cysts in Sediment Beneath Mussel Socks

On both sampling dates, concentrations of *Scrippsiella* sp., *Alexandrium fundyense* and Grey cysts did not differ significantly within each species between sediment samples collected throughout the site ($P > 0.05$, Fig. 4). In September, *Scrippsiella* sp. cysts numerically dominated the sediment with a mean concentration of 596 cysts·cm^{-3} (±335.6 SD) compared with the mean *A. fundyense* concentration of 89 cysts·cm^{-3} (±88.7 SD). Grey cysts were scarce (mean 4 cysts·cm^{-3} ±6.8 SD). In October, mean *Scrippsiella* sp. concentrations were 306 cysts·cm^{-3} (±144.2 SD), *A. fundyense* mean concentrations were 142 cysts·cm^{-3} (±137.3 SD), and very few Grey cysts were found (mean 0.6 cysts·cm^{-3} ±1.3 SD).

In September, there were significantly more putative *A. ostenfeldii* cysts in the sediment beneath sock 1 than elsewhere in the site ($P = 0.008$). In October, however, there were no significant differences in putative *A. ostenfeldii* cyst concentrations in the sediment beneath the mussel socks ($P > 0.05$).

Sediment trap data collected in October as part of a concurrent study (McKenzie et al. 1998) suggested that an *A. fundyense* bloom began during mid- to late-October. Post-bloom, there were significantly fewer *A. fundyense* cysts·cm^{-3} beneath sock 1 than

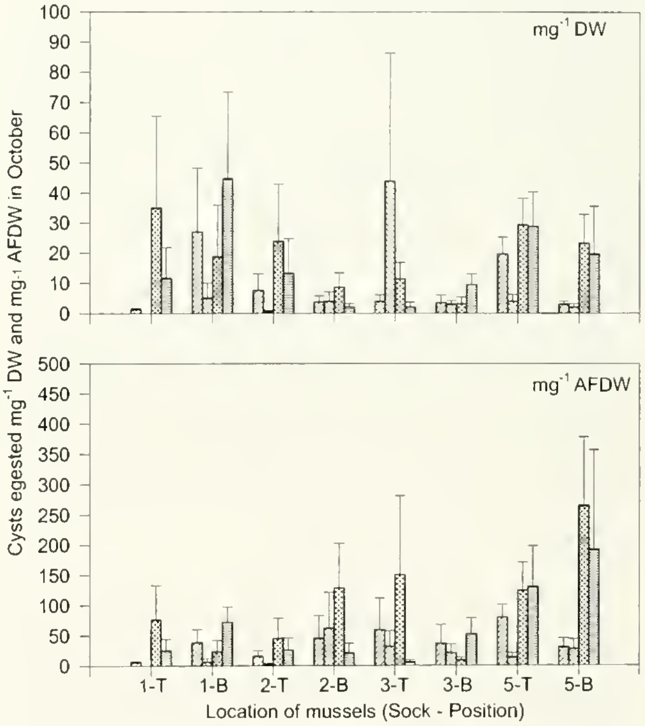

Figure 3. Concentrations of dinoflagellate cysts egested in mussel feces collected from the tops (T) and bottoms (B) of socks in October. Values are standardized per mg dry weight of feces and per mg ash-free dry weight. Each bar is the mean ± standard error ($n = 4$). Legend as in Figure 2.

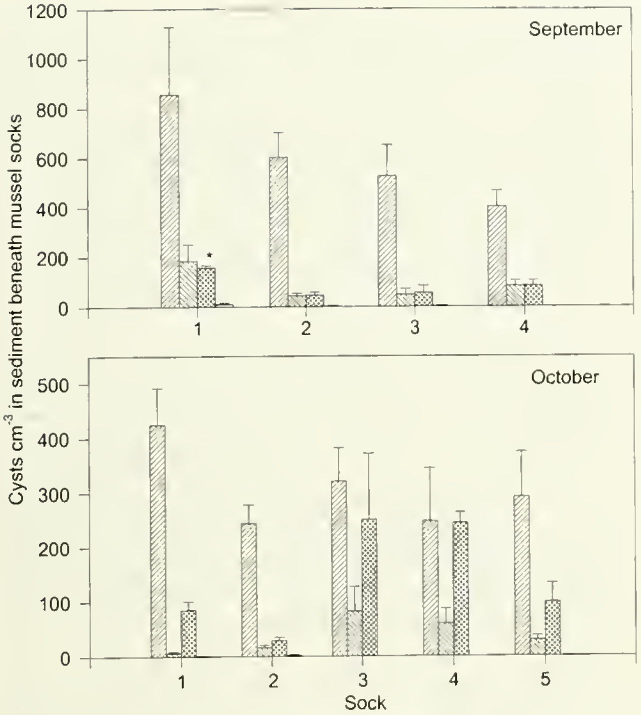

Figure 4. Concentrations of dinoflagellate cysts per cm^3 in the sediment beneath mussel socks sampled in September and October. Sock numbers refer to designations on Figure 1. Each bar is the mean ± standard error ($n = 3$). The asterisk (*) indicates a significant difference between the concentrations of putative *A.ostenfeldii* cysts beneath sock 1 and concentrations beneath socks 2–4 in September ($P = 0.008$). Legend as in Figure 2.

elsewhere ($P = 0.001$). In contrast, there was significantly more putative *A. ostenfeldii* cysts beneath socks 3 and 4 in October than there were in September ($P = 0.006$ and $P = 0.02$, respectively).

DISCUSSION

Significance of Mussel Position in the Water Column with Respect to Cyst Contamination

Vertical repositioning of mussels in the water column may limit their exposure to toxic dinoflagellate cells (Desbiens et al. 1990). To reduce PSP contamination, Desbiens and Cembella (1993) placed mussels near the bottom of the water column (13–15 m deep) during a bloom of vegetative *Alexandrium excavatum* cells and raised the mussels close to the surface when *A. excavatum* was concentrated near the bottom of the water column. Mussels placed near the bottom were the least toxic for most of the exposure period.

In this study, we hypothesized that mussels suspended close to the sediment would egest more dinoflagellate cysts than mussels suspended near the top of the water column. We found that this depended on the location of the mussel sock in the site and the sampling time. In September, mussels collected from the bottom of socks near a barrier island and in the northeastern cove of a former aquaculture site at Barred Island Cove, Newfoundland, egested more putative *A. ostenfeldii* cysts than mussels collected from the top of the socks. In October, however, similar numbers of *A. ostenfeldii* cysts were egested regardless of the proximity of the mussels to the underlying sediment and their location in the site. No differences were found in either sampling date within the concentrations of *Scrippsiella* spp., *A. fundyense* or Grey cysts in feces from mussels collected from the tops and bottoms of the socks.

It is not possible to establish a direct relationship between the concentration of cysts beneath a mussel sock and the concentrations in feces egested by the overlying mussels. Mussels may have been ingesting cysts from other sources (e.g., horizontal advection, newly formed cysts in the water column resulting from encysting vegetative cells), or cells may have encysted within the digestive tract. For example, Grey cysts were numerous in mussel feces collected in October, but few were present in the underlying sediment. Samples from the water column are needed to determine the source of the egested cysts, but it is clear that suspending cultures near the top of the water column does not reduce the exposure of mussels to cysts at this site.

The depth of sediment erosion may be a factor in the resuspension of dinoflagellate cysts, depending on the depth distribution of the cysts of each species. White and Lewis (1982) demonstrated that there are as many *A. tamarense* (*Gonyaulax excavata*) cysts in the top 3 cm of sediment as in the flocculent surface layer, but it is not known whether there are vertical gradients in the abundance of other types of cysts within the sediment. There is no clear relationship between the density of dinoflagellate cysts directly beneath the mussel socks and the number of cysts passing through the overlying mussels.

Distribution of Dinoflagellate Cysts in Bottom Sediments

Concentrations of *Scrippsiella* sp., *Alexandrium fundyense* and Grey cysts did not differ significantly within each species throughout Barred Island Cove. Sediment core samples collected in November and December of 1992 showed the highest concentration of *A. fundyense* (1,130 cysts·cm^{-3}) in the shallow sediment on the eastern edge of the site (McKenzie & Schwinghamer 1994). In October 1993, the highest concentration of *A. fundyense* (118 cysts·cm^{-3}) was detected near the barrier islands (McKenzie 1994). In September 1996, the highest concentration of *A. fundyense* cysts was found beneath sock 1, near the barrier islands (371 cysts·cm^{-3}); in October, the highest concentration was found in the northeastern edge of the cove beneath sock 3 (214 cysts·cm^{-3}). Similarly, the highest concentrations of putative *A. ostenfeldii* were found in the sediment beneath sock 1 in September (182 cysts·cm^{-3}) and beneath sock 3 in October (580 cysts·cm^{-3}). The distribution and concentrations of dinoflagellate cysts in the sediment are clearly variable both temporally and spatially within this site.

Between September and October 1996, concentrations of *A. fundyense* cysts either did not change or significantly decreased in sediments beneath the mussel socks samples. In contrast, there were significantly more putative *A. ostenfeldii* cysts beneath socks in the northeastern cove in October than there were in September. In the interim, a bloom of *Alexandrium* vegetative cells began (McKenzie et al. 1998). Although this bloom was attributed to a proliferation of *A. fundyense* cells, it is possible that it may have been caused by both *A. fundyense* and *A. ostenfeldii* blooming concurrently, triggered by the same environmental conditions; *A. ostenfeldii* does not tend to form monospecific blooms (Cembella et al. 1998). Vegetative cells of *A. fundyense* and *A. ostenfeldii* are difficult to distinguish under the light microscope, the method used by McKenzie et al. (1998). In material fixed in Lugol's iodine, Hansen et al. (1992) were unable to distinguish between the two species.

Blooms of motile, cyst-forming dinoflagellate species can increase cyst deposition in the underlying sediments. For cysts to accumulate, the deposition rate must exceed the rates of loss through regeneration, excystment and transport (Cembella et al. 1988). In this study, we did not observe a post-bloom increase in cyst concentrations of *A. fundyense* in the surface layer of the sediment, but there was an increase in putative *A. ostenfeldii* that may have been the result of transport of cysts within the site or encysting of vegetative cells. Vegetative cells were still in the process of encysting in the water column, as various encystment stages of *A. fundyense* were observed in sediment trap samples (McKenzie et al. 1998).

Previous studies at this site enumerated only those cysts that appeared exactly like *A. fundyense* (McKenzie 1993, McKenzie 1994, McKenzie & Schwinghamer 1994). In 1996, putative *A. ostenfeldii* cysts were as abundant as *A. fundyense* in the sediment. The vegetative cells of *A. ostenfeldii* have been documented in the St. Lawrence Estuary and Gulf (Levasseur et al. 1996) and in coastal Nova Scotia (Cembella et al. 1998). This species has not been recorded in Newfoundland, possibly because it is difficult to identify. Maximum concentrations of *A. fundyense* (371 cysts·cm^{-3}) and putative *A. ostenfeldii* cysts (580 cysts·cm^{-3}) in the sediment from this site are comparable with values from other identified cyst beds in eastern Canada. Sediments in the lower St. Lawrence estuary can contain 400–1,500+ *A. excavatum* cysts·cm^{-3}, depending on the time of the year (Cembella et al. 1988, Cembella 1990). The southern Bay of Fundy, particularly to the east and northeast of Grand Manan Island, is rich in *A. fundyense* (*G. excavata*) cysts, ranging from 2,000–8,000 cysts·cm^{-3} sediment (White & Lewis 1982). In Newfoundland, sediments at Harbour Grace, Conception Bay, contain between 30–150 *A. fundyense* (*G. excavata*) cysts·cm^{-3} (White & White 1985).

Estimated Biodeposition Rate of Cysts from Suspended Mussel Cultures

The replenishment rate of bottom sediments by putative *A. ostenfeldii* cysts egested by mussels can be estimated from the rate of biodeposition, the process by which feces and pseudofeces settle to the bottom (Haven & Morales-Alamo 1966). Since this study was not designed to investigate the biodeposition rates of dinoflagellate cysts, mussels were not continuously exposed to the cells of interest. Values from the following calculations later may, therefore, underestimate the actual rates of biodeposition since the mussels collected in this study were isolated from the seston and were not permitted to feed continuously.

Over the 15 h incubation period in September, mussels (n = 30) egested an average of 333 putative *A. ostenfeldii* cysts·mg^{-1} DW feces. In October, mussels (n = 32) egested an average of 2.4 cysts·mg^{-1} DW feces over 19 h. Navarro (1983, as cited in Navarro & Thompson 1997) reported that biodeposition rates of ~53 mm shell length *Mytilus chilensis* ranged from 10–38 mg DW·mussel^{-1} d^{-1}. Using an intermediate value of 20 mg DW·mussel^{-1} d^{-1}, the biodeposition rate of putative *A. ostenfeldii* cysts by an individual mussel was 6,660 cysts·mussel^{-1} d^{-1} in September and 47 cysts·mussel^{-1} d^{-1} in October. Comparison of these values with the concentrations of cysts in the bottom sediments requires information on the stocking density of mussels. In Newfoundland, the average mussel farm has an estimated 30.9 mussels·m^{-2} bottom (C. Couturier, pers. comm.), therefore in September approximately 2.1×10^5 putative *A. ostenfeldii* cysts·m^{-2} d^{-1} were transferred to the sediment in the form of biodeposits. In October, approximately 1.5×10^3 cysts·m^{-2} d^{-1} were deposited on the bottom.

Mean concentrations of putative *A. ostenfeldii* cysts in the sediment, expressed as numbers·cm^{-2} integrated through the upper 3 cm, were 2.55×10^6 cysts·m^{-2} in September and 4.26×10^6 cysts·m^{-2} in October. Approximately 8% of the putative *A. ostenfeldii* cysts in the sediment were being replenished daily in September by fecal pellet deposition from the overlying mussel stocks. The replenishment rate in October was considerably lower, less than 1% of the cysts in the sediment being deposited in the form of mussel feces.

The egestion of putative *A. ostenfeldii* hypnozygotes by *M. edulis* in feces provides a mechanism for the recycling of dinoflagellate cysts to the sediment. An approximate daily replenishment rate of 8% may be a considerable contribution to the maintenance of cyst populations, and could alter the composition of the sediment over time. Before the contribution of mussel aquaculture to dinoflagellate cyst recycling within a site can be determined, the number of cysts in biodeposits must be compared with other inputs and outputs of cysts, such as horizontal advection or natural sedimentation during blooms. Future research should include the determination of concentrations of cysts and vegetative cells in the water column of aquaculture sites and comparison with appropriate reference areas. The hydrographic characteristics of each site must also be considered as these can clearly play an important role in distribution of cysts.

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REPRODUCTIVE CYCLE OF THE CHOLGA PALETA, *ATRINA SEMINUDA* LAMARCK, 1819 (BIVALVIA: PINNIDAE) FROM NORTHERN PATAGONIA, ARGENTINA

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ABSTRACT *Atrina seminuda* (Bivalvia:Pinnidae) is distributed naturally along the Atlantic coast of America from North Carolina to the Valdés Peninsula. At the San Matías gulf (North Patagonia), its southern distributional limit, it inhabits subtidal areas of fine and coarse sandy bottoms, on depths ranging from 4 to 30 m. The reproductive cycle of a population of *A. seminuda* was studied from October 1999 to October 2000. Histologic samples of gonad tissue were obtained from adult individuals collected at Piedras Coloradas (40°53′S, 65°04′W). Six stages of gonad development were described on the basis of histo-morphologic characteristics: indifferent, early active, developing, mature, spawning, and spent. This study indicates that *A. seminuda* exhibits an annual reproductive cycle. Gametogenesis starts at the beginning of autumn and is prolonged during the winter. A high synchronism in maturation and spawning occurs at November and December. The spent stage begins at mid-summer and concludes at the end of that season. Muscle index (MI) fluctuates inversely to the gonad index (GI) along the reproductive season, suggesting that this species uses reserves stored in the adductor muscle for gonadic maturation. *A. seminuda* is a gonochoric stable species that exhibits, on a 1.05% of the individuals, two types of functional hermaphroditism

KEY WORDS: bivalves, Pinnidae, *Atrina seminuda*, reproductive cycle

INTRODUCTION

Species belonging to the family Pinnidae are commonly known as pen-shell, hacha, rompechinchorro, wing-shell, razor-shell, and locally as cholga paleta. They are distributed along sheltered sites of warm and temperate seas, at depths up to thirty meters.

The cholga paleta, *Atrina seminuda* Lamarck 1819, is a member of the Pinnidae family and is the only Pinnidae present at the Patagonian coast. This species is distributed from North Carolina, United States, to the San José Gulf, Argentina (Scarabino 1977, Lodeiros et al. 1999). *A. seminuda* has been cited by the synonyms *Pinna seminuda*, *Pinna listeri*, and *Atrina (Servatrina) seminuda* (Scarabino 1977).

At the San Matías Gulf (40°40′ S, 63°30′ W) (Fig. 1) this species inhabits sandy bottoms at depths ranging from five to thirty meters. The cholga paleta lives in a vertical position with the anterior portion of its body deeply buried on the substrata, to which it is attached by large and strong byssal threads.

The biology of *A. seminuda* had not been studied until present. However, several studies have dealt with other members of the family Pinnidae, focusing reproductive biology (Noguera & Gómez–Aguirre 1972, Coronel 1981, Yongqiang & Xiang 1988, de Gaulejac et al. 1995a, de Gaulejac et al. 1995b, Maeda–Martínez et al. (unpublished manuscript), Rodríguez–Jaramillo et al. 2001, Ceballos–Vázquez et al. 2000), ecology and population structure (Butler & Brewster 1979, Keough 1984, Butler 1987, Warwick et al. 1997, Richardson et al. 1999), fisheries, and aquaculture (Sung & Myong 1984, Cendejas et al. 1985, Bishui et al. 1987, Yoo & Yoo 1984, Yoo et al. 1988, Maeda–Martínez et al. 1996, Reynoso–Granados et al. 1996, Guo et al. 1999).

Studies on reproductive biology and gonadic cycle are important tools in fisheries and aquaculture management of valuable commercial species. Several methods can be used to estimate the reproductive cycle in bivalves (gonadal macroscopic appearance or frottis, dynamics of embryos and larvae in the plankton, timing of larval recruitment, physiologic indexes behavior, etc) being the histologic study of gonadal tissues the most reliable method.

In this study we describe the reproductive cycle of *Atrina seminuda* from Northwest San Matías Gulf (Patagonia, Argentina) through histologic analyses and the role of the adductor muscle as a storage organ of reserves used in gonad maturation.

MATERIALS AND METHODS

Samples were collected at Piedras Coloradas (40°53′ S, 65°04′ W), a site located on open waters of the NW coast of the San Matías gulf (see Fig. 1). The sampling site has bare bottom, composed of coarse sand and gravel, covered with sparse bivalve shell fragments. Tidal current speed is 0.3–0.5 $m.s^{-1}$, and tides are semidiurnal (average height: 6.31 m) (Servicio de Hidrografía Naval 1994). Monthly average sea surface temperature fluctuates between 10.4°C in August, and 19.8°C in February, and salinity varies between 34.1‰ and 35.1‰ (Fernández 1989).

Sampling was carried out from October 1999 to October 2000, on a monthly basis with the exception of the period October to February when samples were collected fortnightly. Individuals were randomly collected by diving along a straight transect, at depths ranging from 8–12 m. Surface seawater temperature was recorded at each sampling date.

Seventy individuals of sizes ranging from 150–250 mm total length were selected from the sampled lot for histologic treatment and to estimate muscle index (MI).

Histologic Treatment

The visceral mass of each individual was separated from the adductor muscle. A section of gonadal tissue (approximately 1 cm^3) from the posterior-dorsal area was excised from each individual. Each tissue section was fixed in Davidson's solution, stored in 70% alcohol and dehydrated in consecutive baths of ethanol and 1:1 mix of 100% ethanol and xylene. Tissue samples were embedded in paraffin (56/58°C). Five to seven μm sections

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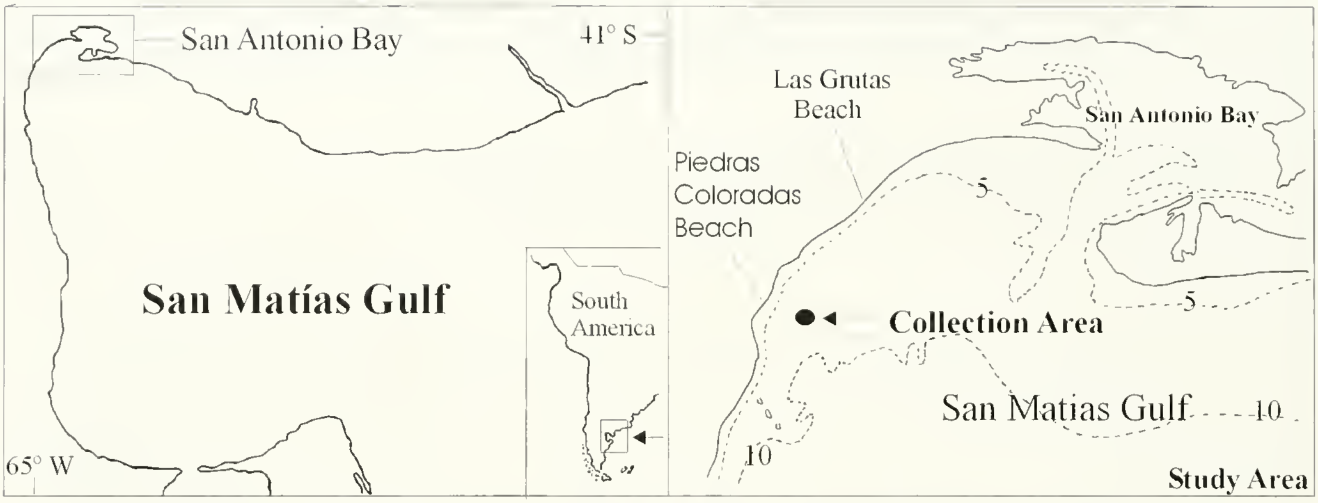

Figure 1. Location of the study area at the Northwest coast of the San Matías Gulf, (Patagonia) Argentina.

were made with rotary microtome and stained with hematoxilin and eosin.

Analysis of the Reproductive Cycle

Histologic sections were examined under light microscope. Sex was determined for each animal.

A scale of gonadal maturity was developed using as a basis a scale of maturity stages described for other Pinnidae, namely *Atrina maura* (Maeda–Martínez et al. unpublished manuscript) and *Pinna rugosa* (Noguera & Gómez–Aguirre 1972, Coronel 1981, Ceballos-Vázquez et al. 2000). Relative frequencies of gonadal developmental stages were obtained throughout the annual cycle. Sex ratio was estimated during the period over which mature and spawning individuals appeared. A *Chi-square* test was used to assess differences from the 1:1 sex ratio.

Frequencies Distribution and Mean Size of Oocytes

Oocyte size was determined from histologic sections using an eyepiece graticule calibrated with a stage micrometer (×400). Five to eight females were randomly selected from the lot sampled during each collection date, and at least 100 oocytes (among those with a clearly visible nucleoli) per female were measured through the longest axis, transverse to the nucleus. Individuals with scarce oocytes and extensive phagocytosis (spent and indifferent individuals) were not analyzed, following the criteria recommended by Grant and Tyler (1983a, 1983b).

Gonad Index

A gonad index (GI) that represents the reproductive activity was estimated using a numerical grading system based on the maturity stages described from each monthly collection data (Heffernan et al. 1989). Three categories were established on the basis of gonad development: 1 = Indifferent (I) + Spent (VI), 2 = Early Active (II) + Developing (III), and 3 = Mature (IV) + Spawning (V). The GI was estimated by multiplying the number of individuals belonging to each category by the category score (1 to 3), then summing the values and dividing the resulting value by the total number of animals in the sample.

Muscle Index

Dry weight of both posterior adductor muscle and remaining soft tissues from 40 individuals on each collection date was obtained using a drying chamber (85°C) until constant weight was reached (~72 h). Muscle index was estimated as MI = (adductor muscle weight/soft tissue weight) × 100. Mean values were calculated for each collection date.

RESULTS

Sex Ratio

A total of 378 individuals were studied (mean shell length = 194.37 mm; SD = 21.49 mm) of which, 175 were females (46.3%), 124 were males (32.8%), 4 were hermaphrodites (1.05%) and 75 (19.8%) could not be sexed (Fig. 2). Sex ratio during the spawning season (1.15 females: 1 male, *n* = 90) did not differ significantly ($P \geq 0.05$) from the expected 1:1 ratio.

Histologic Analyses

Macroscopic and histologic analysis of gonadal tissues, allowed the authors to establish the following stages according to the characteristics exhibited during development.

Indifferent

No evidence of gametic development, acini small with total absence of gametes. Connective tissue is abundant. In this stage it is not possible to determine sex (Fig. 3).

Externally the gonad is brownish, watery, and flaccid.

Females

Early Active: Acini irregular in shape containing oogonias, giving place to previtellogenic pear shaped oocytes (average = 7.6 μm SD = 3.2 μm) attached to the walls of acini. Interfollicle connective tissue decreasing (Fig. 4A).

Macroscopically, gametogenic activity is evidenced due to color and swelling changes. Gonad acquires a pale pink color in the region next to the digestive gland.

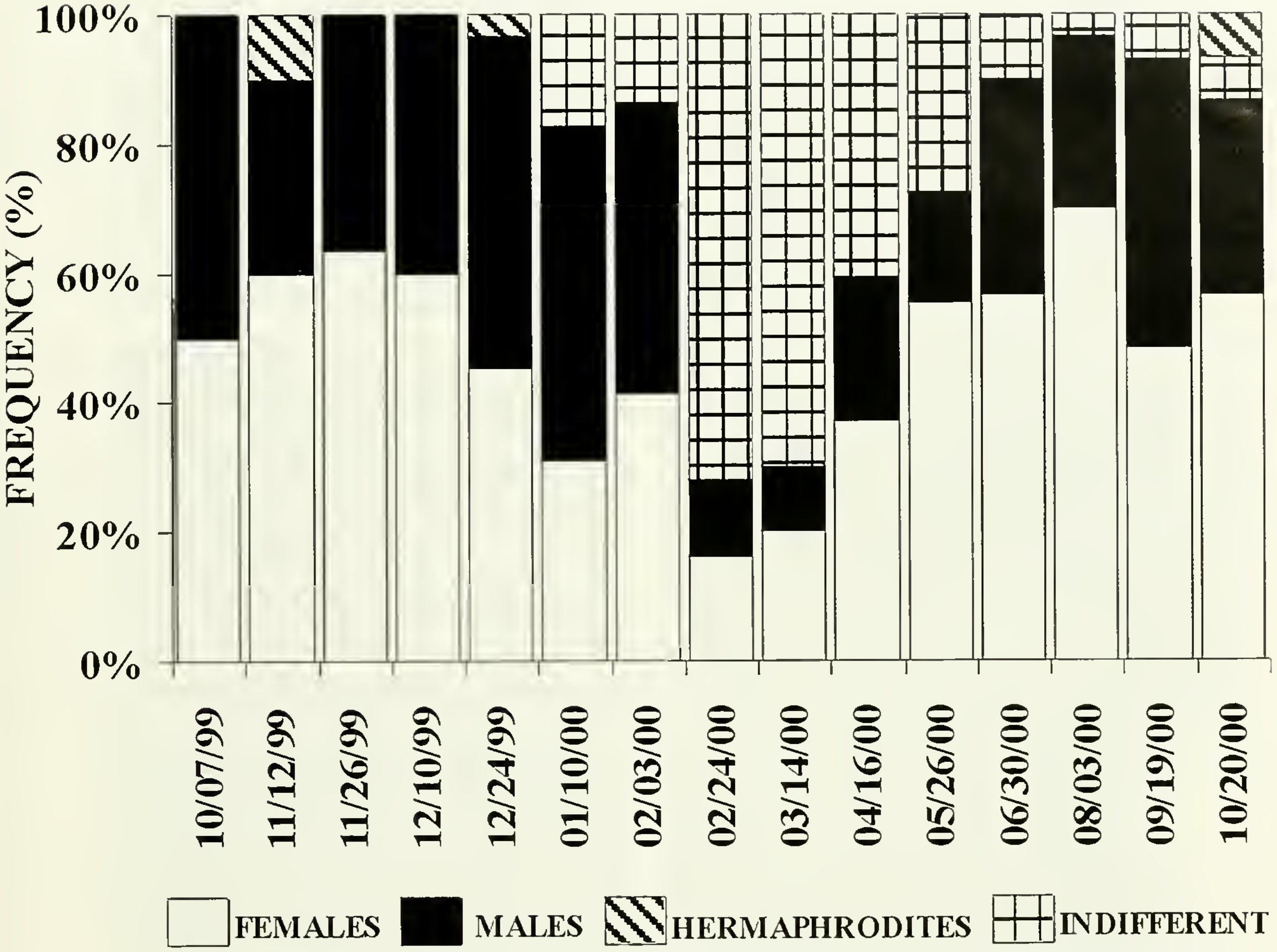

Figure 2. Frequency distribution of male, female, hermaphrodites and undifferentiate individuals of *A. seminuda* in the period October 1999 to October 2000.

Developing: Acini increase in size as a result of oocyte accumulation and growth. Previtellogenic oocytes are found attached to the wall of acini, also several vitellogenic oocytes can be found dispersed in the lumen (average = 31.43 μm, SD = 12.4 μm). Acini walls are well defined and interfollicle connective tissue has decreased (see Fig. 4B).

As gonadic tissue develops, it invades the digestive gland, extending towards the posterior region. Gonadal tissue becomes orange in color.

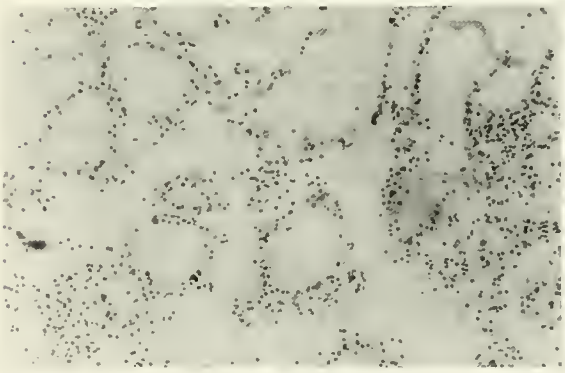

Figure 3. Photomicrograph of gonadal stages: indifferent stage; scale bar = 50 μm.

Mature: Acini are distended and fulfilled with ripe polygonal shaped oocytes (average = 45.6 μm; sd = 23.7 μm); some oocytes remain attached to the walls of acini. Intrafollicle area is reduced to a minimum. The boundary of acini is not distinguishable. Connective tissue is reduced or it is absent (see Fig. 4C).

Gonad reaches its largest area, gonadal tissue is swollen and is red colored.

Spawning: Acini partially spawned containing few oocytes that expand and lose the polygonal shape. Phagocytes are present. Little connective tissue is present. A new proliferation of oocytes attached to the walls of acini can occur (see Fig. 4D).

Externally the gonad exhibits red color, begins to decrease and loses swelling.

Spent: Acini are collapsed as a result of oocyte release. Residual oocytes are irregular in shape; are free in the lumen and begin to be absorbed. Phagocytes proliferate during this stage. Connective tissue abundant. A new proliferation of oocytes can occur attached to acini walls (see Fig. 4E).

Macroscopically, the gonadal tissue shows a pink-red color and watery aspect.

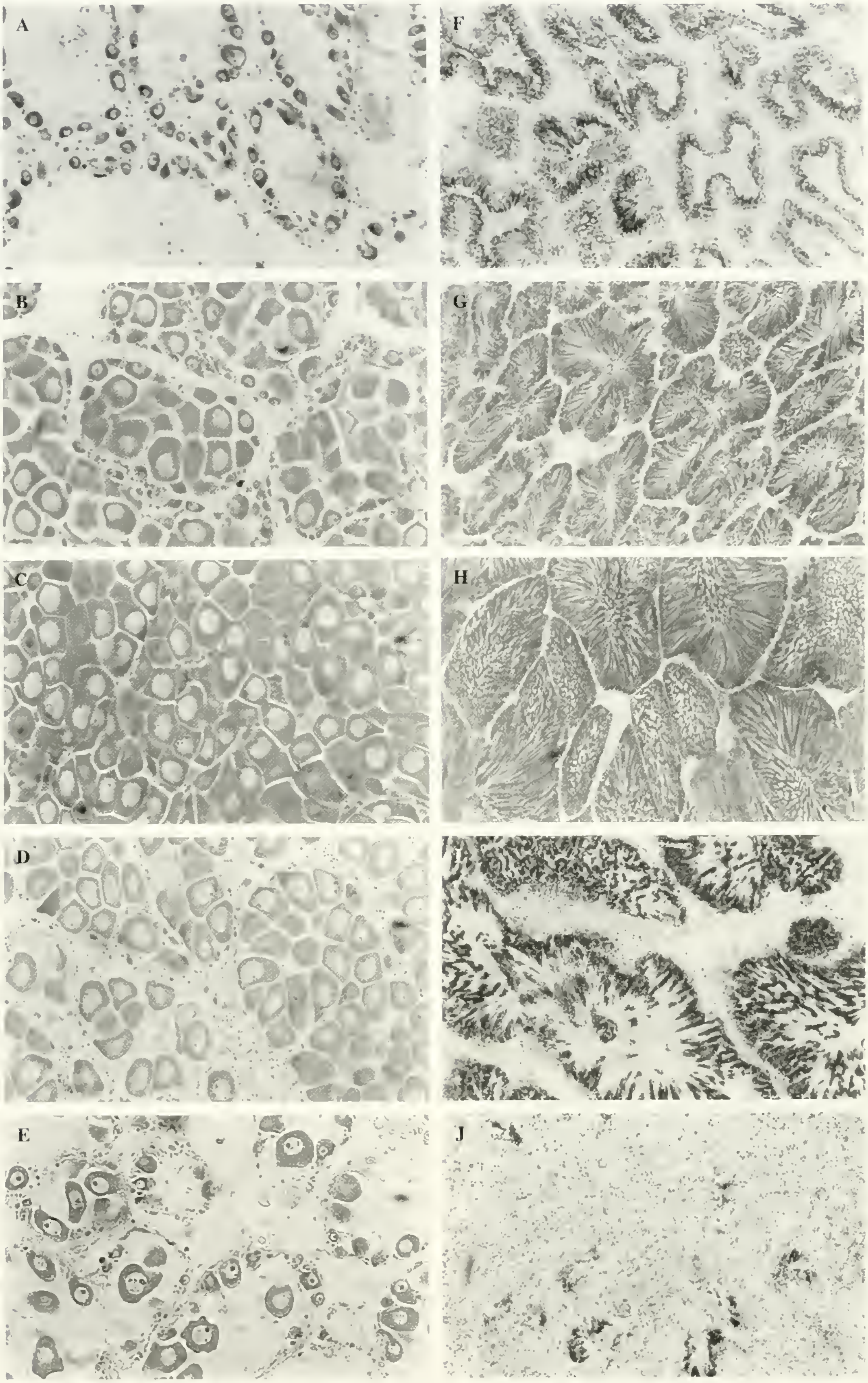

Figure 4. Photomicrographs of gonadal stages of *A. seminuda*. Females: (A) early active, (B) developing, (C) mature, (D) spawning (E) spent; and males: (F) early active, (G) developing, (H) mature, (I) spawning and (J) spent; scale bar = 50 µm.

Males

Early Stage: Several primary germinal cells are attached to the wall of the acini. A variable quantity of spermatogonia is located along the internal wall of the acini arranged in bands of several cells. Connective tissue is decreasing (see Fig. 4F).

Externally, this stage is evident due to color change and swelling of gonadal tissue. It acquires a grizzly white color.

Developing: Spermatogenesis exhibits a centripetal evolution from the internal wall towards the lumen. Spermatocytes and spermatids are free and filling the lumen. Connective tissue continues decreasing as gamete accumulation proceeds (see Fig. 4G).

Gonadal tissue grows invading the digestive gland, and extends towards the anterior area. Gonadal tissue acquires a whitish color.

Mature: Acini are distended, filled with spermatozoa with their tails pointing towards the acini lumen. Spermatocytes and spermatides conform a thick layer against the follicular walls. Connective tissue is absent. Boundaries between acini are not distinguished (see Fig. 4H).

Gonadal tissue is swelling and has a white color.

Spawning: Acini are partially empty, showing a marked decrease in the number of spermatozoa. Connective tissue is increasing (see Fig. 4I).

Externally, gonadal tissue begins to loose size and swelling, and has a grizzly color.

Spent: Acini collapsed with few residual spermatozoa, connective tissue is abundant. No evidence of active gametogenesis. Phagocytes proliferate during this stage (see Fig. 4J).

Gonadal tissue shows a watery aspect and a whitish-grizzly color.

Hermaphrodites

A. seminuda exhibits two types of functional hermaphrodites, both equally represented.

Type 1: Gonads exhibit separated female and male acini (Fig. 5A). Externally, it is possible to distinguish male gonadal tissue and clear reddish female "patches".

Type 2: Female and male gametes arranged inside the same acinus. Oocytes area located at the periphery of the acinus and spermatozoa in the center of the acinus (see Fig. 5B). This type was not detected macroscopically during sampling. The individuals exhibit the appearance of a typical female gonad.

Seasonal Change of Reproductive Cycle

During the period of study, superficial water temperature exhibited its highest value (21.2°C) in January 2000 and the lowest value (9.7°C) on September 2000 (Fig. 6).

The annual reproductive cycle of the total population of *A. seminuda* is summarized in Fig. 7a. In October 1999 only early active (12.5%) and developing individuals (87.5%) are represented. The highest proportion of mature individuals is found in late November (82%), sharply decreasing by middle December. Spawning peaks by the end of December (84%) while, in the following months, individuals at spent and indifferent stages gradually increase. From the end of February to March, the majority of the population is in the undifferentiated stage (72%). Gonad differentiation begins at middle March involving a small portion of the population. Intense gametogenic activity is evidenced through the winter with individuals showing early active and, in lower proportion, developing gonads. By October 2000, as opposed to the former year, the majority of the population has developing gonads (60%) (see Fig. 7a).

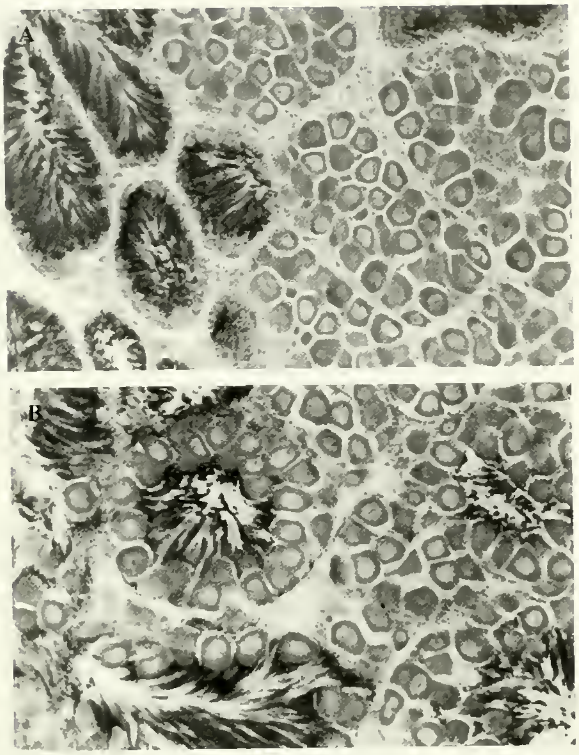

Figure 5. Photomicrographs of hermaphroditic individuals of *A. seminuda*. (A) type 1: separate male and female acini, (B) type 2: male and female gametes in the same acini bar = 50 μm.

Females

In October 1999 all females show developing gonads. The highest proportion of mature individuals is found at the end of November (86%), time at which spawning begins. Spawning peaks in December (86%) and extends through the summer involving low numbers of individuals (10% in January). Gonad proliferation begins in March, with gonads in early active and developing stages represented until October (see Fig. 7b).

Males

In October male gonads are mainly at the developing stage (75%), during November and December gonads at the stages developing, mature and spawning coexist in the population. Evacuation begins in November and, at the end of this month, 78% of mature individuals are detected. Spawning mainly occurs in December, even when spawning extends along the summer until May. Spent individuals are found from January to April. Gonads development begins on April and proceeds throughout the winter and spring months (see Fig. 7c).

Frequencies Distribution and Mean Size of Oocytes

In October 1999 mean oocyte diameter (MOD) was 27.07 μm (s = 11.63 μm). During November and December oocytes grow,

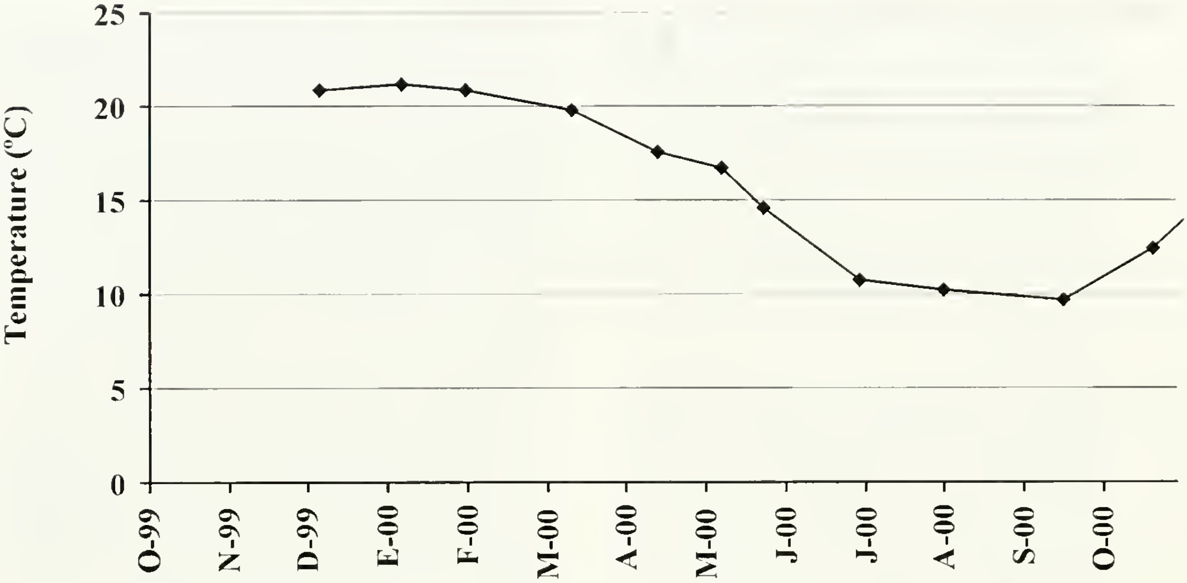

Figure 6. Surface seawater temperature recorded at each sampling date at Piedras Coloradas.

showing the maximal MOD on November 26 (45.63 μm; s = 23.74).

In early December oocytes ranging 35–65 μm decrease in numbers, while oocytes of sizes ranging 5–35 μm, increase. MOD decreases at this time, reaching 29.47 μm (s = 15.84). By the end of December the pattern inverts and the MOD again increases reaching 41.52 μm (s = 12 μm) on December 24. In January and February 2000 the mode represented by 35–65 μm oocytes have diminished markedly and simultaneously a new mode of proliferating oocytes arises in individuals showing spawning and spent gonads. This new proliferation is reflected by an abrupt decline in MOD values that reach 16.11 μm (s = 12.65) in January, and 14.4 μm (s = 12.55) in February. This new summer proliferation does not seem to culminate in a spawning event due to the fact that these oocytes do not reach the typical mature oocyte size in the season (Fig. 8). By the end of February there is a total absence of oocytes in the gonad.

At the beginning of March, MOD shows the minimal values of the annual cycle (mean = 8.08 μm; s = 3.98), and the low dispersion of the data shows the debut of gametogenic activity (see Fig. 8).

Gonad Index

Gonad index exhibits a marked seasonal pattern (Fig. 9). GI increases at mid spring reaching its highest values by the end of November and December as a result of the higher numbers of mature and spawning individuals. Minimal is found by the end of February when most individuals have undifferentiated gonads. GI increases again in March indicating the debut of gametogenesis (see Fig. 9).

Muscle Index

MI also exhibits a seasonal pattern, showing an increase of 73.6% from the end of November, when it has its lower value (20.64%), to August when it reaches its highest value (35.8%) (see Fig. 9). The MI shows, during the reproductive season (October to February), the opposite pattern to the GI, decreasing whilst this increases. This behavior is held until March when both indexes increase and remain stable during the winter months and the debut of spring.

DISCUSSION

A. seminuda exhibits an annual pattern of reproduction at the Northwest of San Matías Gulf. Gametogenesis initiates at the beginning of autumn, when surface seawater temperature (≈19°C) begins to decrease. A high synchronism in maturity and spawning is recorded. Spawning takes place during the end of spring (November to December) when seawater temperature is around 20°C. By the end of December, most of the population was spawning or had already evacuated their gonads. Females exhibit a higher synchronism in maturation and spawning than males. Oogenesis begins earlier in autumn and ends earlier in summer than spermatogenesis. This fact suggests that the small amount of undifferentiated individuals found from June to October can be mainly attributed to males whose spent stage is displaced on time compared with females.

The analysis of oocyte size supported and facilitated the interpretation of the reproductive cycle of *A. seminuda*. Oocyte proliferation and growth could be easily identified, suggesting that the first cohort (December oocytes) is released; meanwhile the new cohort of oocytes that proliferates during spawning or in spent individuals (February oocytes) does not reach maturity and is probably not released. These summer oocytes are most likely reabsorbed.

Gametogenesis is defined by exogenous (mainly temperature and food) and endogenous factors and the relation among them shapes the pattern of each reproductive cycle (Sastry 1979). *A. maura* from La Paz, Mexico, behaves as many tropical and subtropical species, maturing during the warmer months and spawning when temperature decreases (Maeda–Martínez et al. unpublished manuscript). On the other hand, *P. rugosa*, from Bahía Concepción, Mexico show the inverse pattern, with reproductive activity occurring during the warmer months (March to November) and reproductive inactivity in winter (November to February) (Ceballos–Vázquez et al. 2000). Both species show a protracted reproductive period. *A. seminuda* shows a similar reproductive pattern to that of *A. pectinata japonica* from Yoja Bay, Japan (Yoo & Yoo 1984), which shows a protracted developing period and a shorter spawning season that coincides with the warmer months.

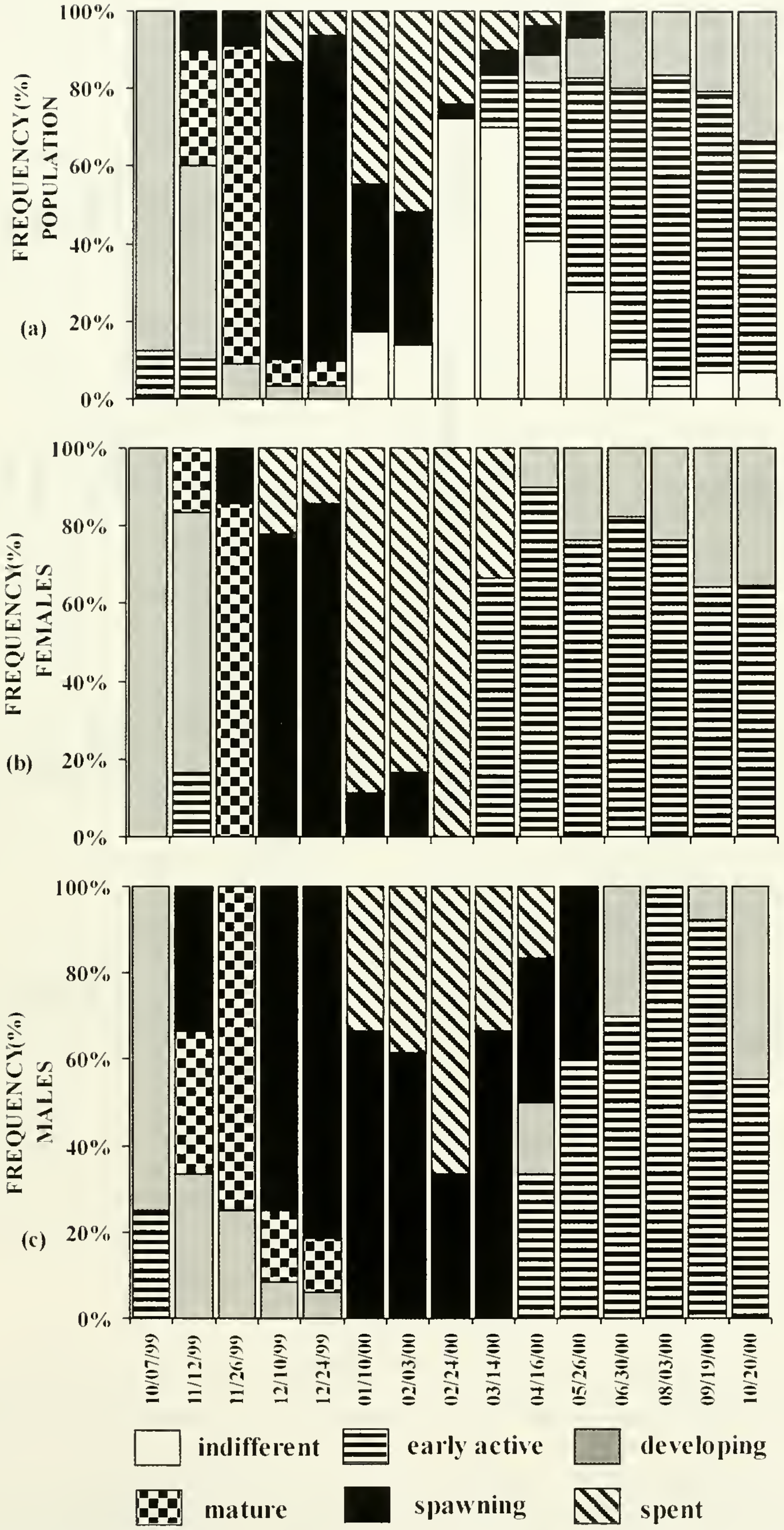

Figure 7. Reproductive cycle of *A. seminuda* at Piedras Coloradas. Relative frequencies of gonadal stages from October 1999 to October 2000. (a) Total population, (b) females, and (c) males.

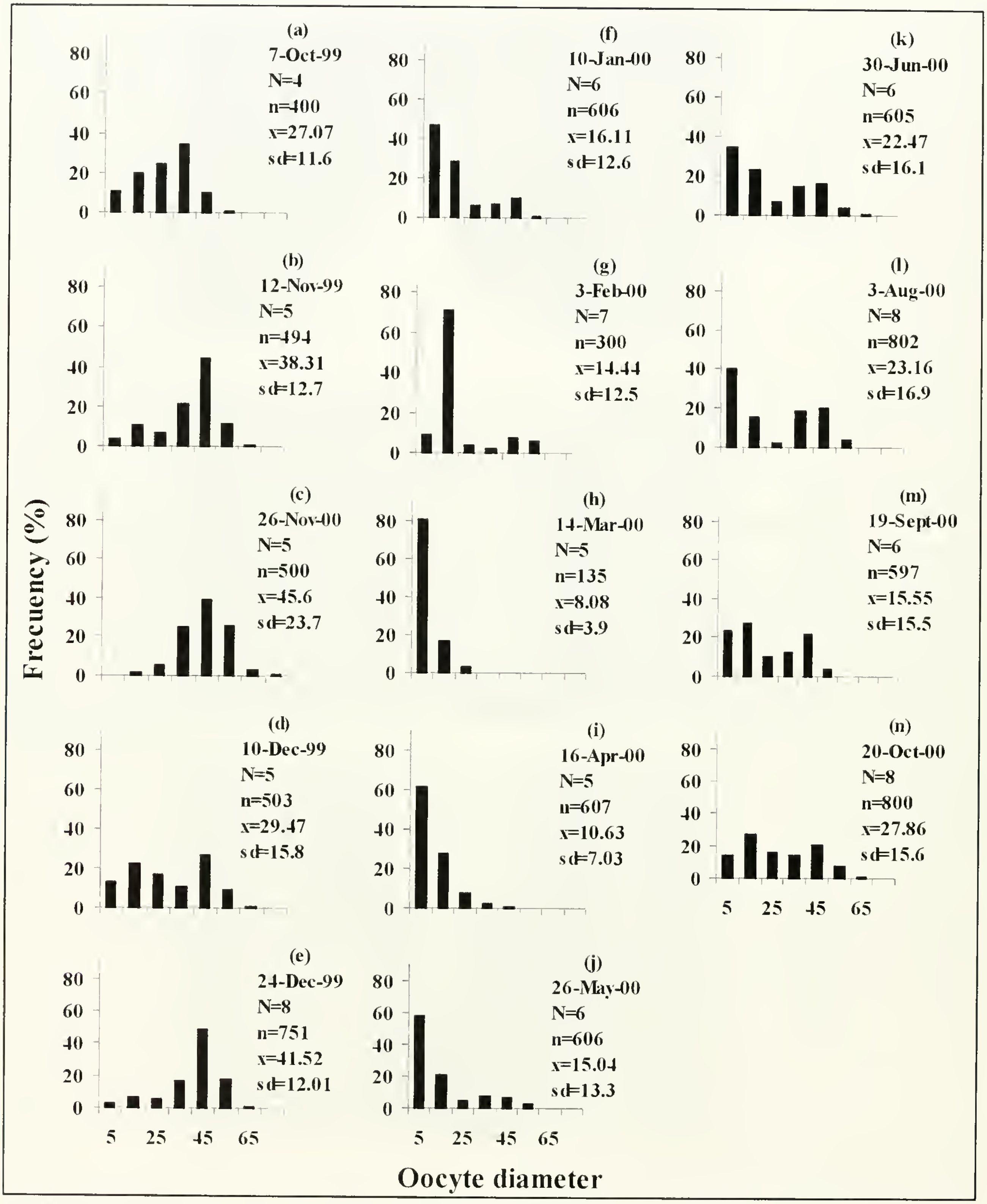

Figure 8. Frequency (%) distribution of oocyte diameter (μm). N = number of females; n = number of oocytes; x = mean oocyte diameter.

This pattern is also shared by other Northern Patagonian bivalve species, as *Amiantis purpurata* (Morsán 2000), *Aequipecten tehuelchus* (Narvarte 2001), *Mytilus edulis platensis* (Trancart 1978) and *Ostrea puelchana* (Morriconi & Calvo 1979).

The adductor muscle of *A. seminuda* (locally called "callo") shows marked seasonal changes becoming light in weight and watery after spawning. The MI sharply decreases during the start of the spawning season, suggesting that this specie uses for reproduction the reserves stored in the adductor muscle. As the proportion of non-spawned individuals in the population decreases, the

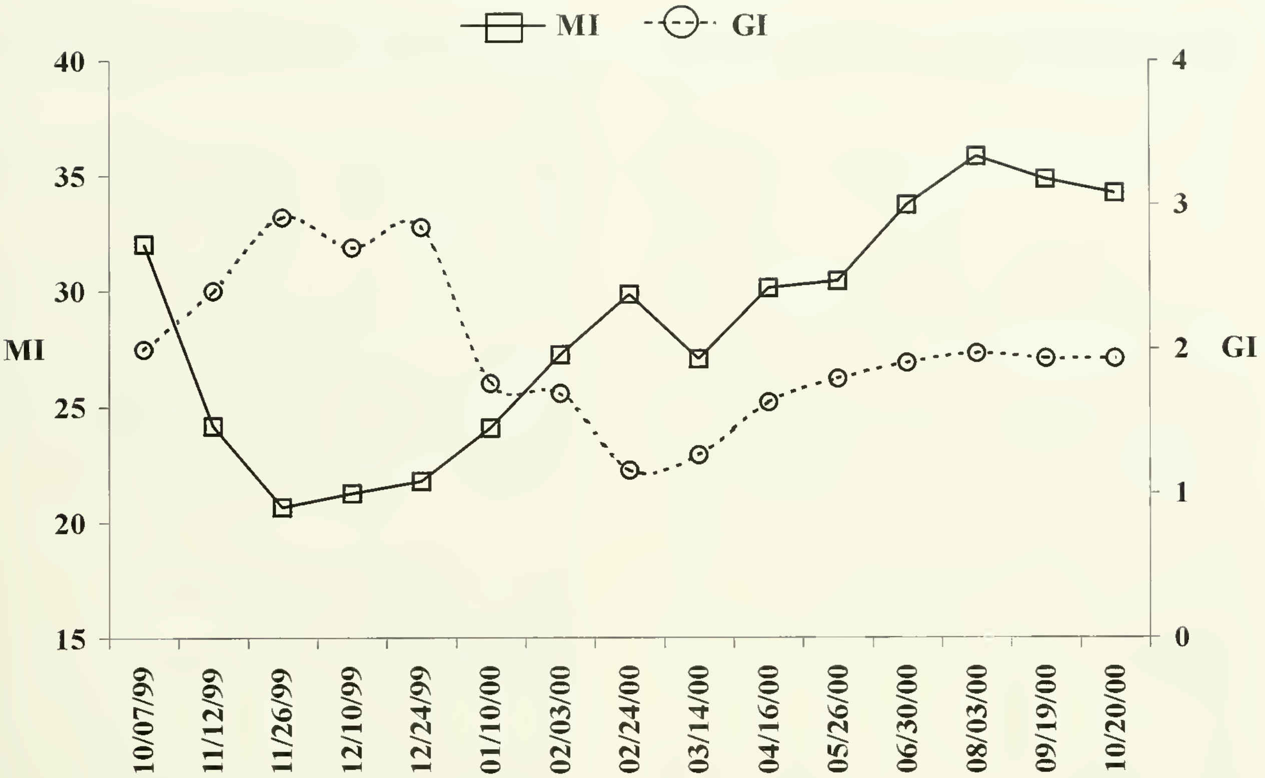

Figure 9. Variation of O = mean gonad index (GI), and □ = muscle index (MI) from October 1999 to October 2000.

MI initiates a gradual increase. This relation among the MI and the reproductive cycle has not been described for other members of the Pinnidae. Maeda–Martínez et al. (unpublished manuscript) found no relation between the MI and the reproductive cycle for *Atrina maura*, a warm temperate sea species that has a different reproductive behavior than *A. seminuda*.

Pelecypods exhibit a wide variation in the expression of their sexuality ranging from strictly gonochoric species to those that are invariably functional hermaphrodites (Sastry 1979). Hermaphrodite individuals are often found in normal gonochoric species. The cholga paleta *A. seminuda* is a stable gonochorist (Giese & Pearse 1974) that shows a low frequency of hermaphrodites (1.05%).

The most common form of hermaphroditism in pelecypods is "type 1" where separate female acini and male acini occur in the same individual (Sastry 1979), although the occurrence of both sexes in the same acini can also exist. The cholga paleta shows both types of functional hermaphroditism. In the case of "type 2" hermaphroditism, the male products are arranged in the lumen and the oocytes are arranged on the walls of acini, suggesting a protandric pattern of gonad development.

Ceballos–Vázquez et al. (2000) documented 20.9% of hermaphroditism in *P. rugosa*, and Butler (1987) reported 0.1% of hermaphrodites in *P. bicolor* from Australia. Coronel (1981) described for *P. rugosa* a similar "type 2" hermaphroditism as the described in our study. Hermaphroditism "type 1" was reported in *Aulacomya ater* (Tortorelli 1987), *Mytilus californianus* (Young 1941, Young 1945) and *Mytilus edulis* (Lubet 1959).

Hermaphrodites have not been reported for cogeneric species *A. maura* (Maeda–Martínez et al. unpublished manuscript), and *A. pectinata japonica* (Yongqiang & Xiang 1987).

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THE PHYSIOLOGICAL ECOLOGY OF BLACK-RIBBED MUSSELS, *SEPTIFER VIRGATUS* (WIEGMANN) (BIVALVIA: MYTILIDAE) ON A SUB-TROPICAL WAVE-EXPOSED SHORE IN HONG KONG

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ABSTRACT Oxygen consumption and ammonium excretion by *Septifer virgatus* were measured *in situ* on an exposed rocky shore monthly from January to December 1999. Condition index and reproductive cycle were determined simultaneously. Spawning was diphasic, the first phase being recorded in spring (March to April). The second spawning was more prolonged (July to October), but less intense than the first. Oxygen consumption was temperature-dependent and demonstrated a seasonal pattern with a peak being recorded from summer to early autumn and with lowest values in winter. Seasonal variation in excretion rate was small with a major peak in summer, possibly associated with post-reproductive stress and high temperatures. O:N ratio reflected energy metabolism and utilization with a peak in spring and early summer (>30) when growth and gametogenesis were resumed. In late summer, spawning was initiated and temperatures rose high and became lethal. O:N decreased to minimum values.

KEY WORDS: *Septifer*, oxygen consumption, excretion, O:N ratio, mussels

INTRODUCTION

Numerous studies have been reported on the relationship between environmental variables and physiological processes of bivalves and corresponding mechanisms whereby the organisms are functionally acclimated to their environments (Bayne 1976, Gosling 1992, Dame 1993). Among various factors, exogenous ones such as food, temperature, and salinity, and endogenous ones such as size, age, and reproductive activity are important in controlling an individual's physiological processes (Bayne 1976, Bayne & Widdows 1978, Bayne & Newell 1983, Huang & Newell 2002).

Representatives of the Mytilidae are highly successful colonizers of intertidal habitats, playing a significant role in energy flow and nutrient cycling due to their dense populations and filter-feeding patterns (Smaal & Prins 1993, Gili & Coma 1998). Among various mytilids, northern temperate/boreal species of *Mytilus* have received most attention (Seed & Suchanek 1992). Black-ribbed mussels, *Septifer virgatus* (Wiegmann) are distributed throughout the Indo West-Pacific and dominate the mid-littoral zone, forming a continuous band on exposed rocky shores including Hong Kong (Iwasaki 1995, Morton 1995). As the ecological equivalent of *Mytilus edulis* and *M. galloprovincialis* on northern Atlantic shores and *M. californianus* on northwestern Pacific shores (Morton 1995), it is surprising that very little is known about this species. In Hong Kong, *S. virgatus* matures at a shell length of ~15 mm and lives for 4–5 y, although older individuals, possibly up to 12 y of age, occur as solitary organisms lower down the shore. Two groups of narrowly spaced growth bands are deposited annually, one during winter (December to March) and another in summer from July to October (Morton 1995, Richardson et al. 1995). Most adult individuals remain mature year round. Spawning is divided, however, into two phases, one in spring (February to March) and another in autumn (September to December). Such a diphasic pattern is suggested to be the result of intense stress in summer when high rock temperatures at mid-day coincide with low spring tides (Liu & Morton 1994, Morton 1995). Such conditions also cause mass mortalities of mussels, barnacles and limpets (Williams & Morritt 1995). In spite of the above research on *S. virgatus* in Hong Kong and elsewhere (Tsuchiya 1983, Iwasaki 1995), no systematic data have been reported upon seasonal variations in physiological responses and their relationships with exogenous and endogenous factors. The objectives of this study are to determine the seasonal changes in physiological responses (oxygen consumption, ammonium excretion) under ambient temperature and food conditions as well as body condition and reproductive cycle. The results provide interesting comparisons with data for other bivalves, particularly the ecologically equivalent mytilids from temperate/boreal regions.

MATERIALS AND METHODS

Sample Collection and Determination of Hydrographic Conditions

S. virgatus occurs as a continuous band in the higher intertidal zone of a wave-exposed shore at Cape D'Aguilar on the southeastern extremity of Hong Kong Island, Hong Kong. About 50 individuals of *S. virgatus* of shell lengths 14–66 mm were collected monthly from the shore from January to December 1999 (except in November when sampling was prevented by strong waves). Epibionts on the shells were removed and individuals were kept for three days in an outdoor fiberglass tank supplied with seawater pumped continuously from the study site.

Food conditions of the seawater, i.e., total particulate matter (TPM: mg l^{-1}), particulate organic matter (POM: mg l^{-1}), particulate inorganic matter (PIM: mg l^{-1}) and organic content (f), were determined using the filter and ash method. Six samples of seawater (200 ml each) were filtered through ashed and pre-weighed 25 mm glass fiber filter papers (Whatman GF/C), rinsed with isotonic ammonium acetate, dried at 90°C for 24 h, weighed to the nearest 0.1 mg, then ashed in a muffle furnace at 450°C for 6 h and reweighed. The concentrations of TPM and PIM were thus measured directly, POM was calculated by subtracting PIM from TPM and f was estimated as $f = POM/TPM$. Temperature (°C), dissolved oxygen (mg l^{-1}) and salinity (‰) were also measured during each visit.

Oxygen Consumption and Nitrogen Excretion

To determine oxygen consumption rate ($\dot{V}o_2$: μg h^{-1}), each *S. virgatus* was placed in a separate sealed perspex chamber (300–

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550 ml, depending on the size of the animal). For individuals smaller than 20 mm, four to six were put in a container and the calculated oxygen consumption was divided by their number. Two empty chambers were used as controls. The sealed chambers were bathed in a fiberglass tank supplied with seawater pumped continuously from the sea to reduce the effect of temperature on the respiratory activity of the contained animals. Each chamber was sealed for about 60 min, the time chosen according to a preliminary study such that the oxygen concentration declined neither too excessively to affect the normal respiration, nor too slightly to accurately determine variations in oxygen concentration (Wong & Cheung 2001). Initial and final dissolved oxygen values were determined with a YSI DO meter. The oxygen consumption rate was measured as $\mu g\ h^{-1}$ after correction with the control.

Ammonium excretion rate (V_N: $\mu g\ NH_4\text{-}N\ h^{-1}$) was determined using the phenolhypochlorite method (Parsons et al. 1984). Individual *S. virgatus* were maintained in separate glass beakers filled with 200 ml filtered seawater (GF/C) for 1 h and the initial and final concentration of ammonium measured. Similar to the oxygen consumption rate measurement, 4–6 individuals smaller than 20 mm were used and the calculated ammonium excretion rate divided by the number in each beaker. Two beakers without animals served as controls.

Oxygen consumption and ammonium excretion rates were transformed to atomic equivalents and the ratio of oxygen to nitrogen (O:N ratio) computed.

Condition Index and Size-Standardization

After determining respiration and excretion rates, the shell length of each individual was measured with vernier calipers to the nearest 0.01 mm. Body tissues were then dissected out and soma and gonad dried separately at 80°C to constant weight. Tissue dry weight was measured by an electronic balance to the nearest 0.1 mg. Body condition was represented by the condition index (CI) calculated from tissue (TDW: mg) and shell (SHDW: g) dry weights according to the equation CI = TDW/SHDW (Brown & Hartwick 1988). Dry gonosomatic index (DGSI), representing the reproductive cycle, was calculated as the ratio of gonad dry weight (GDW: mg) to total tissue dry weight (TDW: mg), i.e., DGSI = GDW/TDW (Lee 1985).

Respiration and nitrogen excretion rates were plotted against shell length and body weight following the allometric equation, $Y = aX^b$, where Y is the physiological parameters, X is body size, and a and b are coefficients. To facilitate comparisons of physiological rates in different seasons, physiological rates were size-standardized according to the following equation:

$$Y_S = (S_S/S_O)^b \times Y_O \quad \text{(Strychar \& MacDonald 1999)}$$

Where, Y and S were the physiological parameters and bivalve body size, respectively and standard and observed measurements refer to the subscripts 's' and 'o', respectively. Analysis of covariance (ANCOVA) indicated that the slopes of the monthly allometric equations were unequal, (i.e., no all-year pooled slope might be regressed) b, therefore, was the coefficient in the above allometric model derived from monthly data (Packard & Boardman 1987).

Physiological parameters were frequently standardized with body weight. One defect of body weight, however, was its seasonal flexibility compared with shell length, especially when large variations in the condition index occur (Navarro et al. 1996), to which the current study conformed. Moreover, it is widely accepted that ventilation rates, one of the factors regulating respiration, is dependent on ctenidial area, which is highly correlated with shell length (Jones et al. 1992, Hughes 1969; Bayne et al. 1976). We, therefore, used shell length instead of tissue weight to standardize the physiological parameters (Iglesias et al. 1996, Labarta et al. 1997). Physiological rates were standardized to a 40 mm individual of *S. virgatus*, the dominant size at the study site.

Statistical Procedures

To obtain functional relationships between physiological responses and environmental conditions, a set of regression equations was fitted to experimental data, following standard least-squares procedures. Regression analyses were performed by simple linear and non-linear procedures, depending on the most appropriate function to be fitted in each case (Zar 1999). Multiple regression analysis was conducted when physiological parameters were correlated with more than one environmental condition, and the collinearity between independents was tested with collinearity statistics of SPSS measured with tolerance value and condition index (Belsley et al. 1980, SPSS Inc. 1999a, SPSS Inc. 1999b). Independent(s) highly correlated with others was (were) eliminated from the independent list and the regressive model was reconstructed until all the intercorrelations between the independent variables were removed. Residuals were also analyzed to check normality, constant variance of predicted dependents and other necessary assumptions of the regression model. Data were transformed if necessary to meet the regressive requisites, (i.e., normality of datum distribution and homogeneity of variances).

Analysis of variance (ANOVA) or analysis of covariance (ANCOVA) was used, depending on whether covariance existed or not, for comparisons among the groups of data. Prior to analysis, raw data were diagnosed for normality of distribution and homogeneity of variances using the Kolmogorov–Smirnov test and Levene test, respectively. All statistical procedures were performed with software SPSS, release 9.0 (SPSS Inc. 1999a, SPSS Inc. 1999b, SPSS Inc. 1999c).

RESULTS

Hydrography

Seasonal variations in hydrographic parameters including seawater temperature, dissolved oxygen, salinity and seston characteristics are presented in Table 1. Temperature underwent seasonal variations with high values (~29°C) being obtained in summer, decreasing gradually to low ones (~17°C) in winter. Dissolved oxygen level was quite constant throughout the year (except in December 2000) and was centered around 6.5 mg l^{-1}. Salinity was fully marine throughout the year except in summer (June to August) when rainfall was highest and value fell to 26‰. Monthly changes in food concentration (TPM) were small but an exceptionally high value was obtained in January, which was six times that of the lowest value (5.11 mg l^{-1}) obtained in June and was probably caused by strong winter monsoon winds and consequent strong waves (Morton & Morton 1983). The value of f varied from 0.20 in April to 0.50 in October with higher values being obtained in summer to autumn (June to December).

DGSI and CI

Temporal variations in total tissue, soma, and gonad dry weight of a 40 mm mussel are shown in Figure 1 and DGSI and CI in

TABLE 1.

Seasonal variations in seston characteristics (±SD) including TPM (mg l^{-1}), POM (mg l^{-1}), PIM (mg l^{-1}), and f, and temperature, dissolved oxygen (DO) and salinity from January to December 1999.

Month	TPM (mg l^{-1})	POM (mg l^{-1})	PIM (mg l^{-1})	f	Temperature (°C)	DO (mg l^{-1})	Salinity (‰)
Jan	33.82 ± 1.33	7.74 ± 0.14	26.08 ± 1.42	0.23 ± 0.01	17.1	5.90	33.6
Feb	9.07 ± 1.58	2.05 ± 0.40	7.02 ± 1.29	0.26 ± 0.04	18.2	7.60	33.8
Mar	5.74 ± 0.65	1.13 ± 0.14	4.61 ± 0.56	0.23 ± 0.03	22.1	7.07	34.0
Apr	7.67 ± 1.79	1.48 ± 0.29	6.19 ± 1.51	0.20 ± 0.02	24.4	6.99	33.8
May	9.47 ± 3.23	1.96 ± 0.18	7.51 ± 3.08	0.23 ± 0.06	25.5	6.13	33.7
Jun	5.11 ± 0.39	1.85 ± 0.17	3.26 ± 0.39	0.37 ± 0.04	28.9	6.47	24.5
Jul	5.67 ± 0.91	2.12 ± 0.46	3.55 ± 0.42	0.38 ± 0.05	29.6	6.30	27.4
Aug	6.75 ± 0.91	2.99 ± 0.79	3.76 ± 0.91	0.44 ± 0.09	28.6	6.87	27.0
Sep	6.47 ± 1.10	2.84 ± 0.97	3.63 ± 0.57	0.44 ± 0.07	28.0	5.61	32.5
Oct	6.89 ± 1.32	3.36 ± 0.75	3.52 ± 0.79	0.50 ± 0.05	25.7	6.25	32.0
Dec	7.21 ± 0.73	2.59 ± 0.29	4.62 ± 0.57	0.35 ± 0.05	17.6	9.90	33.0
Annual mean	9.47 ± 8.05	2.76 ± 1.79	6.71 ± 6.50	0.33 ± 0.11	24.3 ± 4.5	6.81 ± 1.13	31.3 ± 3.3

Figure 2. Soma dry weight remained constant throughout the year with a single peak in February. Two peaks, however, were identified for the gonad with a major one occurring from February to March and a smaller but extended one from May to September. Seasonal changes in the reproductive cycle were represented by DGSI for which two peaks were identified, one in winter and the other in summer (Fig. 2). DGSI was positively correlated with CI (Pearson correlation coefficient r = 0.442, *n* = 402, $P < 0.001$). CI was also correlated with both temperature (Pearson correlation coefficient, r = –0.265, *n* = 402, $P < 0.001$) and TPM (r = 0.054, *n* = 402, $P = 0.276$) but not f (r = 0.039, *n* = 402, $P = 0.436$).

Oxygen Consumption, Ammonium Excretion, and Oxygen: Nitrogen (O:N) Ratio

Allometric equations relating oxygen consumption ($\dot{V}o_2$) and shell length are presented in Table 2. $\dot{V}o_2$ was significantly correlated with shell length for all months. The slopes of the regressions were significantly different (ANCOVA, $F_{10,380} = 5.82$, $P < 0.001$) and varied from 1.30 in September to 2.68 in February. $\dot{V}o_2$ of a standard-sized (40 mm) *S. virgatus* varied significantly with time (ANOVA, DF = 10, 391, F = 34.51, $P < 0.001$) with high values in summer (May to September) and lowest one in January (Fig. 3). The regression analysis showed that $\dot{V}o_2$ was significantly affected by temperature and the relationship between respiration rate, temperature and shell length can be described by the following equation:

$$\dot{V}o_2 = 0.0177 \times SL^{1.948} \times e^{0.0780T}\ (r^2 = 0.726,\ F_{2,399} = 529.54,\ P < 0.001)$$

With temperature as the covariate, salinity did not significantly affect $\dot{V}o_2$ (ANCOVA, $F_{1,399} = 1.098$, $P = 0.295$). There was a negative correlation between $\dot{V}o_2$ and body condition with a Pearson correlation coefficient of –0.14 (*n* = 402, $P < 0.001$).

Allometric relationships relating ammonium excretion (V_N) and shell length are presented in Table 3. V_N was significantly correlated with shell length for all months with the slopes of the regressions varying significantly with months (ANCOVA, $F_{10,380} = 10.09$, $P < 0.001$). The regression coefficient ranged from 1.13 in September to 2.89 in January. V_N of a standard 40 mm *S. virgatus* varied significantly with time (ANOVA, DF = 10, 391, F = 33.13, $P < 0.001$) with the lowest value being obtained in April and a maximum one in September (Fig. 3). As compared with $\dot{V}o_2$, the correlation between V_N and temperature was weak ($P < 0.001$) with a Pearson correlation coefficient (r) of 0.21. Using temperature as covariate, salinity did not significantly affect V_N (ANCOVA, $F_{1,399} = 1.407$, $P = 0.237$). V_N, however, was positively correlated with $\dot{V}o_2$ (r = 0.138, *n* = 402, $P < 0.01$) and negatively with CI (r = –0.176, *n* = 402, $P < 0.001$).

O:N of a standard 40 mm *S. virgatus* varied significantly with

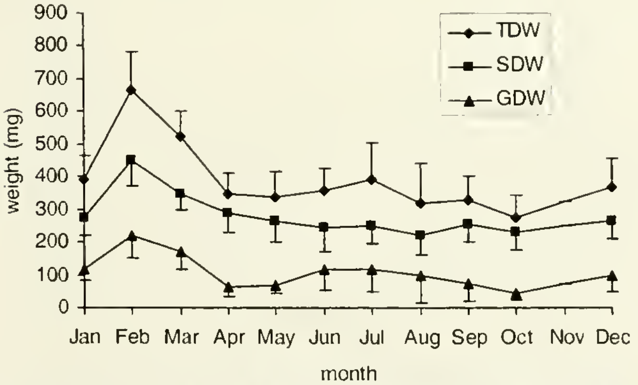

Figure 1. ***Septifer virgatus*. Monthly variations (±SD) of the total tissue (TDW: mg), soma (SDW: mg), and gonad (GDW: mg) dry weight from January to December 1999.**

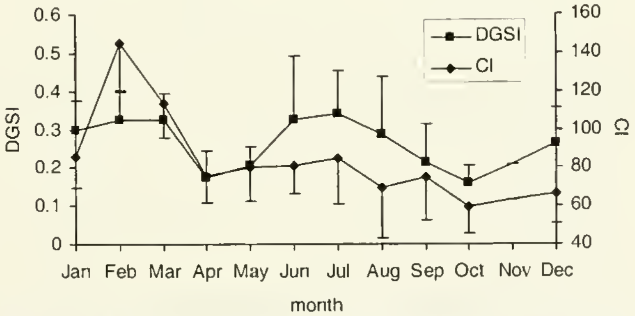

Figure 2. Monthly variations (±SD) of condition index (CI) and the dry gonosomatic index (DGSI) of a standard 40 mm *Septifer virgatus* from January to December 1999.

TABLE 2.

Allometric relationships between respiration rate ($\dot{V}o_2$: µg h^{-1}) and shell length (SL: mm) as well as monthly variation of $\dot{V}o_2$ of a standard 40 mm SL *Septifer virgatus*.

Month	Equation	Regression sum of square	Residual sum of square	F value	*P* value	r square	Standard $\dot{V}o_2$ (mean ± SD)
Jan	$\dot{V}o_2 = 0.0063 \times SL^{2.56}$	11.149	3.871	$F_{1,33} = 95.05$	<0.001	0.74	83.2 ± 25.2
Feb	$\dot{V}o_2 = 0.0053 \times SL^{2.68}$	8.274	2.098	$F_{1,24} = 94.66$	<0.001	0.80	107.3 ± 34.2
Mar	$\dot{V}o_2 = 0.087 \times SL^{1.92}$	9.928	1.929	$F_{1,36} = 185.32$	<0.001	0.84	107.5 ± 26.1
Apr	$\dot{V}o_2 = 0.739 \times SL^{1.44}$	4.748	4.407	$F_{1,28} = 30.17$	<0.001	0.52	162.7 ± 65.2
May	$\dot{V}o_2 = 1.346 \times SL^{1.38}$	5.488	2.402	$F_{1,36} = 82.25$	<0.001	0.70	228.8 ± 65.3
Jun	$\dot{V}o_2 = 0.605 + SL^{1.61}$	9.453	2.089	$F_{1,35} = 158.40$	<0.001	0.82	233.0 ± 49.2
Jul	$\dot{V}o_2 = 0.062 \times SL^{2.21}$	20.458	8.779	$F_{1,37} = 86.22$	<0.001	0.70	240.4 ± 116.8
Aug	$\dot{V}o_2 = 0.075 \times SL^{2.19}$	27.245	4.189	$F_{1,40} = 260.149$	<0.001	0.87	251.0 ± 77.5
Sep	$\dot{V}o_2 = 1.895 \times SL^{1.30}$	7.339	4.578	$F_{1,36} = 57.71$	<0.001	0.62	243.1 ± 80.2
Oct	$\dot{V}o_2 = 0.477 \times SL^{1.52}$	7.568	8.261	$F_{1,38} = 34.81$	<0.001	0.48	142.8 ± 62.3
Dec	$\dot{V}o_2 = 0.011 \times SL^{2.55}$	19.168	4.031	$F_{1,37} = 175.96$	<0.001	0.83	142.5 ± 46.9

time (ANOVA, DF = 10, 391, F = 9.15, $P < 0.001$) with high values being obtained from April to August and low ones from September to March (Table 3, Fig. 4). The annual mean value of O:N was 24.0 with highest and lowest values being 36.1 and 10.5, respectively. O:N was significantly correlated with temperature (Pearson correlation coefficient r = 0.236, $n = 402$, $P < 0.001$) but not with salinity and food conditions.

DISCUSSION

Morton (1995) showed through histological studies that individuals of *S. virgatus* from the same site as the present study remained mature year round. Spawning, however, was limited to two periods in spring (February to March) and autumn (September to December). This matches with our data on DGSI for which the values decreased in March to April and July to October, showing that they reflect reproductive cyclicity satisfactorily. The increase in somatic growth in winter to spring was probably an artefact as the gonad in *S. virgatus*, like in other mussels, was not a discrete organ with gonoglands ramifying throughout the body. Complete separation of gonadal and somatic tissue, therefore, is impossible. This is further confirmed by the suspension of growth from December to March with a winter growth ring formed (Morton 1995). Lee (1988) also found the coupling of gametogenic event and DGSI for another local mytlid, the green mussel *Perna viridis*. The bimodal pattern of spawning and recruitment demonstrated in this study is common to other local mytilids (Morton 1991) and is correlated with seasonal changes in hydrography in Hong Kong of which high summer temperatures (possibly in conjunction with lowered salinities as a result of enhanced summer rain) dividing an otherwise single pattern of summer breeding into pre- and post-summer phases (Morton 1991, Morton 1995). Although DGSI showed two major peaks with similar amplitude, only one was identified for CI in February with a less obvious decrease being obtained from August to December. As growth was suspended in winter to spring (Morton 1995), a higher CI value indicated that reproductive output in spring was much higher than in summer. An extended spawning in summer may help alleviate the problem of post-reproductive stress and increase survival when high rock temperatures at mid-day coincide with low spring tides (Morton 1995). Similar observations were also documented for another locally dominant mussel, *Perna viridis*, inhabiting a sheltered harbor (Cheung 1993a).

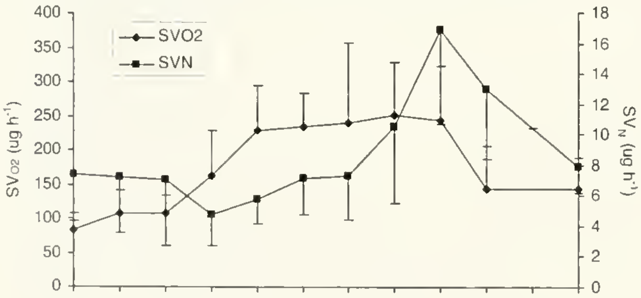

Figure 3. Monthly variations (±SD) of oxygen consumption (SV_{O2}: µg h^{-1}) and ammonium excretion (SV_N: µg h^{-1}) of a standard 40 mm *Septifer virgatus* from January to December 1999.

Food availability is a major factor determining gonadal growth and reproductive cycle (Bayne 1976, Newell et al. 1982, Kang et al. 2000). Food availability in this study was the lowest when the gonad was developing from April to July (Table 1). It was, however, also the time for body growth that was diphasic, with spring growth occurring from March/April to June and autumn growth from September/October to December/January (Morton 1995). A trade-off for limited resources between growth and reproduction may help explain a lower reproductive output in summer than autumn/winter when food availability was highest (October to February).

The oxygen consumption rate of *S. virgatus* demonstrated a seasonal pattern with peaks recorded from summer to early autumn and low values in winter. A similar seasonal pattern has been documented for a number of bivalves (Bayne & Newell 1983, Cheung 1993b, Hummel et al. 2000, Huang & Newell 2002) with temperature, food availability, and reproductive condition being the major determining factors (Babarro et al. 2000). This study indicated that oxygen consumption in *S. vigatus* was most significantly affected by temperature. The effect of food availability was insignificant, although numerous reports have demonstrated that oxygen consumption was reduced under low values of food quality (Babarro et al. 2000) as the result of a lowering of digestive activity and growth (Bayne & Widdows 1978, Bayne et al. 1989).

TABLE 3.

Allometric relationships between NH_4-N excretion rate (V_N: µg h^{-1}) and shell length (SL: mm) as well as monthly variation of excretion rate (SV_N) and O:N ratio of a standard 40 mm *Septifer virgatus*.

Month	Equation	Regression sum of square	Residual sum of square	F value	*P* value	r square	SV_N (mean ± SD)	O:N
Jan	$V_N = 0.000155 \times SL^{2.88}$	14.215	11.624	$F_{1,33} = 40.36$	<0.001	0.55	7.39 ± 3.08	14.38 ± 20.11
Feb	$V_N = 0.000209 \times SL^{2.79}$	8.990	9.511	$F_{1,24} = 22.68$	<0.001	0.49	7.24 ± 3.70	17.82 ± 13.43
Mar	$V_N = 0.000456 \times SL^{2.55}$	17.532	20.885	$F_{1,36} = 30.26$	<0.001	0.46	7.05 ± 4.32	22.61 ± 21.76
Apr	$V_N = 0.0259 \times SL^{1.39}$	4.390	5.488	$F_{1,28} = 22.40$	<0.001	0.44	4.73 ± 2.05	36.10 ± 19.09
May	$V_N = 0.0270 \times SL^{1.45}$	6.008	2.402	$F_{1,36} = 90.23$	<0.001	0.71	5.80 ± 1.66	34.50 ± 15.63
Jun	$V_N = 0.0118 \times SL^{1.73}$	10.847	4.359	$F_{1,35} = 87.10$	<0.001	0.71	7.13 ± 2.42	32.03 ± 13.73
Jul	$V_N = 0.00307 \times SL^{2.09}$	18.248	5.531	$F_{1,37} = 122.07$	<0.001	0.76	7.29 ± 2.91	34.88 ± 32.92
Aug	$V_N = 0.00391 \times SL^{2.11}$	25.270	12.492	$F_{1,40} = 80.92$	<0.001	0.67	10.53 ± 4.96	29.92 ± 30.37
Sep	$V_N = 0.245 \times SL^{1.13}$	5.520	5.946	$F_{1,35} = 33.42$	<0.001	0.48	16.93 ± 6.26	14.90 ± 9.50
Oct	$V_N = 0.00365 \times SL^{2.20}$	15.892	4.290	$F_{1,38} = 140.76$	<0.001	0.79	12.96 ± 4.51	10.52 ± 5.04
Dec	$V_N = 0.0295 \times SL^{1.51}$	6.710	1.791	$F_{1,37} = 138.62$	<0.001	0.79	7.91 ± 1.68	16.04 ± 4.58

Such a discrepancy may be due to small fluctuations in food availability in this study when particulate organic matter was shown to vary from 1.13 to 3.36 mg l^{-1} (except in January). Significant correlation between reproductive activity and oxygen consumption have been reported by a number of authors. Bayne and Widdows (1978) obtained a significant correlation between oxygen consumption rate and gametogenic index and not with temperature for *Mytilus edulis*. Similar results were reported for the cockles *Cerastoderma edule* by Newell and Bayne (1980), whereas other studies have reported significant correlation between oxygen consumption and temperature and reproductive period (De Vooys 1976, Iglesias & Navarro 1991, Smaal et al. 1997). As the gametogenesis of *S. virgatus* covaried with temperature and growth was resumed in spring, it is difficult to distinguish the relative importance of temperature, growth and reproductive activity in determining the oxygen consumption rate in spring. Oxygen consumption rate, however, was lower in winter when the gonads were developing.

Unlike oxygen consumption, the correlation between excretion rate and temperature was weak. A number of workers have documented a close relationship between excretion rate and reproductive cycle (Bayne & Scullard 1977, Bayne & Widdows 1978, Smaal et al. 1997, Babarro et al. 2000). Such correlations were suggested to be the result of a heavy reliance on protein catabolism for energy when mussels are reproductively ripe and have low glycogen content (Bayne & Scullard 1977). Spawning started in August when excretion rate increased abruptly. As glycogen stores are low during that time, energy for maintenance was derived from substantial protein and ammonium production increases. This is further confirmed by the coupled oscillation between DGSI and O:N ratio from June to December (Fig. 4, see later). The problem of post-reproductive stress is aggravated by high rock temperatures at midday coinciding with low spring tides (Morton 1995). Such stresses also resulted in high mortality of limpets (Williams & Morritt 1995), barnacles and *S. virgatus* (Liu & Morton 1994). Temperature started to decrease in October and there was a suspension of spawning and a resumption of growth; excretion rate also started to decrease and O:N ratio increased.

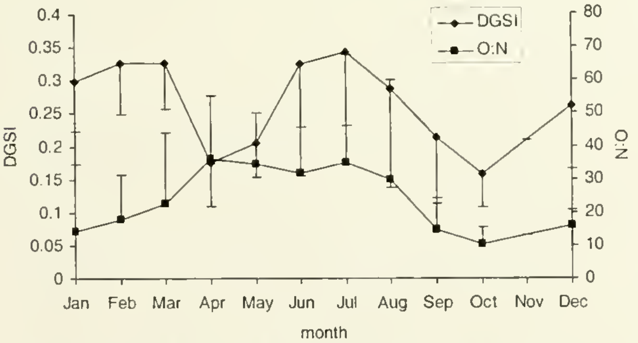

Figure 4. Relationships between temporal variations (±SD) in DGSI and O:N ratio of a standard 40 mm *Septifer virgatus* from January to December 1999.

A low O:N ratio (<20) has been used as an indicator of nutritional stress for marine bivalves (Bayne et al. 1985) as it shows an increased reliance on protein as a catabolic substrate rather than carbohydrates and lipids (Huang & Newell 2002). It may also be related to the greater demand for dietary carbon than nitrogen so as to preserve carbon based energy for utilization in seasons when food resources are low (Kreeger 1993, Huang & Newell 2002). High O:N ratios (>30) coupled with gametogenesis and body growth in spring (April to June), resource demands for gametogenesis, therefore, were mostly derived from temporal nutrition acquisition instead of body reserves. As stress became intense in summer, growth was suspended and spawning was initiated and there was a heavy reliance on protein catabolism for energy resulting in a low O:N ratio (Fig. 4). When growth and gametogenesis resumed in autumn as temperatures decreased, the O:N ratio increased gradually and peaked in spring.

A number of studies have demonstrated the significance of reproductive activity to oxygen consumption, ammonia excretion and O:N ratio for species with a well-defined reproductive cycle such as *Mytilus edulis* (Bayne & Widdows 1978, Widdows et al. 1984, Smaal et al. 1997) and *Mytilus galloprovincialis* (Navarro et al. 1991, Babarro et al. 2000). Being a subtropical species with a diphasic spawning pattern, seasonal variations in energy metabolism and utilization by *S. virgatus* are satisfactorily reflected in O:N ratio and are closely related to reproductive cycle, in addition to environmental influences such as temperature and food availability. Although oxygen consumption is most significantly affected by temperature, excretion rate is mainly influenced by growth and nutritional stress associated with intense heat and reproductive activity.

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MICROALGAL FOOD OF THE RIBBED MUSSEL *AULACOMYA ATRA* (MOLINA, 1782) IN GOLFO NUEVO (PATAGONIA, ARGENTINA)

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ABSTRACT Stomach contents of the ribbed mussel *Aulacomya atra* and phytoplankton samples from the west coast of Golfo Nuevo (Chubut Province, Argentina) were analyzed monthly between August 1997 and December 1998. Twenty-five taxa of microalgae ranging from 9 to 231 μm in size were found in stomach contents along with other miscellaneous items. A marked seasonal variation of microalgal food was not observed in *Aulacomya atra*. Diatoms such as *Paralia sulcata* and *Thalassiosira* sp. were observed year round. Variation in the benthic to total species ratio revealed that during most of the year benthic and pelagic microalgae contributed equally to the diet of the ribbed mussel. In contrast, during dinoflagellate blooms, as occurred during late spring and summer, dinoflagellate cells dominated the ribbed mussel stomach contents. This finding is of special importance in relation to the recurrent blooms of the PSP causative agent, *Alexandrium tamarense*, in the Golfo Nuevo.

KEY WORDS: ribbed mussel, *Aulacomya atra*, food resources, phytoplankton, Southwest Atlantic, Patagonia

INTRODUCTION

Mussels are suspension feeders. Quality and abundance of food affects growth rate, gonad development and survival of bivalve mollusks (Bayne & Widdows 1978, Newell et al. 1982, Berg & Newell 1986). It is generally assumed that suspension-feeding species rely on phytoplankton as their main source of energy. Yet it has been demonstrated that benthic species can also play an important role in the feeding ecology of various bivalves (Mikulich & Tsikhon–Lukanina 1981, Tsikohn–Lukanina 1982). In fact, it is possible to find benthic and pelagic food species equally represented in the guts of bivalves from shallow waters (Shumway et al, 1987). However, several authors have pointed out that food selection occurs within in bivalves (Shumway et al. 1985, Sidari et al. 1998).

The ribbed mussel, *Aulacomya atra* (often incorrectly cited as *Aulacomya ater* according to Cazzaniga, 1994) (Molina, 1782), locally named cholga, supports shellfisheries in the northern Patagonian gulfs of Southwest Atlantic (Ciocco et al. 1998). It is one of the most common bivalve species around the coasts of southern South America (Fig. 1), ranging northwards to San Matías Gulf (41°S) on the Atlantic and to Perú along the Pacific (15°S).

The quantity and quality of food available is a major limiting resource for suspension feeding organisms (MacDonald & Thompson 1985, MacDonald & Thompson 1986, Chauvaud et al. 1998). Until now information on the specific food items used by *Aulacomya atra* was only available for the Chilean coasts (Guzmán & Campodónico 1975, Osorio et al. 1982). This is the first report on feeding in *Aulacomya atra* along the Argentina coast.

Golfo Nuevo, situated at 43°S on the Patagonian coast of Argentina (Fig. 1), is a rough elliptical, semi-enclosed body of water in contact with the Southwestern Atlantic Ocean through a 17-km wide strait. Phytoplankton is dominated by diatoms and dinoflagellates (Gayoso 2001). Recurrent blooms of the toxic dinoflagellate, *Alexandrium tamarense* (Lebour) Balech, have been documented, resulting in human health hazard (Esteves et al. 1992, Ciocco 1995, Gayoso 2001). Other harmful dinoflagellate species including *Prorocentrum lima* (Ehrenberg) Dodge (Gayoso & Ciocco 2001) and *Dinophysis acuminata* Claparède & Lachmann have also been found in the area adding potential risks for human health and marine resources. In this study we describe the stomach contents of ribbed mussels in order to: (1) characterize the nature of the food items; (2) assess seasonal variation of food items; and (3) provide preliminary information about the relationships between microalgal species in water samples and algal cells in the stomachs of the ribbed mussel.

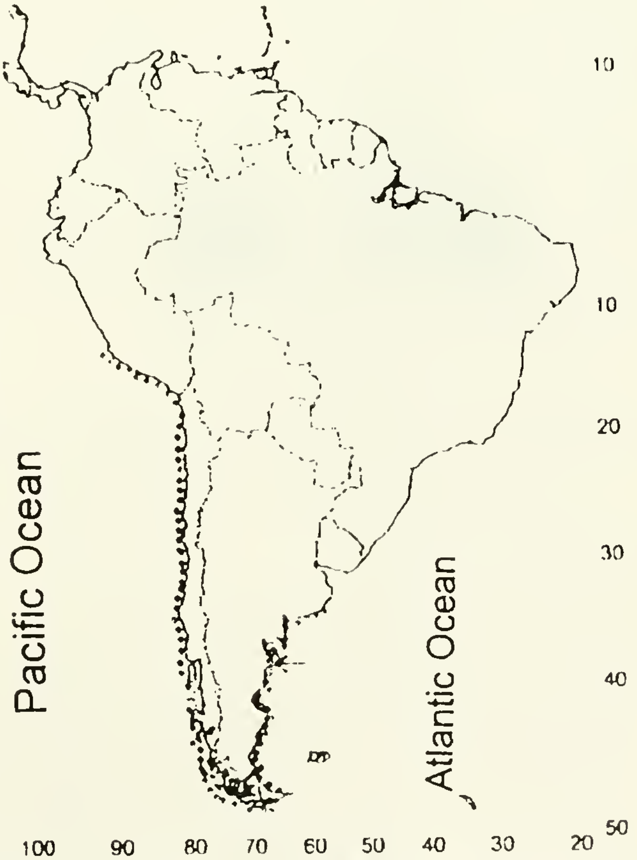

Figure 1. Map of South America showing distribution of *Aulacomya altra* (gray circles) and location of sampling area (arrow).

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TABLE 1.

Seasonal variation in relative abundance of microalgae stomach content of ribbed mussel *Aulacomya atra* from Nueva Bay, Nuevo Gulf (Period: 1997–1998)

Species	Aug 20	Sep 8	Oct 1	Oct 13	Oct 28	Nov 11	Dec 18	Jan 2	Feb 3	Feb 18	Mar 31	Apr 14	May 14	May 29	Jun 10	Jun 30	Jul 14	Aug 4	Sep 1	Oct 21	Nov 17	Dec 2
Actinoptychus vulgaris (B) (33–35)	+	+	+								+				+			+	++			
Coscinodiscus spp. (B/P) (75.5–196)	+	+				+		+	+						+		++	++	+			
Cyclotella sp. (P) (32–35)	++	++	++	++	+	+			+					+		+						
Ditylum brightwellii (P) (33–50.6)	++	++			+	+												+	+			
Paralia sulcata (B/T) (9–34)	+	+	++	++	++	+++	+	++	+		+	+	++	++	++	+++	++	++				
Thalassiosira spp. (P) (26.4–75.5)	++	+++	++	++	+	+		+		+		+	+	+++	++	++	+++	+++	+++	++	+	
Unidentified centric diatoms (P) (35–55)	++	++	++			+	+	++	++		+	+				++				+++	++	
Cocconeis sp. (B) (48–60)	++	++	++	++		+	+												+++			
Grammatophora sp. (B) (60–76)	+	+	+	+																		
Licmophora gracilis (B) (66–120)	+																		+			
Navicula spp. (B) (74.8–82)	+	+			+						+	+		++		++	+		+			
Nitzschia spp. (B) (123–150)	+	+		++				+														
Pleurosigma sp. (B) (178.2–231)	+	+	+					+			+				+				+			
Pseudo-nitzschia pungens (P) (105.6–125.4)	+											+		++	+		++			+		
Rhabdonema sp. (B) (77–82)						+																
Thalassionema nitzschioides (P) (55–136)						+						+	+	++							+	
Unidentified pennate diatoms (B) (24.2–66)	+++	++	++	++	++	+++	++	+++			+	+++	++		+++	++		+++	++	++	++	
Alexandrium tamarense (P) (28.5–30)	+	+							+	+	+									+	++++	++++
Ceratium tripos (P) (53.0–59.0)																	+					
Dinophysis acuminata (P) (33–39.6)									+													
Prorocentrum micans (P) (33–35.2)		+++	++	++	+	+++	++	+++++	+++++	+++++	+++									+	+++	++++
Pyrophacus horologium (P) (55–80)										+	+											
Dictyocha speculum (P) (26.4–32)	+	+								+		+				+						

+ 1–4 cell/stomach (mean of 6 stomachs).

++ 5–10 cells/stomach.

+++ 11–20 cells/stomach.

++++ 21–100 cells/stomach.

+++++ >100 cells/stomach.

Between parenthesis habitat (B = benthic; P = pelagic; T = tychopelagic) and size (μm) of the species.

MATERIALS AND METHODS

Samples of phytoplankton and ribbed mussels (*Aulacomya atra*) were collected monthly from August 1997 to December 1998 at a station located in the west coast of the Golfo Nuevo (42°46'S; 65°02'W). On each sampling date, several adult ribbed mussels (length range: 55.2–116.4 μm) were collected by divers from the bottom (approximately 18 m depth). The mussels were transported to the laboratory and the digestive gland of six randomly chosen individuals were dissected. For microscopic analysis, stomach contents were diluted with filtered seawater and observed in a Sedgwick–Rafter chamber using a compound light microscope. Microalgae present in the stomach contents were identified and counted. Water samples (200 mL) were collected with a Van Dorn bottle at 0 and 15 m depth and immediately fixed in Lugol's iodine solution. For quantitative phytoplankton analyses, 50 mL sub samples, using mixed 0 m and 15 m depths, were settled in a chamber and counted using an inverted microscope.

RESULTS

Twenty-three taxa of microalgae, ranging in size from 9–231 μm, were found in the stomach contents of ribbed mussels (Table 1). The diatoms *Paralia sulcata* (Ehrenberg) Cleve and a *Thalassiosira* species characterized by linear areolae arrays and a diameter ranging from 26.4 to 32 μm, were the most frequent species (77% of all samples). A group of unidentified benthic pennate diatoms that included more than one species also showed high frequency. The dinoflagellate *Prorocentrum micans* Ehrenberg was the most abundant organism found in the ribbed mussel stomachs. During the summer (January to February, 1998), mean densities up to 225 cells per individual mussel were observed. On November 17 and December 2, 1998, cells of the toxic dinoflagellate, *Alexandrium tamarense*, were found in great number (mean values up to 105 cells per individual mussel). Both dinoflagellate species were observed mostly as intact cells. Additional items were observed including macroalgal fragments, foraminifera and empty tintinnid loricae (mainly *Stenosemella avellana* Meunier, *Helicostomella subulata* Ehrenberg and *Tintinopsis gracilis* Kofoid & Campbell). Broken cells of the diatom *Ditylum brightwellii*, (T. West) Grunow ex Van Heurck, and fragments of pennate diatoms were also frequently found.

Phytoplankton seasonal variation was characterized by two diatom peaks, spring and autumn blooms (Fig. 2A), dominated by *Chaetoceros socialis* Lauder, *C.curvisetus Cleve*, *C.diadema* (Ehrenberg)Gran and *C. didymus* Ehrenberg, *Skeletonema costatum* (Greville) Cleve, *Pseudonitzchia pungens* (Grunow ex Cleve) Hasle and *Guinardia delicatula* (Cleve) Hasle. Dinoflagellates tended to be abundant during spring and summer (Fig. 2B), when *Prorocentrum micans* was the most abundant species. Other dinoflagellates included *Pyrophacus horologium* Stein and *Dinophysis acuminata*. *Ceratium horridum* (Cleve) Gran, *C. fusus* (Ehrenberg) Dujardin and *C. tripos* (O. F. Müller) Nitzsch were also present during the autumn. The toxic dinoflagellate *A. tamarense*, responsible for PSP toxicity in the area, was found in phytoplankton samples taken in November to December, 1998 (up to 1.5×10^3 cells L^{-1}). Phytoplankton was dominated during late spring (late October to December 1997) by small forms of phytoflagellates, less than 10 μm in diameter, including *Phaeocystis* spp., coccolithophorids and small-unidentified monads.

All the microalgal taxa identified from the guts of ribbed mussels were also found in water samples. Many of them, however, were true benthic species and only occasional component of the phytoplankton community. The average ratio of benthic species to total species numbers from 6 stomachs per sampling date (Fig. 2C) widely varied throughout the year; it varied from less than 0.1 in summer (February 1997) and late spring (November to December 1998), during *Prorocentrum micans* and *Alexandrium tamarense* blooms, to a ratio of almost 1 in May 1998. Percentages between 0.4 to 0.5 were commonly registered (Fig. 2C). Moreover, some of the more representative phytoplankton taxa and algal groups, such as *Chaetoceros* spp., *Skeletonema costatum* and phytoflagellates, were never found in the stomach contents.

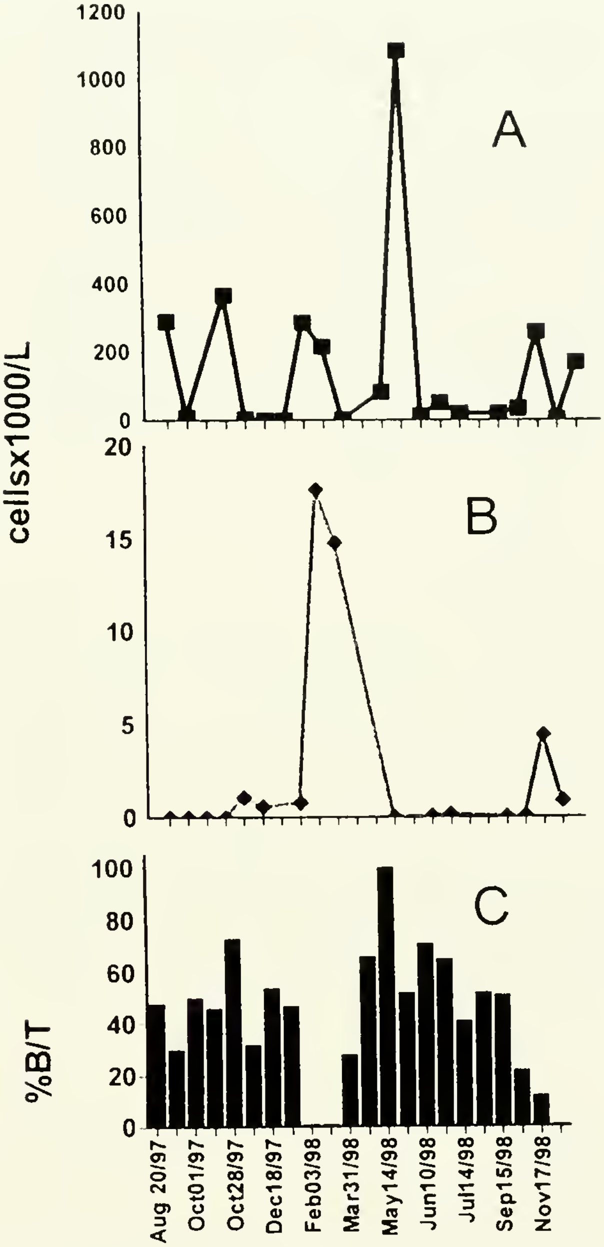

Figure 2. Cell number variation of diatoms (A) and dinoflagellates (B) in the water samples of Golfo Nuevo and ratio of benthic species over total species of *Aulocomya altra* guts in each sampling date (C).

DISCUSSION

The size range of the food items detected in the stomach contents in this study (9–231 μm) is similar to that reported for *A. atra* from Arica, Chile (21–270 μm, exceptionally up to 725 μm; Osorio et al. 1982) and other species such as *Mytilus edulis* Linnaeus (10–250 μm; Newell & Shumway 1993) and inshore *Placopecten magellanicus* (Gmelin)(8–240 μm; Shumway et al. 1987) from the Gulf of Maine. A wider size range of food items were reported in offshore *Placopecten magellanicus* (10–350 μm; Shumway et al. 1987) and *Patinopecten yessoensis* (Jay)(9–950 μm; Mikulich & Tsikhon–Lukanina 1981). Silicoflagellates, foraminifera, and specially tintinnids were other items observed in ribbed mussel stomachs in this study. The three groups are also miscellaneous items reported in *A. atra* from Arica, Chile (together with abundant detritus and other items; Osorio et al. 1982) and gut contents of other bivalves such as *Chlamys varia* (Linnaeus) (foraminifera, Hunt 1925), *Placopecten magellanicus* (silicoflagellates, Shumway et al. 1987), *Aequipecten tehuelchus* (d'Orbigny)(silicoflagellates, Vernet de Hall 1977), *Mytilus edulis* (silicoflagellates, Newell et al. 1989) and *Mytilus galloprovincialis* Lamarck (silicoflagellates, Sidari et al. 1998).

Two diatom peaks are observed as a recurrent feature of the annual phytoplankton cycle in Golfo Nuevo (Gayoso 2001). This common characteristic was not reflected in the annual variation of microalgal food of the ribbed mussel. Among the most representative species of the diatom bloom, *Skeletonema costatum*, *Chaetoceros socialis*, *C.curvisetus*, *C.diadema*, *C. didymus* and *Pseudo-nitzchia pungens*, only the latest, was observed in the ribbed mussel gut (Table 1). Absence of dominant planktonic microalgae has been reported for other bivalve species such as the absence of *Chaetoceros spp.* in *Placopecten magellanicus* (Shumway et al. 1987) and *S. costatum* in *Aequipecten tehuelchus* from Golfo San José, Argentina (Vernet de Hall 1977). These cases suggest a probable selective feeding capacity as indicated for others bivalves (Field 1922, Vernet de Hall 1977, Shumway et al. 1985, Shumway et al. 1987, Sidari et al. 1998). The absence of phytoflagellates in *A. atra* stomach contents may be explained by quick digestion of some small (mainly <10 μm in diameter) algal species (Shumway et al. 1987), reduced retention efficiency of particles less than 7 μm (Mühlenberg & Riisgard 1978) and/or difficulties in identification of micro-flagellates in bivalve stomach contents due to the fragility of these small cells (Vernet de Hall 1977).

A marked seasonal variation was not observed in the microalgal food of *Aulacomya atra*. Diatoms such as *Paralia sulcata* and *Thalassiosira* sp. were observed year round. Variation in the ratio of benthic over total species demonstrated that during most of the year benthic and pelagic microalgae contributed equally to the diet of the ribbed mussel in the Golfo Nuevo. In contrast, during dinoflagellate blooms, as occurred during late spring and summer, the percentage of benthic species dropped to less than 10% (Fig. 2C). The fact that *P. micans* and *A. tamarense* were found mostly as intact cells in the stomachs suggests, however, that they are not assimilated by the ribbed mussels. The capacity of concentrate large amounts of dinoflagellates was also observed in *Mytilus galloprovincialis* during a bloom of *Dinophysis* (Sidari et al. 1998). In *A. atra* from Magellanic Region (Southern Chile) Guzmán and Campodónico (1975) identified *Alexandrium catenella* (Whedon & Kofoid) Balech (formerly *Gonyaulax catenella*) in *A. atra* from Punta Arenas, Chile. Lembeye (1981) reported the toxic dinoflagellates *Alexandrium catenella* and *Dinophysis acuta* Ehrenberg (DSP causative agent) in stomach contents of *A. atra* from Chilean Magellan waters. Great densities of *Alexandrium tamarense* can explain the high toxicity of the ribbed mussels when the species is blooming in the Golfo Nuevo (Esteves et al. 1992, Andrinolo et al. 1999). Toxicity values up to 1,750 μg STXeq/100 g were reported in *A. atra* from Golfo Nuevo by Andrinolo et al. (1999).

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TRIPLOID INDUCTION OF *MYTILUS EDULIS* USING 6-DIMETHYLAMINOPURINE

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ABSTRACT The induction of triploidy in *Mytilus edulis*, using 6-dimethylaminopurine (6-DMAP), was investigated as a potential method of providing high quality sterile product in Prince Edward Island (PEI), Canada. Initial results indicated induction by blocking the second polar body would require a treatment starting time of 21 min post-fertilization at 20°C. Poor yields in subsequent spawns caused a re-examination of these factors by investigating the meiotic events post-fertilization. The resulting description was used to develop a series of suitable treatment time windows for testing inducing techniques. Triploids were successfully produced with the use of 6-DMAP at various concentrations and at various times post-fertilization. The optimal investigated procedure for induction of triploidy in *Mytilus edulis* was the treatment of eggs at 20°C with 400 μmol/L of 6-DMAP starting at 24 min post-fertilization for a treatment time of 10 min. This treatment yielded 83.1% triploids in induced samples with a survivorship of 1.39% to the D- veliger stage. The investigated methods for producing triploid *Mytilus edulis* are not yet optimized to commercial levels. This work forms a basis for further work in the optimization of this technique.

KEY WORDS: 6-dimethylaminopurine, meiosis, mussel, *Mytilus edulis*, triploid

INTRODUCTION

The Prince Edward Island (PEI) mussel aquaculture industry is a large contributor to Canada's aquaculture sector, accounting for greater than 80% of all Canadian mussels over the past two decades. One area of concern to the PEI mussel aquaculture industry is the harvesting and marketing of mussels during, and just after the spawning season. Ripe mussels (near spawning) can spawn due to the stress of shipping. Also, mussels that have recently spawned have significantly reduced meat yield making them less appealing to the consuming public. In PEI, the marketing of non-spawning triploid mussels during this period would alleviate these problems and allow the industry to market a high quality product year round.

Triploids are organisms with three sets of chromosomes, differing from most other sexually reproducing organisms that have two sets (diploids). Triploids also do not normally undergo meiosis, as the three sets of chromosomes cannot properly synapse. Therefore triploids have poorly developed gonads and produce far fewer gametes than diploids (Allen 1988). Triploidy is an aberrant genetic state in molluscs, and most organisms, and therefore must be produced artificially. The main objective of this study is to develop and optimize a triploid induction strategy. The important considerations for developing a triploid-inducing technique for commercial use are effectiveness and safety. 6-dimethylaminopurine (6-DMAP) is far less dangerous than cytochalasin B (CB) and equally effective in most cases (Dèsrosiers et al. 1993), therefore it was selected as the chemical induction agent for testing in this study.

In molluscs, triploid induction occurs through the use of an external treatment on a fertilized egg. The goal of commercial triploid induction of shellfish is to produce a high percentage triploid cohort with high survivorship throughout the hatchery stage (Allen et al. 1986). Imperative to the induction of both a high percentage of triploids and a high survival rate in a cohort of triploid mussels is the determination of the proper time at which to begin triploidy induction, and the treatment duration. In the past, the stage of development when 50% of the eggs showed polar body formation has been used as a common cue to start triploid induction treatment in oysters (Allen et al. 1989). Although the duration of the treatment varies between hatcheries, this treatment has a finite duration, the optimal time of which is determined by the timing of meiotic events. The time, from which a triploid induction treatment may be initiated until it is no longer effective, may be referred to as the induction window. One of the objectives of this study is to determine the optimal time for triploid induction initiation in *Mytilus edulis* embryos. Another objective of this study is to define the induction window for *Mytilus edulis* using an effective concentration of 6-DMAP and a standardized set of parameters. This work serves as the foundation for the future optimization of the triploid-inducing technique (using 6-DMAP) in *Mytilus edulis*.

MATERIALS AND METHODS

All experiments were conducted at the Ellerslie Shellfish Hatchery (ESH), in Ellerslie, PEI. Flow cytometry, to define ploidy level in samples, was performed at the University of Washington in Seattle, Washington, and at the Whiskey Creek Shellfish Hatchery in Tillamook, Oregon. Broodstock were obtained from a mussel lease located in Lennox channel, near Lennox Island, PEI. These animals were conditioned for 6 weeks, during which time they were batch fed a mix of cultured microalgae at water changes every 2 days. Spawning was induced by a +10°C thermal shock. Fertilization occurred within 2 h of gamete collection so as not to compromise gamete quality.

Description of Polar Body Extrusion Timing

Temperatures of 15°C and 20°C were investigated. 20°C was selected as it is an easy achievable temperature in most shellfish hatcheries, and 15°C was selected, as it is closer to the temperature during the natural local spawning in this species, and also common in many shellfish hatcheries. Approximately 3 million eggs were pooled from 3 females for use in each temperature treatment. Sperm was also pooled from 4 individuals for egg fertilization.

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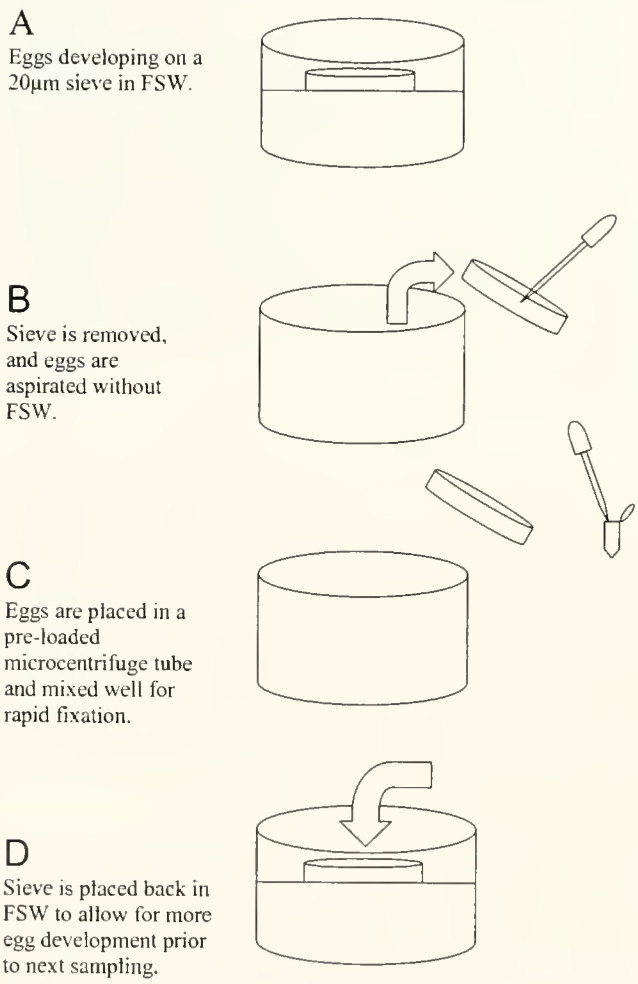
Figure 1 illustrates the sampling procedure used for this experiment. Eggs were placed on a 20 μm sieve within a 20 L bucket of 1 μm filtered sea water (FSW). This bucket was placed in a water bath to keep the eggs and surrounding sea water at 20°C. Eggs were sampled by raising the screen out of the water and aspirating eggs from the screen (without an excess of sea water) and immediately returning the screen to the bucket (Fig. 1). The aspirated eggs were immediately placed into a pre-loaded 2-ml microcentrifuge tube containing 2% buffered formalin fixative. The sample was mixed well so as to fix the eggs rapidly. The eggs were sampled prior to fertilization, at fertilization, and at subsequent 3-min intervals up to 2 h post-fertilization. Simultaneous to this sampling, the same procedure was being followed using the same egg and sperm pools in a water bath of 15°C. Under light microscopy, polar bodies in the fixed egg samples were visible using a 0.5% aceto-orcein stain (Guo 1991). For each sampled time, the number of eggs (out of the first 50 visualized on the slide) showing visible polar bodies was recorded. When this number reached 25 (50% of eggs), the target had been reached.

Effect of 6-DMAP Concentration on Percent Triploid Induction

The sperm from 6 males was pooled for this experiment. The sperm was used to fertilize the pooled eggs from 4 females. The pooled eggs were counted and loaded into 1L plastic beakers at a density of 100,000 eggs per beaker (100 eggs per ml of FSW). Treatments were replicated three times in this experiment and consisted of eight concentrations of 6-DMAP, at each of the two investigated temperatures. The tested 6-DMAP concentrations were similar to those used by Dèsrosiers et al. (1993) who ranged concentrations from 0 to 600 μmol/L. The same range was selected for experimentation in this case as the resolution in their results gave clear indications of the effect of chemical concentration on percent triploid induction. For each treatment the appropriate amount of 6-DMAP was weighed and placed in a labeled aluminum foil packet. When a treatment was initiated the chemical was emptied into the beaker and rapidly mixed into the water until it was dissolved. Egg suspensions were mixed every few minutes throughout the treatment duration to ensure adequate chemical contact with all eggs. Water baths of 15°C and 20°C were used to keep egg suspensions at the proper temperature. Eggs were allowed to acclimate to the proper temperature for 30 min just prior to fertilization for optimal induction (Allen et al. 1989). All eggs were treated at 21 min post-fertilization for 20 min duration. At the conclusion of the treatment duration the eggs were thoroughly rinsed, placed onto a 20-μm sieve, and resuspended into their respective treatment beakers with new FSW. The egg suspensions were allowed to incubate for 24 h, then samples were taken. Embryos that had grown to the D-veliger stage were collected by rinsing them onto a 64-μm sieve. Sampled embryos were transferred into a 15 ml centrifuge tube filled with FSW. These samples were used for ploidy determination.



A
Eggs developing on a 20μm sieve in FSW.

B
Sieve is removed, and eggs are aspirated without FSW.

C
Eggs are placed in a pre-loaded microcentrifuge tube and mixed well for rapid fixation.

D
Sieve is placed back in FSW to allow for more egg development prior to next sampling.

Figure 1. Schematic of the experimental sampling procedure used in the description of polar body extrusion timing and the description of meiotic events.

Description of Meiotic Events

Eggs from 4 females were pooled for use, and sperm from 7 males was pooled for fertilization. This experiment was only performed on one sample of pooled fertilized eggs held at 20°C. A water temperature of 20°C was used as this was shown in the results of the description of polar body extrusion timing (the first experiment) to have more synchronous meiosis, (therefore it should be more effective than 15°C for triploid induction). Samples of eggs were taken every minute from fertilization until one hour post-fertilization. Each of the first 50 eggs observed for every sample were categorized as being in a particular stage of meiosis. The first 50 readily scored comprised the sample for each time investigated.

Evaluation of Several Treatment Windows

Sperm from 5 males was pooled to fertilize the eggs pooled from 12 females. All treatments were standardized using a temperature of 20°C and a 6-DMAP concentration of 400 μmol/L and 100 eggs/ml of FSW. The treatments investigated included different treatment starting times based on the observed meiotic events in the previous experiment. Four treatment durations of 5, 10, 15, and 20 min were investigated along with four treatment starting times of 24, 27, 30, and 33 min post-fertilization. Survivorship was assessed by taking 15 ml samples of each culture at day 3 and fixing them with Lugol's iodine. A survivor was later scored as a fixed D-veliger that showed food in the gut, under light microscopy. Samples were taken for both ploidy and survivorship.

RESULTS

Description of Polar Body Extrusion Timing

Table 1 summarizes the results. In the 20°C group polar bodies appeared as early as 15 min post-fertilization and showed the mean

TABLE 1.

Percent of polar body 1 extension in the 15°C and 20°C groups.

Time (Min)	15°C	20°C
0	—	—
3	—	—
6	—	—
9	—	—
12	—	—
15	—	2.67 ± 1.15
18	2 ± 2.0	23.33 ± 3.05
21	30 ± 10.58	50.67 ± 4.16
24	38.67 ± 14.05	—
27	63 ± 15.56	—

desired score of 50% polar body display at 21 min (50.67% ± 4.16) post-fertilization. Samples taken from the 15°C group showed a slower development of polar bodies, with none appearing until 18 min post-fertilization. All samples from the 15°C group showed 50% polar body display by 27 min post-fertilization (63% ± 15.56). The 15°C group was also less synchronous, with replicates showing a standard deviation of as much as 15.56% (of eggs showing polar bodies) between them at 21 min post-fertilization. The largest standard deviation in the 20°C group at any given time interval was 4.16%.

Effect of 6-DMAP Concentration on Percent Triploid Induction

Figure 2 is a summary of the results. In all treatments the number of live D-larvae was too low to run each replicate separately for ploidy analysis. Therefore, all three replicates for each treatment were combined for ploidy determination by flow cytometry. There were no triploids in the controls, however with the addition of 6-DMAP, all of the tested concentrations except the 50 μmol/L treatment at 15°C produced triploids. In all cases except the 500-μmol/L concentration (that had an outlier percent triploid of 9.8% and was therefore excluded) the 15°C treatments gave a lower percent triploidy than did the 20°C treatments. For both temperatures, the rate of increase in the percent of triploids induced decreased at concentrations higher than 300 μmol/L. There is little increase in the percent triploids with an increase in 6-DMAP concentration beyond 500 μmol/L.

Description of Meiotic Events

Figure 3 is a summary of the results. Telophase 1 (the stage when polar body 1 is extruded) was visible as early as 12 min post-fertilization. Anaphase 2 (the stage that is a precursor to polar body 2 being extruded) began at 22 min post-fertilization. Telophase 2 (during which polar body 2 is extruded) began at a time of 27 min post-fertilization. Egg cleavage (the first mitotic embryonic cleavage) began at 44 min post-fertilization. Approximately 50% of the scored eggs were going through Anaphase 2, and 50% through Telophase 2 at 32 min post-fertilization.

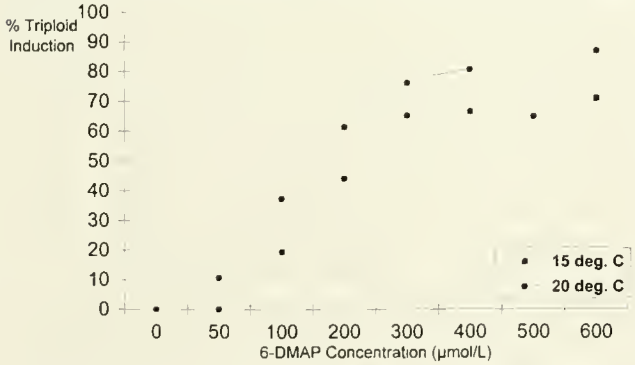


Figure 2. Percent triploid induction of *Mytilus edulis* using 6-DMAP.

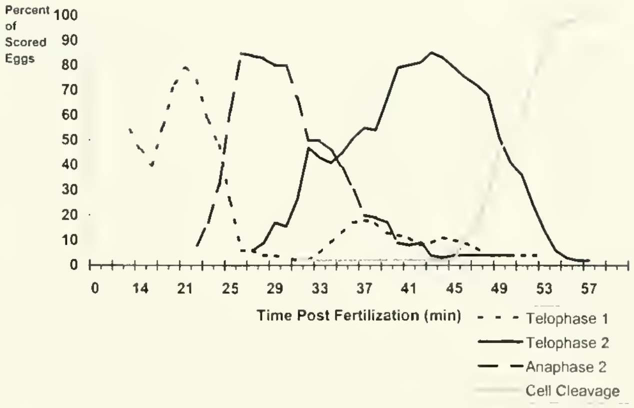


Figure 3. Summarized time-related meiotic events in *Mytilus edulis* at 20°C.

Evaluation of Several Treatment Windows

The investigated treatment windows varied greatly in both the percentage of survivors to the D-stage (survivorship) and also in the percent triploid induced. Table 2 is a summary of the obtained results. The control samples tested showed a 37.6% survivorship to the D-stage, with no triploids present. The highest mean treatment survivorship (9.33%) was found in the treatment begun 24 min post-fertilization for a duration of 5 min. The lowest mean survivorship (0%) was found in the treatment begun at 27 min post-fertilization for duration of 20 min. The highest mean percent triploid induction (83.1%) was found in the treatment begun 24 min post-fertilization for duration of 10 min. The lowest mean percent triploid induction (0%) was found in the treatment begun at 33 min post-fertilization for duration of 15 min. Tetraploid peaks were evident in a number of these treatments from the resulting printout of the flow cytometry (Table 2).

DISCUSSION

Evaluating the Induction Window Using Meiotic Events

The determination of the proper induction window is crucial to triploid induction for a number of reasons. First, the induction treatment is potentially lethal to fertilized eggs when exposure times are too long. Dèsrosiers et al. (1993) found that longer exposures to 6-DMAP interfered with first cleavage and resulted in developmental abnormalities, especially in *Mytilus edulis*. In cases where the induction comes early, although a cohort with a high percentage of triploids might be produced, the survivorship would be low, making the cohort less useful from a commercial perspective. Secondly, poor timing might not only lower survivorship, but also affect the percentage of triploids that would be produced in a given cohort. If the induction window (although the proper length of time) is initiated too late, meiosis II will not be blocked and the production of mostly diploids will result. This would result in a cohort with a high percentage of diploids, and thus a lower per-

TABLE 2.

Results of investigation treatment windows.

Initiation Time (min)	Duration (min)				
	0	5	10	15	20
0	$M_s = 37.6 \pm 10.9$ $M_t = 0$				
24		$M_s = 9.33 \pm 5.7$ $M_t = 56.1 \pm 17.9$	$M_s = 1.39 \pm 1.6$ $M_t = 83.1 \pm 8.8$	$M_s = 0.98 \pm 0.8$ $M_t = 59.3 \pm 18.3$ *	$M_s = 0.28 \pm 0.6$ $M_t = 68.6 \pm 5.4$ *
27		$M_s = 3.90 \pm 2.8$ $M_t = 48.0 \pm 14.2$	$M_s = 0.84 \pm 0.9$ $M_t = 65.8 \pm 12.2$	$M_s = 0.97 \pm 1.0$ $M_t = 76.5 \pm 13.0$ *	$M_s = 0$ $M_t = 49.2 \pm 19.0$ *
30		$M_s = 6.96 \pm 2.9$ $M_t = 51.8 \pm 5.9$	$M_s = 0.98 \pm 1.5$ $M_t = 49.5 \pm 6.6$	$M_s = 3.2 \pm 2.5$ $M_t = 11.6 \pm 4.6$ *	$M_s = 0.42 \pm 0.6$ $M_t = 17.2 \pm 5.8$ *
33		$M_s = 5.58 \pm 2.1$ $M_t = 27.6 \pm 9.3$	$M_s = 2.23 \pm 1.6$ $M_t = 14.9 \pm 13.7$	$M_s = 5.29 \pm 3.0$ $M_t = 0$	$M_s = 3.07 \pm 2.9$ $M_t = 2.20 \pm 3.8$

M_s = mean % survivorship to the D-veliger stage (3 replicates/treatment).
M_t = mean % triploidy of the sampled D-veligers.
* tetraploids were found in the ploidy sample.

centage of triploids. It is important to note that not all eggs are in the same meiotic stage at any time. If the induction window begins too early, the result would be a blocking of meiosis 1 for most of the eggs, resulting in pentaploids (5N) being produced. Pentaploids do not survive to the D-veliger stage. This was shown to occur in *Mytilus galloprovincialis* by Scarpa et al. (1993). The authors exposed eggs to 1mg/L of cytochalasin B from 7 to 35 min after fertilization, blocking meiosis 1. The resulting developing pentaploid eggs showed a subsequent inhibition of first cleavage and died. Therefore the resulting cohort would have a large percentage of triploids at the D-veliger stage, as whatever wasn't a pentaploid would likely be a triploid (having meiosis I completed and being in meiosis II during treatment). This cohort would likely have a lower survivorship however, as the pentaploids would all die.

The first experiment (the description of polar body extrusion timing) was the first attempt to find a starting time for triploidy induction with reference to the time of fertilization. It was assumed at that time that it was more critical to find the initiation time of induction, as the exact duration of the treatment could be refined in further experiments. It was also decided that both temperatures of 15°C and 20°C would be investigated as they are both common, and easily achievable working temperatures in shellfish hatcheries, and they would give some insight as to a temperature effect in the induction window. The main objective of this experiment was to determine the time at which 50% of fertilized eggs developed polar bodies. Fifty percent polar body formation has been used as a common cue to start triploid induction treatment for shellfish, with treatments ending after various periods depending on the species (Baker 1996, Allen et al. 1989). It is important that most of the eggs are highly synchronous in terms of meiotic events. If most eggs are in the same stage during induction, and it is the effective stage, the result would be a higher percent triploidy. Lu (1986) found that meiotic events of the eggs are more synchronous at higher temperatures (not exceeding the physiologic limits). Given this finding, a higher temperature within the physiologic limit would be expected to produce more synchronous meiotic events. This was supported by the finding that more eggs were in the same meiotic stage on average at a given sampling time in the 20°C sample compared with the 15 °C sample in the first experiment.

One observation in related work was that during the production of both triploid and diploid animals for a simultaneous growth trial there was an unexpected high mortality level during repeated spawns. This initiated a reevaluation of the treatment window, particularly the induction initiation timing as determined to be between 21 and 24 min post-fertilization in the description of polar body extrusion timing. One possible explanation for repeated low survivorship (less than 0.01% to the D-veliger stage) may have been that initiation of treatment to induce triploidy might have been too early. If this were the case, polar body 1 extrusion would be blocked in most eggs, forming pentaploids. Pentaploids do not survive to the D-veliger stage. However, any eggs that were past this stage would likely have polar body 2 extrusion blocked and thus become a triploid. The observed trend in the related growth trial spawns was in concordance with this phenomenon as the survivors of these spawns,although few were mostly triploids (all over 80% triploidy, with many over 90%).

Therefore, the description of meiotic events involved a more in-depth study of the meiotic events in *Mytilus edulis* at 20°C. As opposed to attempting to treat at the time just after polar body 1 was extruded, a time-related description of the meiotic events was used. This strategy was shown to be successful in Geoduck clam triploid induction (Vadapolas 1999). Dèsrosiers et al. (1993) found that triploid induction treatment using 6-DMAP was effective at the metaphase I stage. In Geoduck clam induction however, Vadapolas (1999) found that extrusion blocking with 6-DMAP just at the point where 50% of the eggs were in Anaphase 2 and 50% of the eggs were in Telophase 2 produced optimum results.

The description of polar body extrusion timing and the description of meiotic events showed similar results in terms of polar body 1 extrusion times. The description of polar body extrusion timing illustrated that most eggs had extruded, or were in the process of extruding polar body 1 by 21–24 min post-fertilization. The description of meiotic events similarly showed that most eggs were undergoing polar body 1 extrusion, scored as Telophase 1, at a time of 24 min post-fertilization. The fact that both experiments showed a similar time of polar body 1 extrusion infers that if the induction initialization used in the growth trial spawns was in fact early, causing mortality, it was not because the target of first polar body extrusion was improperly identified.

Triploid Induction

The effect of 6-DMAP concentration on percent triploid induction demonstrated that 6-DMAP could be used at a number of different concentrations to induce triploidy in *Mytilus edulis*. Although 15°C was shown to be less synchronous (and thus less optimum for triploid induction) in the determination of polar body extrusion times, these results were not yet obtained at the time of the first induction experiment. In all cases the 20°C samples had a higher percentage of triploids than did the 15°C sample (Fig. 2). This is likely due to the fact that eggs at 20°C are more synchronous in terms of their meiotic stage, resulting in a higher percentage of eggs in the effective treatment stage, and a higher percent of triploids. This is in concordance with the results from the description of polar body extrusion timing that showed a higher degree of meiotic synchrony in eggs at 20°C compared with 15°C. This is also supported in the literature as Lu (1986) found that meiotic events of eggs are more synchronous, and thus should give a higher percent triploid induction, at higher temperatures (not exceeding the physiologic limits). In past studies, treatments with a higher degree of meiotic synchrony allowed for more effective induction treatments as more eggs were in the target stage during treatment, resulting in a higher percent triploid and a higher survivorship (Downing & Allen 1987, Allen et al., 1989, Gérard et al. 1994). In light of these past studies, and given that the 20°C gave both a higher percent triploid induction and a higher degree of meiotic synchrony, the 20°C treatment is considered more optimal than the 15°C treatment.

The evaluation of several treatment windows tested various induction windows (initiation and duration times), which could be compared with the meiotic events described previously. Survivorship was low in all cases. This was likely because a relatively high concentration of 6-DMAP (400 μmol/L) was used. A decrease in the number of normally developed D-larvae (presumed to not be capable of survival) with an increase in 6-DMAP concentration was reported by Gérard et al. (1994). In the case of the current experiment, the relatively high 6-DMAP concentration was required to ensure that a suitably high percentage of triploids was produced, and was based on the results from the effect of 6-DMAP concentration on percent triploid induction (Fig. 2). Treatments initiated at 24 min post-fertilization produced both the highest percent triploid (83.1 at a duration of 10 min) and the highest survivorship (9.33 at a duration of 5 min). Taylor Resources Inc., a hatchery that commercially produces triploid *Mytilus galloprovincialis*, typically obtains 95–100% triploidy with 10–15% survivorship. It is difficult to compare these refined procedures with the results from these preliminary experiments, as much more optimization is required to reach commercial relevance. As these were preliminary experiments, a triploid percentage of greater than 50% with as high a survivorship as possible was the objective. This was based on the caveat that further optimization would improve both the percent triploid induction and survivorship.

Past attempts at inducing triploidy in *Mytilus edulis* have given a wide range of optimal treatments, resulting in a wide range of percent triploid induction and survivorship. Beaumont and Kelly (1989) produced, at best, 25% triploid-producing eggs using heat shock alone (from 15°C to 25°C at 10 min post-fertilization for 10 min). The authors measured the percentage of eggs that were developing, and the percentage of those that were developing normally. They found that, at best, approximately 10% of treated eggs developed. Of those that developed, approximately 60% developed normally and would likely go on to develop as triploid embryos. One treatment had less than 5% development and less than 7% of those showed normal development. However, in the same study, it was found that CB treatments could produce up to 60% triploid larvae in the optimal treatment group (using 1mg/L of CB at 5 min post-fertilization for 15 min). Again there was a significant reduction in the percentage that developed, and of those, which developed normally. Approximately 8% of treated eggs developed, and approximately 63% of those developed normally. The current results (Table 2) illustrate some treatments that showed better induction performance when compared with the aforementioned study, with up to 83.1% triploid induction (the 24-10 treatment) and up to 9.33% survivorship (the 24-5 treatment) to the D-veliger stage. Yamamoto and Sugawara (1988) were able to produce up to 97.4% triploidy by the use of heat shock alone (from 20°C to 32°C at 20 min post-fertilization for 10 min), and found apparently no negative effect upon survival rates. However, these results are considerably higher than those produced by Beaumont and Kelly (1989). As well, Yamamoto and Sugawara (1988) report yields much higher than those found in these experiments that showed results slightly higher than those of Beaumont and Kelly (1989). Yamamoto and Sugawara (1988) gave high yields with heat shock alone despite the fact that 6-DMAP is generally accepted as being a more effective triploid inducer than temperature alone (Dèsrosiers et al. 1993). Yamamoto and Sugawara (1988) also found 2–22% triploids in their controls that they attribute to handling stress. No other known triploid shellfish work has shown triploids in their controls. In the Chilean blue mussel, *Mytilus chilensis*, Toro and Sastre (1995) found an optimum induction treatment using heat alone (from 18°C to 32°C at 10 min post-fertilization for 10 min) to induce 51% triploid with 29% survival to an age of 15 days. The optimum percent triploid induction in their study was lower than in these experiments, the survival rate was much higher however. Scarpa et al. (1994) compared six methods to induce triploidy directly (not including 6-DMAP) and found that cytochalasin B (CB) was the most effective at inducing triploidy in *Mytilus galloprovincialis*, obtaining 86% triploidy to the D-veliger stage. These authors also found that, averaged between their two trials, 71.6% survived the CB treatment. These reported results are higher than the results of this study, however, as mentioned previously, we excluded CB from our trials due its carcinogenic effects.

Although percent triploid induction in this study is comparably high (over 50%) in some treatments when compared with many shellfish studied (Beaumont & Fairbrother, 1991), survivorship is lower than expected. Future optimization of the triploid induction method should focus on increasing survivorship while not compromising percent triploid induction. Improved hatchery techniques and husbandry may also increase survivorship, but cannot help to increase the percent triploidy. When considering a commercial technique, a high percentage of triploids in a cohort are not the only consideration in an induction technique. It has to be balanced with finding a method that produces a high survivorship of the triploids to remain economically feasible.

One further noteworthy result is the incidental production of tetraploid mussel larvae during some of the treatments in the evaluation of several treatment windows (Table 2). Six different treatment combinations produced some measurable number of tetraploids. There may be commercial significance in these findings. Triploids can be produced at a rate of 100% with a high survivorship, and without using chemicals by mating a tetraploid to a diploid (Guo et al. 1996). Therefore, if tetraploids could successfully be produced and reared, they might become commercially important animals in the production of triploid mussels. Scarpa et al. (1993) found that tetraploidy could be induced in *Mytilus galloprovincialis* by suppression of both polar body 1 and 2. This may have been the mechanism for the production of the tetraploids found in these results. However, when these tetraploid-inducing treatments are compared with Figure 3, a pattern that suggests another mechanism is evident. All tetraploid producing treatments but the 24–15 treatment overlap cell cleavage. Theoretically, if an inhibition treatment overlaps cell cleavage, the chromosomes could divide into two sets but the cell would not cleave. The result will be one cell with double the diploid number of chromosomes, and subsequent mitotic divisions will result in tetraploid larvae (Beaumont & Fairbrother 1991). This has been shown as a viable method of tetraploid induction. Guo et al. (1994) attempted to produce tetraploids by inhibiting mitotic cell cleavage with heat shocks, producing up to 45% in one case, however no tetraploids in their study survived past the D-stage of development. Future work on triploid production of *Mytilus edulis* should focus on the production of tetraploid broodstock used to produce 100% triploid larvae without the use of chemicals. Many commercial triploid Pacific oysters are now produced using this method (Chew 2000).

It has been demonstrated that using the puromycin analogue 6-DMAP at various concentrations can produce triploid mussels. At the conclusion of this research the optimum investigated procedure for induction of triploidy in *Mytilus edulis* was to treat eggs at 20°C with 400 μmol/L of 6-DMAP starting at 24 min post-fertilization for a treatment time of 10 min. This treatment yielded 83.1% triploids in induced samples with a survivorship of 1.39% to the D-veliger stage. The current investigated methods for producing triploid *Mytilus edulis* are not yet optimized to commercial levels. These findings are a basis for further work in the optimization of this technique.

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MUSSEL CULTURE AND COCKLE FISHERIES IN THE NETHERLANDS: FINDING A BALANCE BETWEEN ECONOMY AND ECOLOGY

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ABSTRACT In the Netherlands, wild stocks of mussel seed are fished and mussels are cultured on bottom plots. In addition, wild stocks of the edible cockle are dredged for harvest. Two of the areas where these activities are carried out are nature reserves. In 1993, the government implemented a policy in these reserves to ensure the conservation, protection and development of natural values and processes in which human activities should fit in. Fishing for shellfish is considered a traditional activity in these waters. Therefore, it is allowed, but under the restriction that no negative effects are caused. As a result of this policy, fishing for mussel seed and cockles is not allowed in areas with a high potential for the development of mussel beds and seagrass fields. A number of bird species are dependent on shellfish for their food requirements. Therefore, the policy makes use of a reservation system. This means that, in years when mussel and cockle stocks are low, an amount is reserved for the birds and cannot be fished. The government and shellfish industry agreed on co-management, (i.e., the fishermen are responsible for implementing the measures). This task is carried out by Producers' Organizations. An overview of the viewpoints of the interest groups and the role of policy makers and scientists is given.

KEY WORDS: *Mytilus edulis, Cerastoderma edule*, co-management, culture, fisheries, government regulations

INTRODUCTION

Shellfish are an important component of the ecosystem as they filter the water and are a food source for organisms such as birds and humans (Dame 1996). The protection and restoration of shellfish beds can contribute both to preservation of estuarine and near coastal ecosystems and to sustainable economic development of the coastal zone. In the Netherlands, six shellfish species are commercially exploited (Smaal & Lucas 2000). Different species are harvested in different areas (Fig. 1). In the coastal zone fishing is directed at wild stocks of the trough clam *Spisula subtruncata*, and the American razor clam *Ensis directus*. In the Oosterschelde estuary and the saline lake Grevelingen the European oyster *Ostrea edulis* and the Pacific oyster *Crassostrea gigas* are cultured. Fishing for seed of the blue mussel *Mytilus edulis* takes place in the Wadden Sea and on rare occasions in the Oosterschelde estuary. The mussels are cultured on bottom plots located in the Wadden Sea in the North and the Oosterschelde in the South. Fishing on wild stocks of the edible cockle *Cerastoderma edule* takes place in the Voordelta, Western Scheldt, Oosterschelde and Wadden Sea.

This article describes an attempt to combine the exploitation of shellfish stocks and the protection of estuarine habitats in the Netherlands. To reach such a goal agreement among stakeholders is essential. A description of the mussel culture and fishing for mussel seed and cockles is presented. Regulations concerning the management of shellfish stocks and other habitats are summed up. Furthermore, an overview of the viewpoints of the interest groups and the role of policy makers and scientists is given.

METHODS

Mussel Culture in the Netherlands

In the Netherlands, the culture of the blue mussel *Mytilus edulis* depends on seed that is fished twice a year in the most western part of the Wadden Sea (see Fig. 1). Seed mussels are approximately 10–30 mm in length. These mussels are transported to the bottom culture plots, which are leased from the government. The plots are located in the Wadden Sea and the Oosterschelde (see Fig. 1). The mussels are left to grow to market size (>45 mm). Depending on the environmental conditions this size is reached in 1–3 y (Dijkema 1997). The mussel seed catches show large annual fluctuations that reflect the variability in spat fall (Fig. 2a). Experience of the mussel growers shows that a catch of 65 × 10^6 kg seed mussels is needed to sustain a yearly production of 100 × 10^6 kg mussels (see Fig. 2b). This relatively low conversion is caused by heavy mortality as a result of predation by birds and starfish, and losses due to storms. The mussel seed target is not always reached. In fact, during the last ten years the target was reached only once. Thus, mussels are imported from other areas such as Germany (Dijkema 1997). The Netherlands is the fourth largest blue mussel producer in the world, after China, Spain, and Denmark (FAO 2000a, FAO 2000b). In 1999 the total landing value of mussels was Dfl 125 million = 55 million USD (Productschap Vis 2000). Almost 40% of the mussels are exported to Belgium.

Cockle Fisheries in the Netherlands

Dredging for cockles takes place in the Wadden Sea, the Voordelta, the Oosterschelde and Western Scheldt (see Fig. 1). Wild stocks are fished mechanically with suction dredges. In addition, a small group of fishermen makes use of rakes with nets that are deployed by hand. The majority of the cockles is not sold in their shell, but cooked and canned for transportation to Spain. Therefore, landings are expressed in meat instead of fresh weight (Fig. 3). The catches show considerable variability again caused by fluctuations in spat fall. The main harvest area is the Wadden Sea. In 1998 a large cockle stock was available, and the total value of the catch was Dfl 60 million = 27 million USD (Steins 1999). The Netherlands is the number one cockle producer in the world (FAO 2000a).

Producers' Organizations

All groups involved in the mussel and cockle industry (i.e., fishermen, vessel owners and shellfish sellers) are organized in the Mussel Producers' Organization (PO Mussels) and the Cockle Producers' Organization (PO Cockles). The POs depend on voluntary

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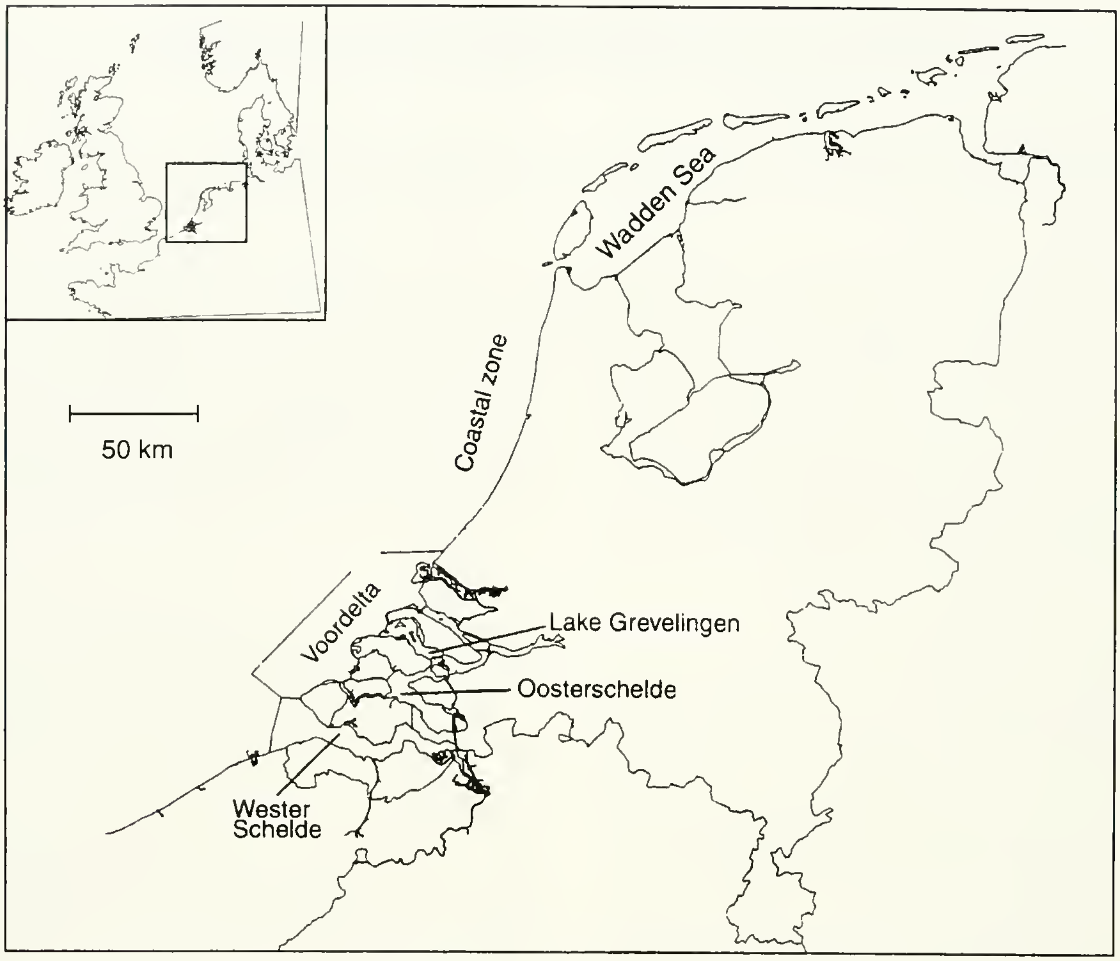

Figure 1. Shellfish fishing and culture areas in The Netherlands.

membership. POs offer attractive facilities to its members and they have legal powers to enforce binding agreements. The POs formulate Shellfish Fisheries Management Plans (e.g., Productschap Vis 2000). These include yearly fish plans, which are based on results of yearly surveys that determine the amount and distribution of mussel seed and cockles. In some cases (e.g., when mussel seed is present on a sheltered location), seed is not fished in fall, but left for next spring.

The Role of the Netherlands Institute for Fisheries Research

Every March, since 1992, the Netherlands Institute for Fisheries Research (RIVO) carries out a subtidal mussel seed survey in the western part of the Wadden Sea. This survey is conducted by order of the PO Mussels. The sampling set-up is stratified (i.e., the sampling grid is intensified at locations where subtidal mussel beds are present). These locations are known from the previous year and qualitative assessments carried out in early spring by fishery inspectors of the Ministry of Agriculture, Nature Management and Fisheries. At every sampling point a 20-cm-wide cockle dredge is used for 150 m. The mesh size is 5 mm. For profitable seed fishery a biomass of 0.1 kg mussels per m^2 is necessary (Fig. 4). The RIVO also monitors the distribution of seedbeds and the development of mature mussel beds in the intertidal with aerial photography surveys in combination with ground truthing.

Each May, since 1990, RIVO carries out a basin-wide cockle survey in the Wadden Sea, the Oosterschelde and the Western Scheldt. The survey is under contract by the Ministry of Agriculture, Nature Management and Fisheries and the PO Cockles. The sampling grid is stratified with a denser grid at cockle beds (Fig. 5). The location of these beds is derived from a qualitative survey that the fishermen carry out just prior to the annual RIVO survey. From the survey data the total cockle stock is determined.

Dutch Regulations

The Wadden Sea and the Oosterschelde do not only function as areas where shellfish fishing and culture takes place, but also as nature reserves. The birds, wetlands and other habitats in these areas are protected under several national and international laws such as the Ramsar Convention, the European Directive on the Conservation of Wild Birds, and the UNESCO Man and Biosphere Program. The areas used to have extensive seagrass meadows and intertidal mussel beds. Renewed development of these habitats is desirable. For migrating birds, the Wadden Sea and the Oosterschelde are important wintering areas or stopover sites for refueling on the flyway between the North Pole and Africa. In addition, a number of resident species, such as oystercatchers and eider ducks, are present (Rosner et al. 1993).

In these protected areas human activities are possible only when they do not cause negative effects. In the early 1990s, low stocks of both cockles and mussels were present in the intertidal

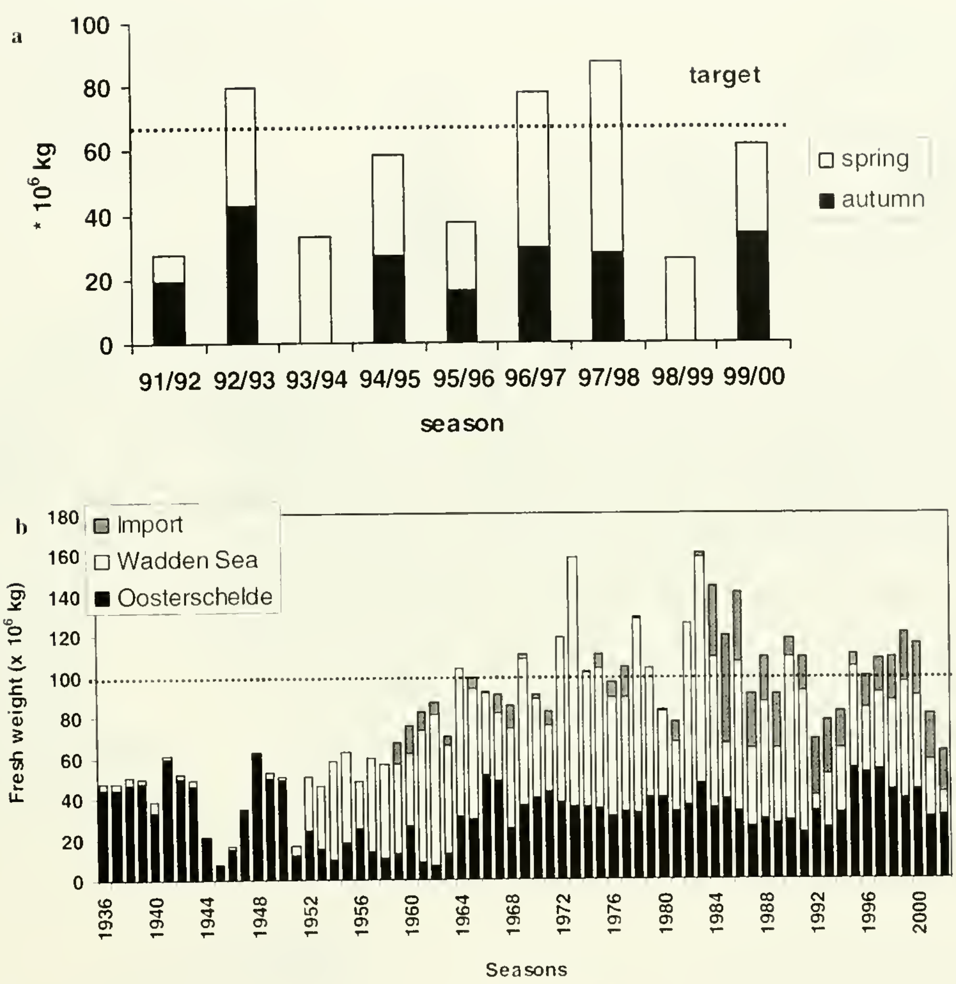

Figure 2. Mussel seed catches and mussel landings in the Netherlands in million kg fresh weight. Targets are indicated with dotted lines.

Wadden Sea (Beukema 1993). This was a result of a number of factors including reduced natural spat fall, storms and unrestricted fishing activities (Dankers 1993). At the same time, high mortality of a shellfish eating bird species was observed (Smit et al. 1998). Thus, the possibility of a link between bird mortality and shellfish fisheries appeared. The public awareness and social commotion caused by these events triggered the formulation of regulations to better protect the area.

In 1993, the government implemented a policy for the period 1993 to 2003 to ensure the conservation, protection and development of natural values and processes in the Wadden Sea and Oosterschelde estuary (LNV, 1993). Human activities must fit into this policy. Targets are restoring bird populations at levels of the 1980s, and promoting development of seagrass beds and 2,000–4,000 ha of stable intertidal musselbeds (LNV 1999). Fishing for shellfish is considered a traditional activity in these waters. Therefore, it is allowed, but under the restriction that no negative effects are caused. The government makes use of co-management (i.e., the fishermen are responsible for implementing the measures). The PO Cockles and the PO Mussels carry out this task. Consumption-sized cockles and mussels are also the preferred prey of oystercatchers and eider ducks (Zwarts et al. 1996, Nehls & Ruth 1994). Therefore, the policy makes use of a reservation system in the shellfish fisheries. In years when mussel and cockle stocks are low, the aim of the policy is to ensure that 60% of the food requirement of birds is reserved. However, the calculations underlying the policy have been questioned by (Ens 2000). Figure 6 shows large annual fluctuations in cockle stock as a result of variability in spat fall. Since the implementation of the food reservation policy in 1993, fishing for cockles was not allowed in the Wadden Sea in 1996 and restricted to the subtidal areas in 1997. In the Oosterschelde fishing was not allowed in 1997, 1998, 1999, and 2000. Another regulation is that fishing for mussel seed and cockles is not allowed in areas with a high potential for the development of mussel beds and seagrass fields. In 1993, 14% of the intertidal flats in the Oosterschelde and 26% of the intertidal Wadden Sea were permanently closed to shellfishing. In 1999, an additional 5% of intertidal flats in the Wadden Sea were closed (Fig. 7). The location of these areas is based on habitat maps for seagrass meadows and mussel beds. The maps are produced with GIS models that include parameters such as exposure time, wave action, sediment characteristics (Brinkman et al. 2002, de Jonge & de Jong 1999). To monitor that shellfish fishing does not take place in the closed areas all vessels are equipped with a black box. This system registers the ship's position at 1-min intervals when the ships are fishing. The data are retrieved from the boxes by an independent agency and the POs give penalties to offenders. In addition to the government measures, the Shellfish Fisheries Management Plan of

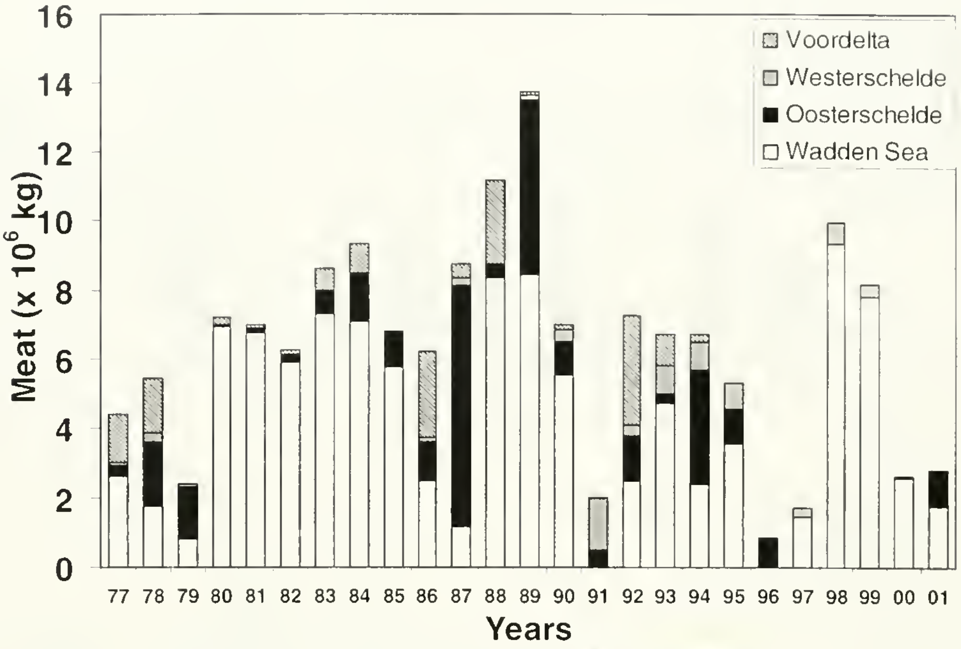

Figure 3. Cockle landings in the Netherlands in million kg meat.

the POs for the period 1993 to 2003 includes a reduction in the number of cockle vessels, a more spread out distribution of the cockle fleet, restricted fishing periods for both cockles and mussel seed, and yearly fishing plans on amounts fished and fish locations based on survey data (Productschap Vis, 2000).

Evaluation Projects

In 1998, the effectiveness of the governmental measures was evaluated for the period 1993 to 1998 in the so-called EVA I project (LNV 1998, Smit et al. 1998). Due to a series of cold

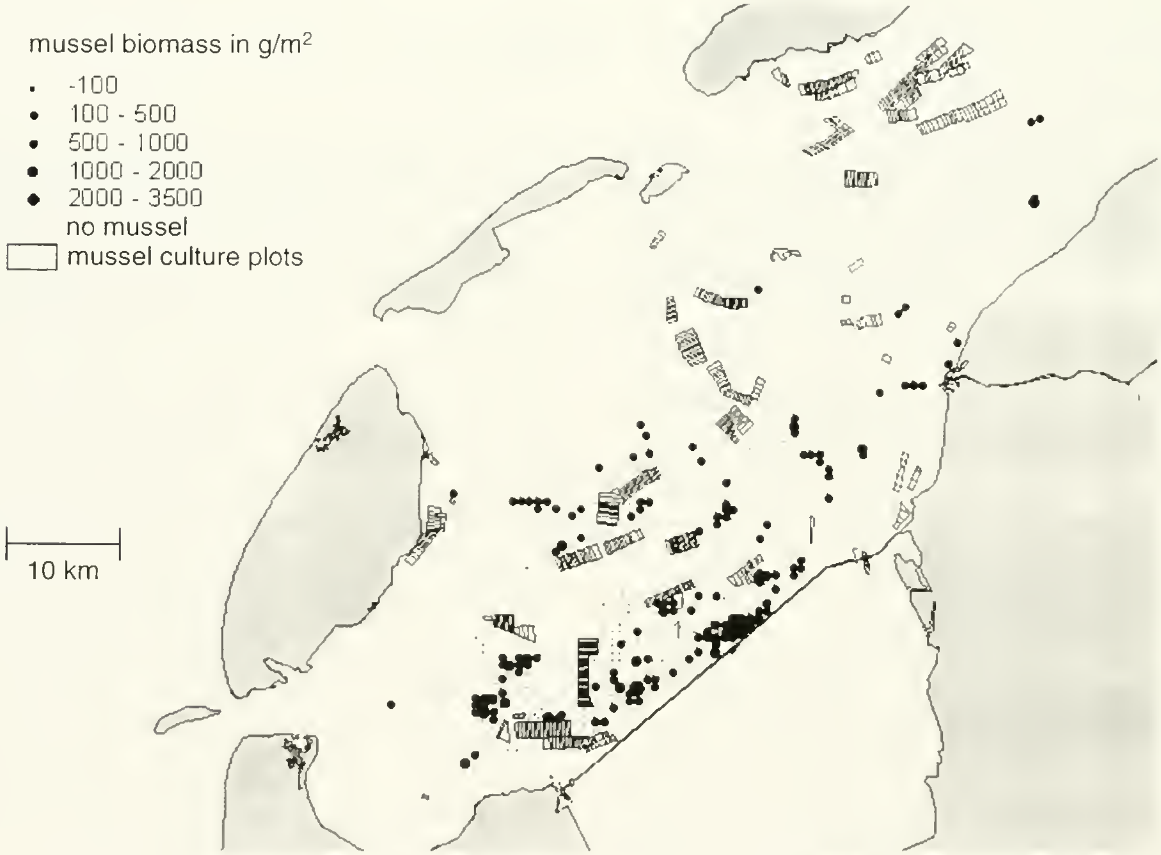
mussel biomass in g/m²
-100
100 - 500
500 - 1000
1000 - 2000
2000 - 3500
no mussel
mussel culture plots
10 km

Figure 4. Subtidal mussel seed biomass in the western Dutch Wadden Sea. Results of RIVO survey in 2000.

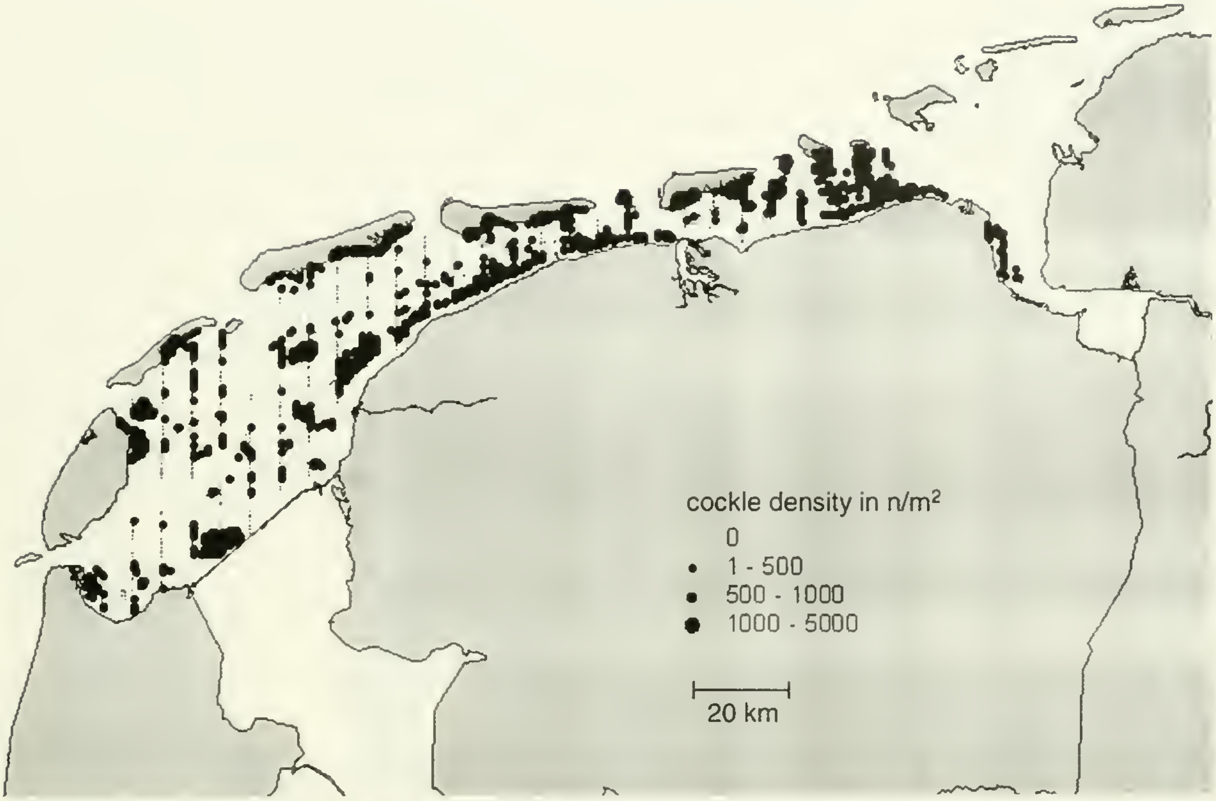

Figure 5. Cockle density in the Dutch Wadden Sea. Results of RIVO cockle survey in 2000.

winters with high shellfish mortality in this period, clear conclusions on effects of shellfisheries could not be drawn from the available dataset. The rate at which intertidal mussel beds developed was lower than expected. Intertidal mussel spat fall occurred in 1994 only (van Stralen & Kesteloo–Hendrikse 1998), and the intertidal mussel area in the Wadden Sea is approximately 1000 ha in 2000 (Kater & den Os 2001) Therefore, an additional 5% of the intertidal flats was closed based on the newly developed mussel habitat map (Brinkman et al. 2002). In 2000, the second phase of the evaluation started. In this EVA II project (Ens et al. 2000) the effects of shellfish harvests on nature in the Wadden Sea and Oosterschelde is evaluated for the entire 10-year period from 1993 to 2003 (LNV & VW 1999). The project is based on the following policy questions (Ens et al. 2000).

- What are allowable effects of shellfish harvests on mussel beds, seagrass meadows and shellfish consuming birds?
- Have the measures taken so far had the expected effect?
- Do we comply with the international regulations on bird protection?
- When effects are negative, what can we do to reduce these effects?

These policy questions were translated into a number of research sub-projects. Effects of mussel fishery and culture on bird populations are studied. The effects of cockle dredging on cockle stocks; stable intertidal mussel beds, other zoobenthos, seagrass meadows, and sediment composition are studied by comparing their development in open (fished) and closed (unfished) areas. In addition, availability of black box data gives the opportunity to further specify the fishing intensity within the open areas. Linking survey information with black box data and catch data gives information on the relationship between density of shellfish, fishing intensity and yields. Another subproject checks the assumptions on which the calculation of the amount of food reserved for birds is based. What is the profitable shellfish density for birds? Does it matter where the food is located (both in the tidal zone and in the horizontal plane)? Which fraction of the total stock is usable by birds? And finally, the slow recovery of both seagrass meadows and mussel beds warrants refinement of the habitat maps. In this way a better selection of closed areas is possible. The results of the evaluation project will play a role in the final decision on the continuation of shellfish harvesting, which the government will take in 2003.

Finding a Balance Between Economy and Ecology

Cockle fishermen and mussel farmers make a living with their activities and represent the commercial economy, while nature conservationists protect birds and habitats, such as seagrass beds and mussel beds, and are concerned with ecology. Both groups agree that the Wadden Sea and Oosterschelde should not be completely closed for human activities (e.g., recreation and fishing) provided that these activities do not have negative impacts. The discussions center on these impacts. Despite the policy to reserve food for shellfish eating birds, a further decline in oystercatcher population is taking place (Fig. 8). In addition, the development of intertidal mussel beds and seagrass meadows is slow. These observations cause tension between the different stakeholders. The different forms of shellfish fishing and farming show different degrees of opposition between the stakeholders. Traditional fishing for cockles by hand does not cause much tension. Fishing for mussel seed in the subtidal area is starting to become an issue, as these stocks also provide food for eider ducks. The main disagreements are on fishing for intertidal mussel seed and mechanical cockle fishing (Piersma et al. 2001, Smaal et al. 2001, Camphuysen et al. 2002).

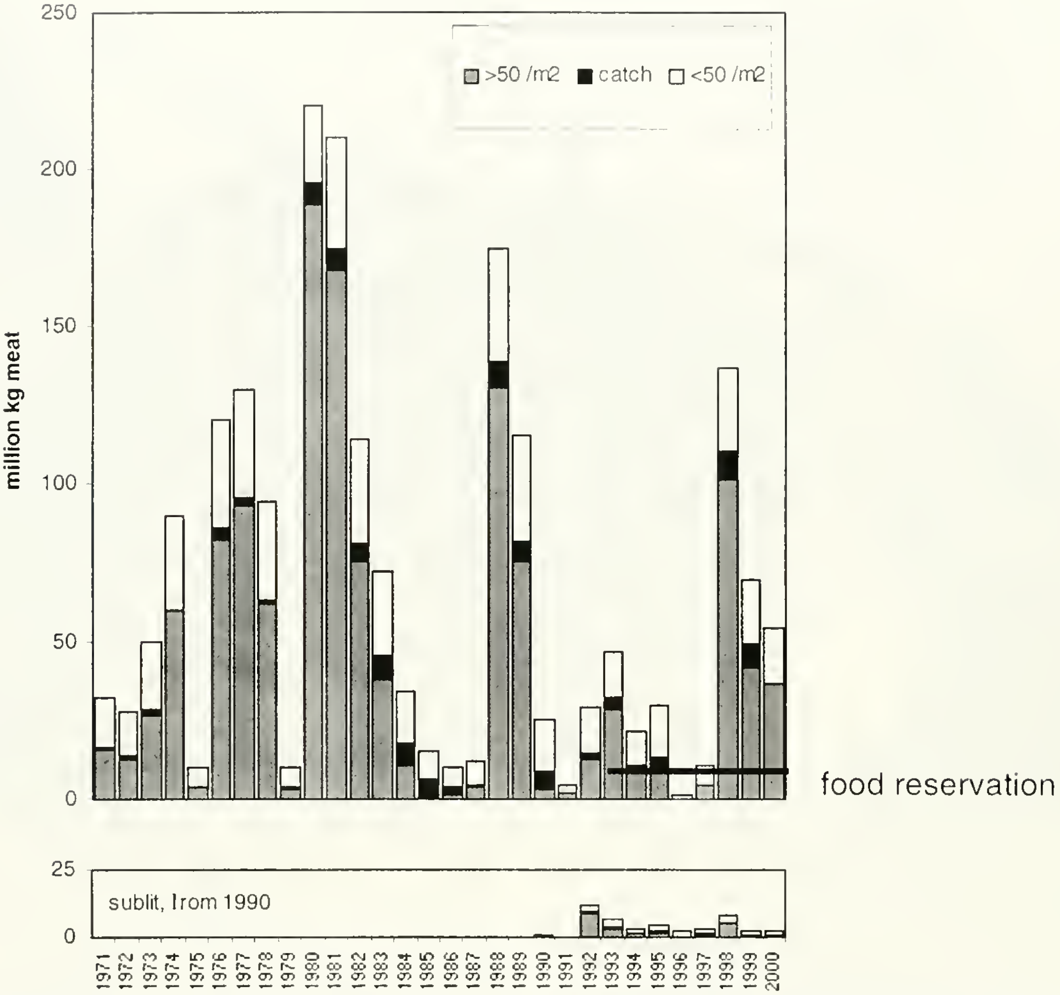

Figure 6. Cockle stock in the Dutch Wadden Sea as determined by extrapolation from various sampling campaigns in the Wadden Sea and the basin wide RIVO surveys from 1990 onwards. It is assumed that both birds and fishermen need a cockle density of more than 50 per m^2 for profitable use of the stock (grey part of the bars). Part of the grey bar is black, which indicates the catch. From 1993 onwards 12.6 million kg meat is reserved as food for birds. This is indicated with a horizontal line.

The cockle and mussel seed fishermen see an increase in restrictions, without the expected restoration of the ecosystem. As they have already put considerable effort in a changed management (e.g., the use of fishing plans and black box systems), they are reluctant to agree to more. Shellfishermen point out that, although bird populations are smaller than in the eighties, they have increased since the seventies. Nature conservation groups see the continued decline of bird populations as a sign to stress the precautionary principle. The definition of the precautionary principle is under debate. The conservation groups define it as: when uncertainty on the effects of a human activity exists, do not allow it. The European Commission have extended this with a balancing of interests: measures taken on the basis of the precautionary principle need to be in relation to the chosen protection level and need to be based on research on possible costs and benefits of carrying out that measure (COM 2000). Both fishermen and environmentalists question the calculated amounts of food needed by the birds. Fishermen ague that the lower level of bird populations in the seventies did not coincide with lower shellfish stocks. Nature groups and ornithologists (Ens 2000, Camphuysen 1996, Camphuysen et al. 2002) point out that not the total shellfish stock, but the stock that is available for birds is of importance. This may lead to higher food reservation values. Furthermore, there is debate about the closure of intertidal areas to promote development of mussel beds. Fishermen have the impression that moderate fishing improves the sediment and enhances survival of mussel seedbeds, while environmentalists are of the opinion that natural processes should not be interfered with. The images that both groups have of each other strongly influence the discussions that are part of the decision-making process (Steins 1999). Thus, securing ongoing communication between interests groups is of great importance. Identification of common interests should play a key role.

In addition to the tension between fishermen and conservationists, there is tension between science and politics. The government wants to keep the shellfish industry and protect nature at the same time. To do this properly they want to make decisions on objective scientific grounds. The scientists are unable to reach consensus on scientific grounds for the presence or absence of a link between shellfishery activities and the recent ecosystem developments (decline in bird populations, and slow recovery of intertidal mussel beds and seagrass meadows). Scientists generally work on a micro scale and can be fairly certain about their conclusions, while politicians need answers on a macro scale. The advice of the scientists needs to be objective and exact. However, when findings from

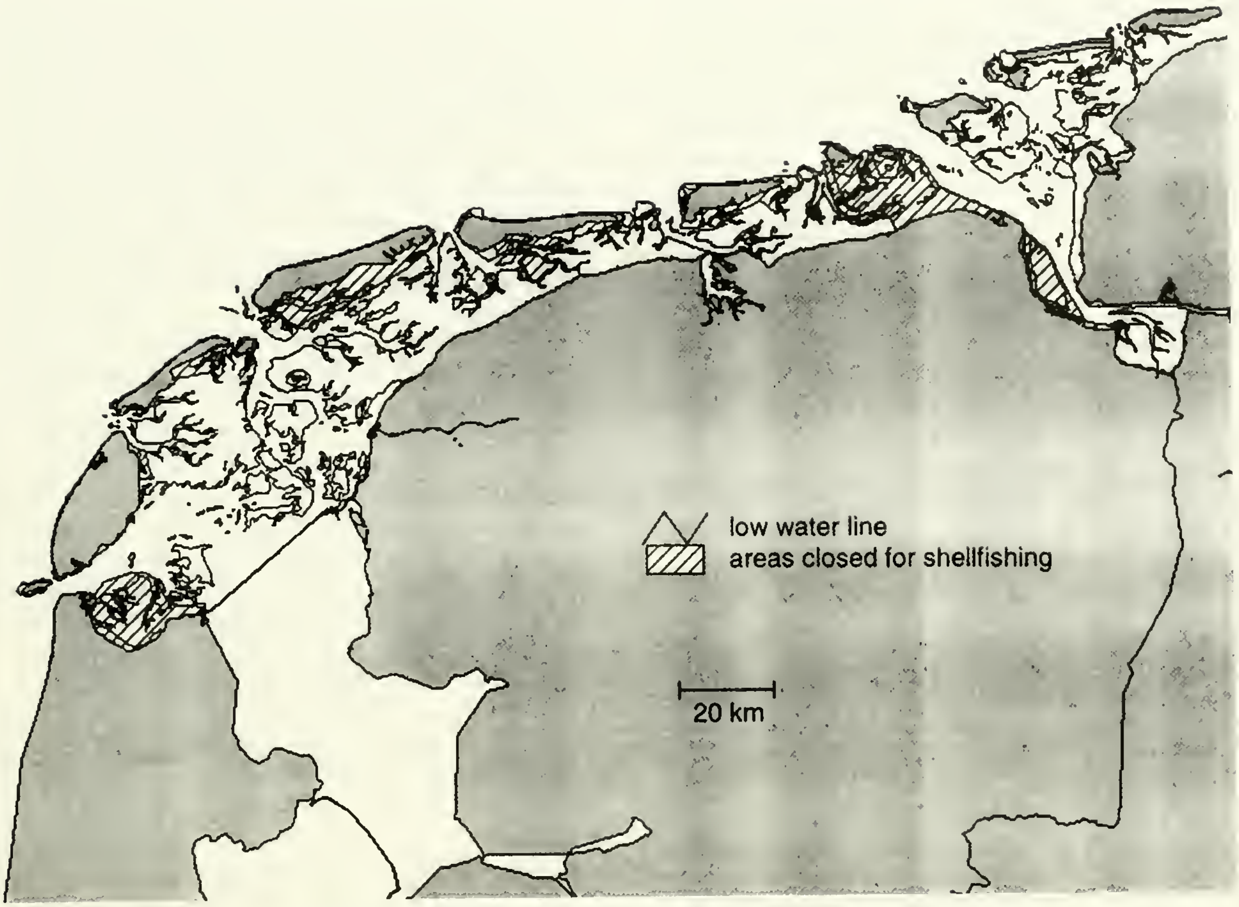

Figure 7. Location of areas that are closed for shellfish fisheries in the Dutch Wadden Sea.

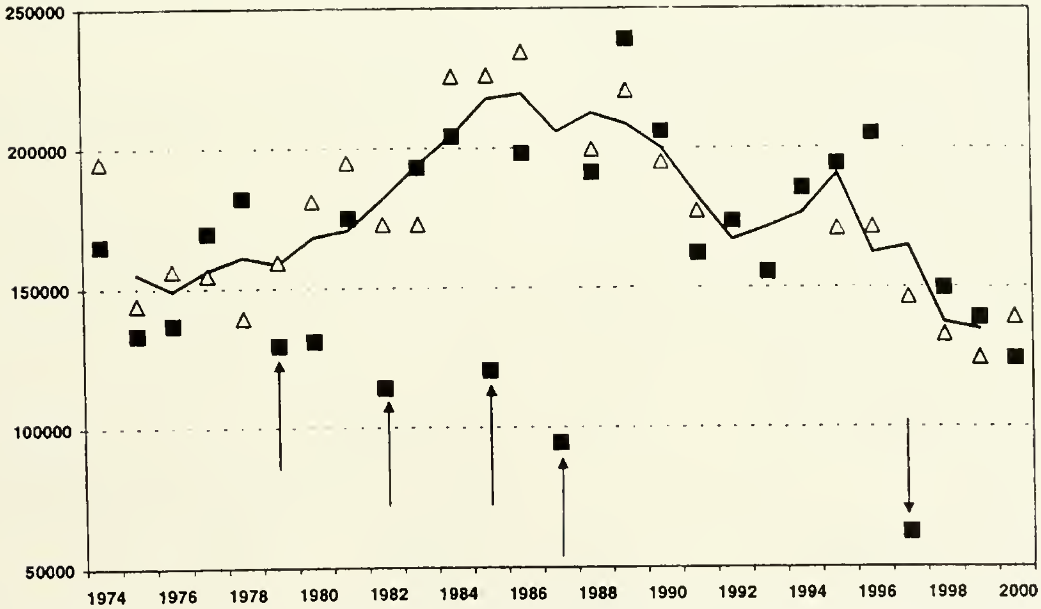

Figure 8. Development of total oyster catcher population in the Dutch Wadden Sea from 1975 to 1999. Counted numbers in fall (open squares) and following winter (closed squares) are indicated. Running mean (black line) is calculated from these counts. Counts in severe winters (closed squares with arrows) are not included in mean (Ens 2002).

small scales are upgraded to larger scales the results will always have a certain degree of uncertainty. To avoid scientific conflicts, and remain credible to society, it would be better to present results in the form of ranges instead of single values (Hauge 2000). However, this causes problems for the managers, as they need single values when drawing up permits. Thus, alternative ways to deal with scientific uncertainties concerning natural resources are needed.

CONCLUSIONS

The balance between economy and ecology is very delicate. In fact, balancing between economy and ecology is a more appropriate way to describe the current situation. Co-management has not brought the solutions the government hoped for. At present, two options dominate the discussion on the future of shellfisheries in the Wadden Sea and Oosterschelde. These are:

- A continuation of the fisheries as before, which seems unacceptable to the nature conservation groups.
- A buy out of the mechanical cockle fishery, which seems unacceptable to the fishermen.

The shellfish-fishermen, -farmers, and -sellers have set up a foundation called ODUS (Development Sustainable Shellfisheries). This foundation proposes the following methods for cockle fishing and mussel farming that aim for effects that are within the restoration capacity of the system and could be more sustainable (ODUS 2001).

- Selecting optimal areas for harvest may enlarge the efficiency of cockle dredges. Newly developed fishing gear may reduce the effects of the dredges on the bottom.
- Management of closed areas can be dynamic instead of static. The location of closed areas could be based on annual variations according to the location of the cockle stock
- Relaying of cockles may reduce mortality and enhance growth. This can be the beginning of a development from fishery to culture.
- Fishing of unstable mussel seed beds will use seed before it is lost in storms.
- Methods should be developed to avoid large seed losses on culture plots. This will enhance the yield and thus reduce the amount of mussel seed needed.
- Optimization of the mussel stock on culture plots to the exploitation capacity of the system will enhance the yield.

In our opinion, these promising options should receive more scientific attention. Conservation of the natural system is a common interest of all stakeholders. Degradation of the system will lead to reduced biodiversity and lower resilience. Both birds and fishermen need a well functioning ecosystem that guarantees the continued presence of shellfish stocks.

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SETTLEMENT OF *POMATOCEROS TRIQUETER* (L.) IN TWO SCOTTISH LOCHS, AND FACTORS DETERMINING ITS ABUNDANCE ON MUSSELS GROWN IN SUSPENDED CULTURE

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ABSTRACT In Scotland, some 2,000 tons of mussels (*Mytilus edulis*) are produced each year from suspended rope cultures. Mussels can act as a settlement substrate for tubeworm, *Pomatoceros* spp. and badly fouled mussels are devalued and may be discarded. The estimated cost to the Scottish rope-grown mussel industry is between £300,000 and £500,000 per annum. Established growers believe the problem is worsening. Collector plates were deployed at two sites in two lochs on the West Coast of Scotland to monitor *Pomatoceros* spp. settlement. In conjunction, *in situ* trials at a mussel farm site assessed tubeworm settlement on rope-grown mussels. The tubeworm *P. triqueter* was found to be the prevalent species settling on the collector plates and on the mussels. The timing of peak tubeworm settlement differed between lochs but was synchronous between sites and different depths within the same loch. Peak settlement in both lochs occurred after the highest seawater temperatures were recorded. Settlement intensity differed between lochs, sites, and depths, indicating the scale of variation in settlement within lochs. Mussel shell size was distinguished as a significant factor influencing *Pomatoceros* spp. settlement as higher numbers of tubeworm settled on larger mussels. In large mussels (mean shell length 60.8 mm ± 0.70 SE) tubeworm settlement was greatest in treatments where conspecific adults were already present and higher levels of settlement were found in mussels stocked at lower densities. In small mussels (mean shell length 33.5 mm ± 1.03 SE) the initial stocking density and tubing system had no effect on tubeworm settlement. Differences in the fouling intensity between mussel stocks were attributed to variation in the abundance of tubeworm larvae in the water column and the size of the mussels at the time of retubing. A 100% mortality could be expected in adult *P. triqueter* after 24.1 h and 35.4 h when exposed to air at 7°C and 13°C, respectively. In areas where tubeworm is a persistent problem consistent annual monitoring of the *Pomatoceros* spp. settlement is recommended as part of a management strategy to avoid heavy fouling on mussel stock. Grow-out strategies to alleviate tubeworm fouling on rope-grown mussels are discussed.

KEY WORDS: *Pomatoceros, Mytilus edulis*, settlement, tubeworm fouling, mussel cultivation

INTRODUCTION

Calcareous tube-dwelling polychaetes from the genus *Pomatoceros* have long been considered to be primary fouling organisms (Crisp 1965, OECD 1967). In Scottish waters, mussels are likely to be colonized by two species, *Pomatoceros lamarcki* (Quatrefages) and *P. triqueter* (L.). *P. lamarcki* is an intertidal to shallow subtidal species, whereas *P. triqueter* is generally considered subtidal, particularly adjoining deep water (Hayward & Ryland 1995). Mature adults are capable of breeding all year round; however, reproductive studies from other countries suggest episodes of peak settlement over relatively short periods (1–3 wk) (Klockner 1976, Castric-Fey 1983). The larval phase is temperature dependent and lasts for approximately three weeks (Seagrove 1941); however, monitoring larval populations from plankton tows has proved unsatisfactory in the past because the soft-bodied larvae do not preserve well (Senz-Braconnot 1968). Newly settled worms can reach full tube length (20–60 mm) and sexual maturity in four months (Castric-Fey 1983) and are therefore capable of rapid colonization. However, the species distribution and settlement of *P. lamarcki* and *P. triqueter* in Scottish lochs has not been described.

The cultivation of *Mytilus edulis* L. in suspended culture in Scotland increased to approximately 2,000 tons in 2000. The Scottish rope-grown mussel industry maintains its competitive edge over the Irish rope-grown mussel and UK dredge industries by trading in premium-quality mussels only. Farmed mussels constitute an excellent substrate for the settlement of many other organisms, which from the perspective of the mussel farmer are collectively termed fouling. Suspended long line cultures are particularly vulnerable to fouling because they are continually submerged and considerable effort is required to clean crop for marketing (Hickman 1992). Both barnacles and tubeworms frequently settle on mussel shells. Barnacles are not as problematic and are usually removed by the brushes used to strip mussels from culture lines, however, tubeworm (*Pomatoceros* spp.) longer than 5 mm are resistant to brushing and result in badly fouled mussels being devalued or discarded. Mussels with more than 7% of the shell fouled are not considered Grade A quality. Tubeworm-fouled mussels are not suitable for sale as "live-in-shell" product to retailers as the fouled shells are considered visually unattractive, the tubeworm can die before the mussel causing an offensive smell and the tubeworm can be released from the tube on cooking and appear unappetizing. In Scotland, it is considered uneconomical to produce Grade B rope-grown mussels because of the higher economic investment involved compared with dredged mussel operations, and the competition for the export market from the larger Irish cultivation industry which produces primarily Grade B stock. The estimated cost of tubeworm fouling to this industry is between £300,000 and £500,000 per annum (P. Marshall SSMG) and established growers believe the problem is worsening.

Previous work has focused on the competition for food resources between fouling suspension feeders and rope grown mussels (Lesser et al. 1992). However, few data exist with regard to reducing the impact of fouling organisms in cultured mussel systems. A survey of Irish rope-grown mussel growers indicated the most important factors in managing tube-worm fouling on their farms were the timing of handling (delaying grading and thinning of stock until after the main tubeworm settlement), the density of mussels on the lines (outer mussels forming a barrier to settlement), the influence of weather and the depth of culture line (Purcell & Cross 1994). All of these factors have been identified by Scottish mussel growers as potentially being of major importance to fouling levels on their sites.

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The objectives of this study were to first describe tubeworm species colonizing Scottish rope-grown mussels, monitor settlement, and determine the timing of peak settlement in two contrasting lochs and at different depths, thereby ascertaining inter and intra-loch variation in settlement abundance. Second, field trials were conducted to assess the effect of mussel density, depth, retubing material, and the presence of adult tubeworm conspecifics on tubeworm settlement intensity in two size classes of mussels. Third, to evaluate the use of aerial immersion as a method to control tubeworm growth on mussels. The data collected provide basic information for the development of husbandry practices to alleviate tubeworm fouling.

MATERIALS AND METHODS

Sample Sites

Two lochs, Loch Striven (N55°59′,W5°07′) and Loch Beag (N56°53′,W5°44′) with established mussel farms, were selected as the rope-grown stock here was known to be prone to annual settlements of tubeworm species (Fig. 1). Loch Striven is a narrow Fjordic loch, almost 13 km in length with the third longest flushing time (12 days) of any sea loch (Edwards & Sharples 1986). In contrast, Loch Beag is not a true fjordic loch but an elongated embayment with greater mixing and fresh water run-off (Howson et al. 1994).

Timing of Settlement

The collector strings consisted of settlement plates (a roofing slate, 40 × 25 mm) attached by cable ties to a length of 6 mm polypropylene rope, at depths of 2, 5, and 8 m. Before deployment, the plates were conditioned for two weeks in aquaria of sand-filtered seawater and kept in darkness to promote a predominately bacterial biofilm (Hammer et al. 2001) because lack of an established biofilm on slates has been shown to limit settlement (Chan & Walker 1998). Fortnightly, from mid-April to mid-December 2001, at each of two sites within Loch Striven and Loch Beag (Fig. 2), four replicate collector strings were deployed and two replicate collector strings, which had been in place for a fortnight, were recovered for tubeworm enumeration. Thus, on every sampling visit, two replicate collector strings were added to those in the water, and they remained there until completion of the monitoring program. A Tinytalk™ II temperature logger was deployed at a depth of 5 m in each loch to record sea temperatures throughout the sampling period. All tubeworm observed on both sides of the 12 slates recovered from each loch, every fortnight, were enumerated with the aid of transparent counting grid.

Pomatoceros *Species Identification*

Tubeworms were identified from slates retrieved at the end of the experiment, which had been placed in the water during the peak settlement period. Individuals (>20 mm tube length) were randomly selected from replicate slates at each depth, site, and loch; removed from their tubes; and identified under a dissection microscope on the basis of opercular structure (Zibrowius 1968), which has been confirmed by previous genetic studies as suitable to distinguish the two species (Ekaratne et al. 1982).

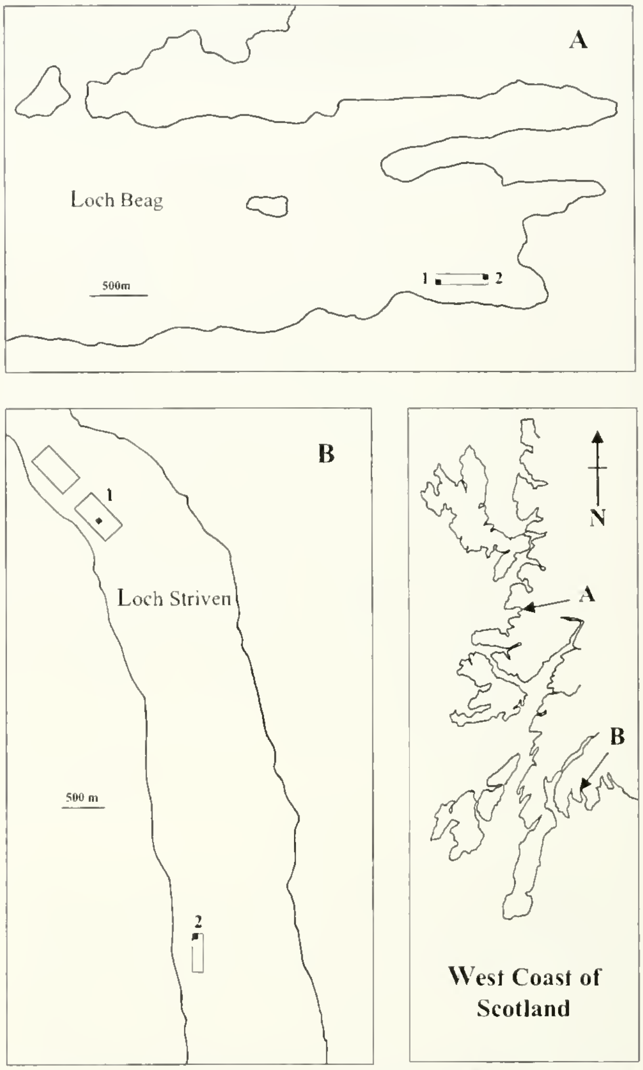

Figure 1. Map of the West Coast of Scotland and sampling locations at Lochs Beag and Striven. Rectangles represent areas of long line mussel cultivation. Small black squares show subsites at which collector plates were deployed (mid-April to mid-December 2001).

Effect of Mussel Size, Density, Retubing System, and Conspecifics on Tubeworm Settlement

At Loch Striven in mid-August 2001, before peak tubeworm settlement, rope-grown mussels, with <2% of individuals fouled with tubeworm (termed "clean"), were graded to create two distinct size classes with 1) large clean mussels (mean shell length 59.9 mm ± 1.05 SE, $n = 50$) and 2) small clean mussels (mean shell length 33.5 mm ± 1.03 SE, $n = 50$). Mussel cultures are allowed to establish on 8-m lengths of polypropylene rope hanging vertically. Before attachment by the byssal threads, the mussels are supported by a mesh tube. Mussels may migrate through different tubing systems at different rates and/or establish different feeding structures on the culture line, which could influence tubeworm abundance on mussels. For this experiment the clean large and small mussels were retubed using two common tubing systems: cotton tubing and plastic "pergolari" mesh, at a high and a low stocking density forming eight different treatments (Tables 1 and 2). A further group 3), large mussels (mean shell length 61.7 mm

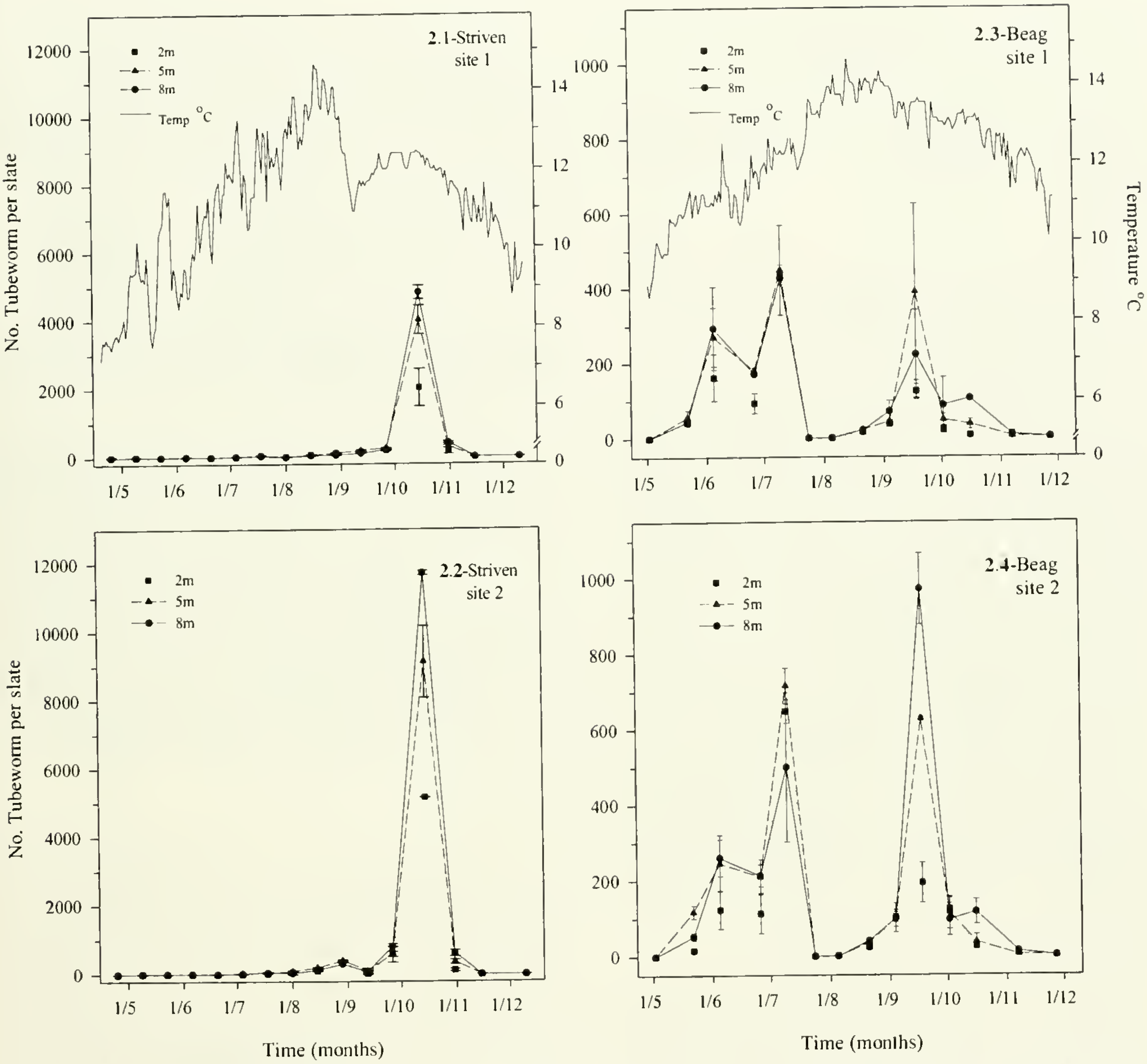

Figure 2. Mean and standard deviation for *Pomatoceros* spp. settlement counts per slate at 2, 5, and 8 m at two sites 4 km apart within Loch Striven (Apr to Dec 2001), 500 m apart in Loch Beag (note difference in scale on the Y-axis), and seawater temperature (°C) at 5 m.

± 0.915 SE, n = 50) with >98% of individuals fouled with tubeworm, were graded and retubed using plastic "pergolari" mesh only at a high and a low stocking density, forming a further two treatments (Tables 1 and 2). The mean shell length was significantly smaller in the small clean mussel treatment than in the two large mussel treatments, which were not significantly different in terms of shell length, thus allowing the inclusion of mussel shell length as a treatment effect (F = 250.2, $P < 0.001$, df = 2, Tukeys $P < 0.05$; Table 2).

Each of the 10 treatments had 5 replicates that were suspended vertically and at random, approximately 40 cm apart, along the horizontal main line. The treatments were removed from the water after 16 wk (mid-December) during which peak tubeworm settlement had occurred and then declined to a base level. The number of *Pomatoceros* spp. that had settled on individual mussel shells sampled from a depth of 4–5 m from each replicate was counted.

TABLE 1.

Treatment characteristics.

Treatments	Replicates	Tubing	Density
(A) Large clean mussels			
1	5	Cotton	High
2	5	Cotton	Low
3	5	Pergolari	High
4	5	Pergolari	Low
(B) Small clean mussels			
5	5	Cotton	High
6	5	Cotton	Low
7	5	Pegolari	High
8	5	Pergolari	Low
(C) Large fouled mussels			
9	5	Pergolari	High
10	5	Pergolari	Low

TABLE 2.

Shell length (mm) and standard error (SE) and stocking densities of the mussel treatments.

Treatment	Length (mm) (n = 50) (±S.E.)	Stocking Density kg/m) High	Low
Large clean	59.9^{a} (±1.05)	5	2
Large fouled	61.7^{a} (±0.92)	5	2
Small clean	33.5^{b} (±1.03)	1.75	0.875

Means with different superscripts in the same column are significantly different ($P > 0.05$, ANOVA and Tukeys).

Mussel Stock Fouling Comparisons

In early August 2001, the fouling intensity of tubeworm on rope-grown mussel stocks with varying treatment histories in Loch Striven was assessed. In Loch Striven the mussel spat generally settles from the plankton in spring (late-April to early-May), by the following spring the mussels are termed 1-y-olds. At intervals over the growing cycle the mussel stocks may be stripped from the culture rope, graded according to size, and resuspended at a lower density. The different groups and depths examined were determined by the stocks (settled in spring 1999 or 2000), which were available at the site. Tubeworm fouling intensity was compared on 1-y-old ungraded mussels (1-y ungraded), 1-y-old mussels that had been thinned in November 2000 (1 y Nov), and in small 2-y-old mussels that had been graded in July 2001 (2 y July). Mussels from 2, 4, 6, and 8 m depth on culture line from each stock were compared. A 1-m length of mussel culture line was removed from five replicate culture ropes at the four different depths and individuals separated and washed. As a measure of mussel density the total weight of individuals per m was recorded to the nearest 0.1 kg. Finally, the percentage of mussels with tubeworm and the number of tubeworm per mussel for a kg sample per replicate were ascertained.

Aerial Immersion Time and Tubeworm Survivorship

In December 2001, rope-grown mussels heavily fouled with tubeworm were collected from Loch Striven and transported in cool boxes to the laboratory aquaria. In each of two temperature-controlled rooms at 13°C and 7°C (both at 95% humidity), groups of five mussels with three or more adult tubeworm per mussel shell were divided among raised trays randomly placed within a continuous flow seawater tank. The mussels with tubeworm were left for five days to allow recovery and acclimatization to laboratory conditions before the start of the experiment. All tubeworms were identified as *P. triqueter*. At time 0, all trays were removed from the seawater, excluding six replicate control trays, which remained immersed for the duration of the experiment. Six replicate trays of mussels were returned to their respective tanks after 6, 12, 18, 24, 30, and 36 h of aerial exposure at either 13°C and 7°C. Forty-eight hours after each tray was returned to the seawater, the percentage of tubeworms alive on each mussel was recorded. Individuals were assumed to be dead when they did not retreat into their tubes when touched by a pin.

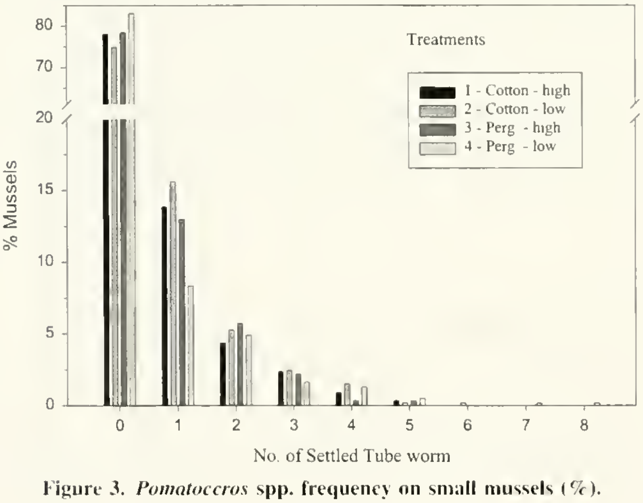

Figure 3. *Pomatoceros* spp. frequency on small mussels (%).

TABLE 3.

Mean and SD *Pomatoceros* spp. counts at peak settlement for each depth at sites in Loch Striven and Loch Beag.

Site	Depth (m)	n	Mean No. *Pomatoceros* spp. per slate (±SD) Loch Striven	Loch Beag
1	2	2	2029^{a} (±552)	124^{a} (±18.4)
	5	2	4019^{b} (±423)	389^{ab} (±236)
	8	2	4825^{b} (±202)	222^{ab} (±120)
2	2	2	5159^{b} (±127)	194^{a} (±53.0)
	5	2	9124^{c} (±1039)	628^{bc} (±5.0)
	8	2	11711^{d} (±65.0)	971^{c} (±94.0)

Means with different superscripts in the same column are significantly different ($P > 0.05$, ANOVA and Tukeys).

Statistical Analysis

ANOVA was used to test for significant differences among treatments and depths, the data having met the assumptions of the test. Test of association (χ^2) was used to compare tubeworm frequency distributions among the treatments. The linear correlation between variables was assessed using Pearson's correlation coefficient (r). Regression analysis was performed to determine the functional relationship between tubeworm survivorship (%) and immersion time (h). ANCOVA and Tukeys pairwise comparison was used to compare regression lines between the different treatments. The statistics package Minitab® version 13.1 was used for all analyses.

RESULTS

Throughout the experimental period, juvenile *Pomatoceros* spp. were the most abundant species observed on collector slates in both lochs. The distribution of settled juvenile tubeworm on the slates tended to be uneven, composed of aggregated groups with a greater proportion of individuals on the lower half of the slate. After two weeks of immersion at each site, the tube length of individuals on the slates was <3 mm.

Pomatoceros *spp. Species Identification*

All individual tubeworms examined on slates from the peak settlement period at Loch Striven and Loch Beag were identified as *P. triqueter*. Therefore, *P. triqueter* was assumed to be the most abundant of the *Pomatoceros* species present and, furthermore, responsible for colonization and fouling of rope-grown mussels in the lochs.

Pomatoceros *spp. Settlement*

The timing of peak settlement differed between lochs but was found to be synchronous between sites and over depth within the same loch. Significant differences in settlement intensity were observed between lochs, sites, and depths. At the Loch Beag sites two periods of peak settlement were observed (26.6.01–10.7.01 and 4.9.01–18.9.01) with the highest mean number of *P. triqueter* per slate (971 ± 94 SD, site 2) recorded in the later peak (Table 3 and Fig. 2). It is not known whether settlement at Loch Beag was in fact two discrete periods or one potentially more intense period disrupted by environmental conditions. A single period of peak settlement was recorded at Loch Striven (26.9.01–15.10.01); with the highest mean number of individual *P. triqueter* per slate (11711 ± 65 SD, site 2), an order of magnitude higher than in Loch Beag (Table 3 and Fig. 2). The Loch Striven and Loch Beag later peak events occurred as summer seawater temperatures declined. In both Loch Striven and Loch Beag at peak settlement, site 2 had significantly greater settlement than site 1 (Loch Striven, $F = 280.0$, $P < 0.001$, $df = 1$; Loch Beag, $F = 27.2$, $P < 0.02$, $df = 1$; Tukeys, $P < 0.05$), indicting the scale on which *P. triqueter* settlement intensity can vary within lochs at sites 500 m to 4 km apart (Loch Beag and Loch Striven, respectively).

In Loch Beag, no effect of depth on settlement intensity was observed at site 1, whereas at site 2, significantly greater settlement occurred at 5 m and 8 m than at 2 m. The effect of depth on *P. triqueter* settlement was more pronounced at Loch Striven. Site 1 had significantly greater settlement at 5 m and 8 m than at 2 m and at site 2 settlement increased significantly with increasing depth.

Effect of Mussel Size, Density, Retubing System, and Conspecifics on Tubeworm Settlement

The number of *Pomatoceros* spp. settling on small mussels was found to be significantly lower than that on the large mussel treatments. Among the small size class treatments, the percentage of mussels with ≥1 tubeworm on the shell ranged between 21.5% and 28.4%, whereas in the large size class treatments, the percentage of mussels with ≥1 tubeworm on the shell ranged between 91% and 100% (Tables 4 and 5, Fig. 3 and 4). Thus, analyses on small mussel treatments were performed separately to the large mussel treatments.

Small Mussel Treatments

The small mussels migrated successfully from the two tubing systems. *Pomatoceros* spp. were observed to have settled predominately along growth checks on the mussel shell exterior with a smaller proportion having settled on the umbo and shell ligament regions.

No significant difference in the mean percentage of individuals fouled was found (ANOVA, $F = 1.24$, $P = 0.329$, $df = 3$) and no significant differences were observed among frequency distribution of *Pomatoceros* spp. fouling between the small mussel treatments (four classes, 0, 1, 2, 3+, $\chi^2 = 10.29$, $P = 0.328$, $df = 9$; Table 4, Fig. 3).

Large Mussel Treatments

Significant differences in number of tubeworm per individual were identified between the large mussel treatments (ANOVA, $F = 17.1$, $P < 0.001$, $df = 5$; Table 5). However, in all large mussel pergolari treatments, the mussels did not establish outside the tube because the mesh size proved too small to allow mussels to migrate through the apertures. Thus, the mussels were confined within the tube for the duration of the field trial, affecting the structure of the feeding colony and preventing comparison between pergolari and cotton tubing systems with respect to tubeworm fouling.

Clean cotton-tubed mussels at low density had a significantly greater number of tubeworm per mussel than clean cotton-tubed mussels stocked at high density. No significant differences were observed between clean cotton-tubed mussels at low density and clean pergolari-tubed mussels at high and low densities. No significant differences were observed between clean cotton-tubed mussels at low density and fouled pergolari mussels at high and low densities. Tubeworm numbers per individual were significantly greater in fouled pergolari-tubed mussels at high and low densities compared with clean pergolari mussels at high and low densities (Table 5).

From the test of association analysis (χ^2), four significantly different distributions among frequency classes of tubeworm on large mussels were identified: 1) clean cotton-tubed and clean pergolari-tubed at high density had the greatest proportion of mussels with low frequencies of tubeworm and the proportion of mussels decreased steeply in higher tubeworm fouling classes (Fig. 4, 4.1 and 4.3); 2) clean pergolari tubed at low density had a large proportion of mussels with low frequencies of tubeworm fouling, which did not decrease as steeply over higher tubeworm fouling classes compared with distribution 1 (Fig. 4, 4.4); 3) clean cotton tubed at low density comprised of an approximately even frequency distribution of mussels throughout the tubeworm fouling categories (Fig. 4, 4.2); 4) fouled pergolari-tubed mussels at high and low density also consisted of an approximately even frequency distribution of tubeworm frequency classes among mussels yet had the lowest proportion of mussels in low tubeworm fouling categories (Fig. 4, 4.5 and 4.6).

TABLE 4.

Mean (%) of mussels with *Pomatoceros* spp. and SE in the small mussel treatments.

Treatment	Tubing	Density	Replicates	Mean % Mussels With *Pomatoeeros* spp (±SE)	CV %
1	Cotton	High	5	22.3^a (±2.65)	26.7
2	Cotton	Low	5	28.4^a (±4.82)	38.0
3	Pergolari	High	5	21.3^a (±2.02)	21.2
4	Pergolari	Low	5	21.5^a (±1.81)	18.8

Coefficient of variation is given (CV%). Means with different superscripts in the same column are significantly different ($P > 0.05$, ANOVA and Tukeys).

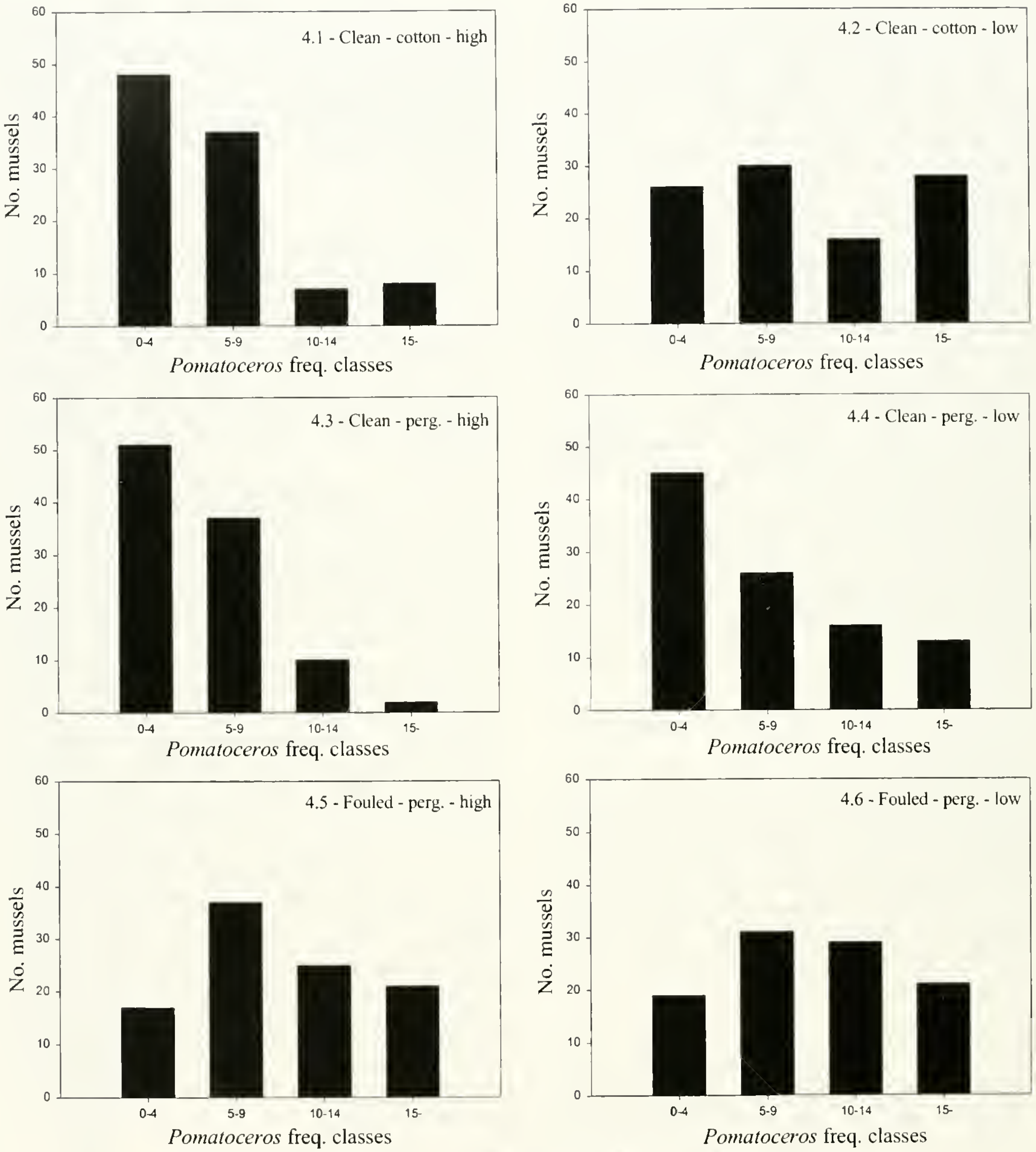

Figure 4. ***Pomatoceros*** **spp. frequency classes on large mussels (100 mussels per treatment).**

Mussel Stock Fouling Intensity Comparisons

The results demonstrate the wide variation in the percentage of mussels fouled that exists among different mussel stocks within the farm site, with the mean percentage mussels fouled among the different mussel stocks ranging between 0.7% (1-y ungraded stock) and 5.9% (1-y Nov-graded stock; Table 6). The stocks—1-y Nov, 1-y ungraded, and 2-y July mussels were all significantly different with respect to levels of tubeworm fouling, with a greater percentage of mussels fouled in the 1-y Nov stock and least in the 1-y ungraded stock ($F = 55.6$, $P < 0.001$, $df = 2$; Tukeys $P < 0.05$).

Variation in fouling intensity was observed to increase as depth increased. However, no correlation between the percentage of fouled mussels and the variables depth (m) and density (mussels kg/m) among individual stocks or stocks combined was found. A negative correlation was found between depth (m) and mussels density when all treatments were combined ($r = -0.532$, $P < 0.001$, $n = 60$), indicating that as depth increases mussel density along the culture line decreases.

TABLE 5.

Mean number of *Pomatoceros* spp. per mussel and SE in the large mussel treatments.

Treatment	Fouling	Tubing	Density	n	Mean No. *Pomatoceros* spp. Per Mussel (±S.E.)	CV%
1	Clean	Cotton	High	100	5.81^{a} (±0.50)	87.1
2	Clean	Cotton	Low	100	10.55^{b} (±0.84)	79.6
3	Clean	Pergolari	High	100	5.07^{a} (±0.38)	75.1
4	Clean	Pergolari	Low	100	7.24^{a} (±0.58)	80.7
5	Fouled	Pergolari	High	100	10.10^{b} (±0.65)	64.4
6	Fouled	Pergolari	Low	100	10.45^{b} (±0.66)	63.3

Coefficient of variation is given (CV%). Means with different superscripts in the same column are significantly different ($P < 0.05$, ANOVA and Tukeys).

Aerial Immersion Time and Tubeworm Survivorship

Although increasing *P. triqueter* mortality was clearly evident, as aerial immersion time (h) increased, no mortalities amongst the mussels occurred during the trials. No *P. triqueter* mortalities were observed in the control treatment, which was never immersed during the experiment (Fig. 5)

The interaction term (time × treatment) was significant (ANCOVA, $F = 5.90$, $P = 0.02$), indicating that the slopes, (*P. triqueter* % survival rates over time) were significantly different at 7°C and 13°C, at the 95% confidence level. Adult *P. triqueter* were predicted to achieve a mean 100% mortality after 24.1 h and 35.4 h at an air temperature of 7°C and 13°C, respectively (Table 7).

DISCUSSION

P. triqueter was the most abundant of any species settling on collector plates and the only species of tubeworm found on the mussels. This is in concordance with the observation that *P. lamarcki* occurs more frequently in intertidal and shallow turbid subtidal waters (Hayward & Ryland 1995) and that *P. triqueter* can out-compete *P. lamarcki* for settlement space at depths of 13 m, although the dominance of one species over the other is thought to be dependent on climatic conditions (Castric-fey 1983).

The pattern of settlement in the two Scottish lochs was consistent with that in other temperate populations of *P. triqueter* (Klockner 1976, Castric-fey 1983), with peak settlement occurring later in the year, possibly the result of the more northerly latitude (Klockner 1976). The timing of peak settlement differed between lochs, presumably as a result of specific biologic and physical differences (Crisp 1974, Dirnberger 1990) but was synchronous within each loch and over depth. Levels of settlement differed between lochs, sites, and over depth within the same loch, giving indication of the scale on which intra-loch variation in environmental factors influence settlement abundance. Such monitoring should be repeated to ascertain year-to-year variation in occurrence of peak settlement within Scottish lochs.

The higher settlement levels observed at Loch Striven may result from a long flushing time combined with comparatively little fresh water input and mixing (Tett et al. 1986); consequentially, the larvae are retained within the loch for longer periods of time and rarely experience lowered salinity, which is reported to reduce settlement (Castric-fey 1983). In addition Loch Striven has had an established mussel site (8 y), whereas the site in loch Beag was recently established (3 y) and is approaching its second harvest. The scales of the operations are also significantly different (standing mussel stock at Loch Striven is 200 tons and Loch Beag is 30 ton). A longer period of mussel production at Loch Striven may have increased the standing population of tubeworm through the accumulation of live mussels and shell material beneath the farm, reported to increase site availability for settlement of calcareous polychaetes (Tenore et al. 1982, Kaspar et al. 1985).

Increasing or peak seawater temperature may act as a direct or indirect cue for optimum gamete production or spawning or create optimal conditions for larval settlement and survival, resulting in discrete and intense periods of tubeworm settlement. At higher temperatures bacterial loading would also be maximal and may also generate a cue for settlement. A high bacterial density has been identified as a major cue for the settlement response of *P. lamarcki* (Hammer et al. 2001), and mussel farms have been shown to possess increased microbial assemblages resulting from elevated levels of organic enrichment (La Rosa et al. 2001).

Speculatively, the exaggerated metabolic output from mussels intensively farmed within a restricted loch environment may enhance or promote a tubeworm larval settlement response. Natural concentrations of ammonia (NH_3), a principle component of excretion in bivalves, released by adult oysters can be sufficient to induce settlement of oyster larvae, particularly at highest temperatures when peak values for total NH_3–NH_4^+ occur (Fitt & Coon 1992). Mussels may therefore be more susceptible to tubeworm

TABLE 6.

Mean length of mussels (mm) with SE mean density of mussels (kg/m) with SE, mean (%) of mussels with *Pomatoceros* spp. and SE for each mussel stock.

Treatment	Length (mm) ($n = 50$) (±S.E.)	CV%	Mean Density kg/m (±S.E.)	Mean % Mussels Fouled ($n = 100$) (±S.E.)
1 y Ungraded	37.8 (±1.45)	27.1	6.0 (±0.80)	0.66^{a} (±0.17)
1 y Nov	40.4 (±0.66)	11.5	4.4 (±0.20)	5.89^{b} (±0.51)
2 y July	41.7 (±0.70)	11.9	4.0 (±0.20)	2.64^{c} (±0.34)

Means with different superscripts in the same column are significantly different ($P < 0.05$, ANOVA and Tukeys).

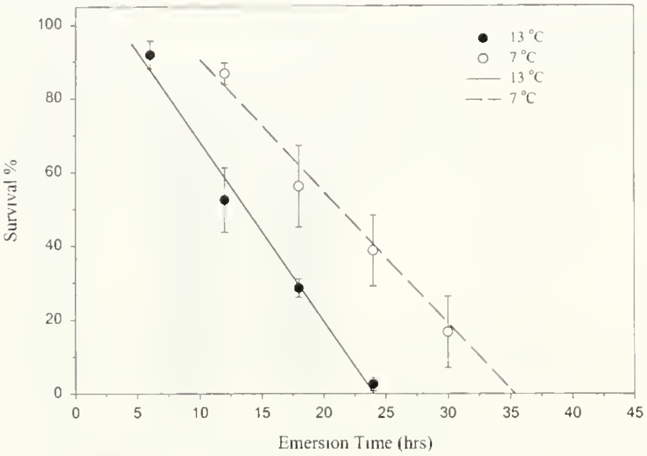

Figure 5. Mean *P. triqueter* survival (%) and standard error against aerial immersion time (h) at 13°C and 7°C after a 48-h recovery period for the linear section of the relationship. Regression lines are fitted.

fouling immediately upon re-immersion after grading, for in addition to offering an unprotected surface for settlement, a pulse of excreted nitrogenous waste, accumulated during immersion and released on re-immersion, could further induce *P. triqueter* larvae to settle. Tubeworm settlement cues with respect to environmental conditions on a mussel farm warrant further research.

The field trial highlighted mussel size (shell length) as an important factor influencing *P. triqueter* abundance on farmed mussels because small mussels had consistently fewer tubeworms. This may be a result of the fast growth rates of smaller mussels, preventing successful tubeworm attachment. Tubeworms were not found on areas of fastest growth such as the posterior shell edge but predominately on shell growth checks and on the umbo indicating settlement to have occurred on regions of relatively slower growth and/or at periods of disturbance and slow growth. The density of smaller mussels established on culture lines is greater than large mussels, having smaller interstices between individuals and less of a surface area for settlement of fouling organisms. As mussels age, a change in the nature of the mussel shell texture or its biofilm may make them more attractive for tubeworm settlement. No effects of initial stocking density or tubing system on tubeworm settlement on small mussels were distinguished, indicating that size has a greater influence on settlement than either of these two factors. Further work should determine the relationship between mussel size and susceptibility to tubeworm settlement.

P. triqueter settlement was greatest in treatments with conspecific adults on the mussels. However, there was no difference in tubeworm settlement levels between high and low densities in the fouled mussel treatments. This suggests that the cue to settle provided by conspecifics was sufficient to outweigh the effects of stocking density. Settlement on or near conspecific adults has benefits; adults derive reproductive benefits from being within aggregations and larvae that settle near adults benefit from choosing a habitat likely to support post larval growth (Pawlik 1992). *P. lamarcki* has been shown to settle in response to chemical substances originating from the body of conspecific adults or juveniles (Chan & Walker 1998). As a result, whenever practical, individuals with fouling should be removed from mussels that are to be retubed for on growing.

Tubeworm numbers were more abundant on the clean large mussels stocked at low stocking densities; this may be attributed to greater shell surface areas exposed for settlement and or a decrease in overall feeding activity in mussels at lower densities on the culture line. In areas of high tubeworm settlement, mussel lines should be stocked at optimum densities for maximal rapid growth and distributed evenly throughout the tubing.

A wide variation in fouling intensity was observed among mussels stocks with different treatment histories at the farm site. Differences in the fouling intensity between the stocks are most likely to be attributed to the settlement intensity of tubeworm and the size of mussels at the time of retubing. The influence of depth on settlement was not as distinct along mussel culture lines when compared with the collector slates. Consequently, adjusting culture line depth to control fouling may prove ineffectual. No correlation could be found between mussel density and degree of fouling. However, counting the number of mussels on a meter of culture rope may have been too insensitive as a measurement of density as mussel density was not always homogenous within a meter of culture line and settlement site selection by tubeworm is influenced over small scales (Pawlik 1992). Further studies of increased precision would determine the true effect of these variables.

As a result of the discrete nature of *P. triqueter* settlement, mussel growers could routinely monitor tubeworm settlement and to avoid practices such as grading and returning mussels to the water in periods of intense tubeworm settlement. Monitoring settlement should be conducted specifically for each loch. Growers should monitor settlement in conjunction with physical variables such as seawater temperature and salinity specific to individual sites to reveal areas and conditions that consistently yield lower levels of tubeworm fouling, and criteria for establishing new mussel farm sites should include hydrographic conditions that minimize tubeworm fouling. Furthermore, mussel-management strategies should adhere to minimizing the time mussel stocks are in the water, particularly in the second growing season when individuals reach a critical size and become vulnerable to excessive tubeworm fouling. In contrast, an alternative approach to limiting tubeworm settlement might be to avoid grading in the second year, thereby

TABLE 7.

The regression coefficients α and β and respective SE, Pearson's correlation coefficient (*r*), probability and predicted aerial emersion times (t_{pred} [hr]) with upper and lower 95% confidence intervals (C.I.), to achieve 100% mortality for *P. triqueter* at 7°C and 13°C after a 48-h recovery period.

Temperature	*n*	Coefficient α (±SE)	Coefficient β (±SE)	*r*	t_{pred} (h)	Lower 95% C.I.	Upper 95% C.I.
7°C	24	116.8 (±6.52)	−4.86 (±0.402)	−0.919 ($P < 0.001$)	35.4	33.0	38.6
13°C	24	126.2 (±9.38)	−3.57 (±0.323)	−0.940 ($P < 0.001$)	24.1	22.3	26.5

leaving mussels undisturbed and allowing a mixed size-class feeding colony to develop.

Adult *P. triqueter* were comparatively resilient with respect to aerial immersion, with a predicted mean 100% mortality after 24.1 h and 35.4 h at an air temperature of 7°C and 13°C, respectively. However, as a result of the lengths of immersion time needed to achieve 100% *P. triqueter* mortality, it may not be practical for growers to use aerial exposure as a method to control tubeworm growth on mussels to be retubed and harvested at a later date. Although no mussels died within the experimental period, aerial immersion over such periods of time may cause undue stress in rope-grown mussels unaccustomed to exposure, resulting in higher mortalities after grading and retubing and delayed growth.

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MUSSEL DREDGING: IMPACT ON EPIFAUNA IN LIMFJORDEN, DENMARK

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ABSTRACT Species composition and population density of epibenthos are described in two areas in Limfjorden, Denmark. Both areas covered both a mussel fishing ground and an area that has been permanently closed for mussel dredging since 1988. Furthermore, mussels were dredged in a part of the mussel fishing grounds in both areas four months before the investigations. The rest of the fishing grounds had not been exploited for at least four years. This study describes the short-term impact (4 mo) and long-term impact (>4 y) of mussel dredging using the permanently closed areas as controls. The data were analyzed by multivariate statistics. In both short-term study areas significant effects of dredging were observed. A number of taxa (sponges, echinoderms, anthozoans, molluscs, crustaceans, and ascidians) had a reduced density or were not observed in fished areas four months after the fishing was ended. In one of the two long-term study areas, significant differences in species composition and density were observed between fished and closed areas, indicating that the fishery may have a long-term impact on the epibenthic community, whereas in the other long-term area no difference was observed between fished and control areas. Significant reductions in the amount of shell debris and gravel were observed in the dredged areas. The impact of the loss of these benthic structural components on ecosystem processes and functions is discussed.

KEY WORDS: long-term impact, short-term impact, fishery impact, benthic epifauna, mussel dredging, seabed structure

INTRODUCTION

The use of dredges, beam-, and otter-trawls in demersal fisheries and the development of heavier gear types with increased fishery effort have raised a growing concern on the impact at benthic ecosystems (see review by Jennings & Kaiser 1998, Hall 1999, Collie et al. 2000). Recent studies have investigated the direct impact of towed-bottom fishing gear on benthic organisms (Bergman & Hup 1992, Eleftheriou & Robertson 1992, Brylinsky & Gibson 1994, Thrush et al. 1995, Currie & Parry 1996, Collie et al. 1997, Tuck et al. 1998, Hall-Spencer et al. 1999, Hoffmann & Dolmer 2000, Dolmer et al. 2001), the impact on seabed topography and seabed composition (Hall et al. 1990, Pranovi & Giovanardi 1994, Kaiser & Spencer 1996, Schwinghamer et al. 1998), and the effect of suspension of bottom sediment and release of oxygen-consuming substances and nutrients (Riemann & Hoffmann 1991, Dyekjær et al. 1995, Pilskaln et al. 1998). Indirect effects of the fisheries include changes in the trophic structure (Babcock et al. 1999) as the reported increase in density of scavengers feeding on injured or discarded species (Gislason 1994, Jennings & Kaiser 1998) or as changes in species behavior and species interactions (Ramsay & Kaiser 1998).

Because the blue mussel is an economically and ecologically important species in many coastal areas, mussel dredging may significantly affect the form and the function of benthic ecosystems. Blue mussels form tridimensional matrices of byssus-thread interattached mussels. These stabilized structures are important for assemblages of associated organisms (Tsuchiya & Nishihira 1985, 1986, Svane & Setyobudiandi 1996, Ragnarsson & Raffaelli 1999). In soft-bottom habitats, solid components such as gravel and biogenic structures such as mussel beds and shell debris substantially increase substratum heterogeneity and complexity. These structures are reported to be important for invertebrates as spatial refuges from predators (Heck & Crowder 1991, Dumas & Witman 1993, Lee & Kneib 1994, Thiel & Dernedde 1994) or as a substratum for settling invertebrates and sessile organisms (Witman & Suchanek 1984, Baker 1997, Lapointe & Bourget 1999). Mussel dredging may destroy mussel bed structures and remove important seabed structuring components such as shell debris and gravel. The fishery thus exerts a potential direct impact on the ecosystem by changing benthic habitat structures and indirectly interfering with invertebrate settling processes and prey–predator interactions.

In Limfjorden, Denmark, an extensive fishery for blue mussels, *Mytilus edulis*, exists. In a previous study in Limfjorden, Dolmer et al. (2001) demonstrated that mussel dredging reduced the density of invertebrates living in the bottom. In particular, the polychaetes were significantly reduced in number. Another investigation in 1997 of the long-term impact of mussel dredging on the epifauna in an area that has been closed for mussel dredging since 1988, however, failed to show any effects of the mussel fishery (Hoffmann & Dolmer 2000). The aim of the present study was to compare the spatial composition of assemblages of epibenthic fauna on mussel fishing grounds four months after a fishery was finished, in areas that have not been fished for more than four years, and in areas that have been permanently closed since 1988 to test the short-term and long-term impacts of mussel dredging on the epifauna. The second objective of the study was to measure the impact of mussel dredging on the amount of shell debris and gravel on the seabed.

MATERIAL AND METHODS

The study was conducted in Limfjorden (Fig. 1). This area is a micro-tidal, eutrophic water system of interconnected enclosures in northern Denmark, supporting a high production of blue mussels. The mean mussel biomass in the part of the area open to mussel dredging (~750 km^2) is about 600,000 tons with large temporal variations (Dolmer et al. 1999). This biomass supports the largest fishery in Europe exploiting natural mussel populations. The annual mussel landing is approximately 100,000 tons or 15% of the mussel stock.

The short-term and long-term impact of mussel fishing on benthic epifauna was investigated in two different areas in Limfjorden: in Løgstør Broad, in the central part of the fjord, and in the Agerø area, in the western part of the fjord (Fig. 1). Part of both areas has been permanently closed to mussel fishing since 1988. In Løgstør Broad, the northern part of the area is closed and in the Agerø area, the central part is closed. During April–May 1999 (4 months before the investigation) mussels were commercially dredged in the area just southwest of the closed area in Løgstør

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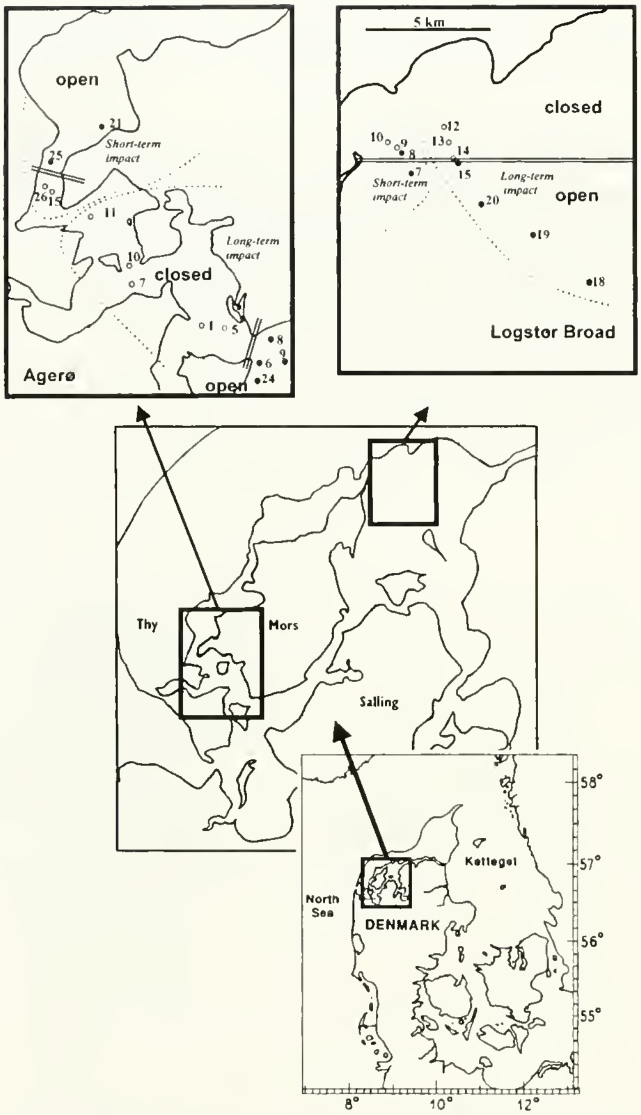

Figure 1. Map of the two study areas and their stations. The double lines indicate the separation between fished areas and areas that have been closed to mussel fishing since 1988. In Løgstør Broad (eastern area), the area north of the double line is closed and the area south of the line is fishing ground. In the Agerø area (western area), the area between the two double lines is closed to mussel fishing. The stations used in the analyses of long- and short-term impact are separated with dotted lines. (○) control stations and (●) stations with mussel dredging.

Broad and in the area just north of the closed area in the Agerø area. Furthermore, data from the mussel stock assessment program, where the mussel biomass at 250–350 stations in the Limfjorden is described annually (Dolmer et al. 1999), indicate that that the area southeast of the permanently closed area in Løgstør Broad and the area south of the permanently closed Agerø area has not been fished at least since 1996. Epifauna in the Løgstør Broad and the Agerø areas was described in September 1999 to test whether the species composition and density differed between permanently closed areas, areas that were fished four months before the study, and areas that had not been fished for more than four years. Replicated stations in the area closed to fishing were contrasted with replicated stations located in adjacent areas that were subject to harvesting practices.

Short-Term Impact (4 Mo)

Stations 7–8 in Løgstør Broad and stations 21 and 25 in the Agerø area were fished by mussel dredge during April–May 1999 and significant impact on the seabed was observed when diving in September. To test the impact of dredging on the epifauna species, composition on the two stations in each area was contrasted with two control stations in the nearby closed areas. Stations 7 and 8 were contrasted with stations 9 and 10 in Løgstør Broad and stations 21 and 25 were contrasted with stations 15 and 26 in the Agerø area (Fig. 1). No signs of dredging activity were observed on the seabed when diving at the control stations.

Long-Term Impact (>4 Y)

In the eastern part of Løgstør Broad, the stations in the closed area (12, 13, 14) were contrasted with the stations in the area open for mussel dredging (15, 18, 19, 20). In the Agerø area, the stations in the closed area (1, 5, 7, 10, 11) were contrasted with the stations in the area open for mussel dredging (6, 8, 9, 24).

At each station, the epifauna (>1) cm was identified and quantified. Colonial species such as hydrozoans and bryozoans were omitted from the study because of difficulties in quantifying these taxa. The sponges were included in the investigation, and here each distinct colony was classified as one individual. At each replicated station, the epifauna in 30 circles of 0.24 m^2 were quantified by use of SCUBA diving. The circles were marked with an iron ring randomly placed on the sea floor. The material in the last 10 circles was collected and the population density of blue mussels and the weights of shell debris and pebbles were measured in the laboratory.

The epifauna species composition and density data was tested with the PRIMER-5-package (Plymouth Routines in Multivariate Ecological Research). Before the analysis, the ring samples without epibenthic organisms were excluded from the datamatrix. Bray–Curtis similarity indices were calculated on 4^{th} root transformed species density data according to Clarke and Warwick (1994). The stations were plotted in a Multi-Dimensinal Scaling plot (MDS) to identify separate clusters of stations. Differences between contrasted stations were tested in two steps by ANOSIM analyses. It was tested if there were differences among replicated stations within each treatment area (fished contra closed) in each area and if differences ($P < 0.01$) were obtained then an ANOSIM analysis tested for differences in species composition between contrasted stations on average ranked data. If no differences were observed among replicated stations then an ANOSIM analysis tested for differences between contrasted stations on the set of data treating each ring observation observations as separate data. The SIMPER procedure identified which taxa contributed most to the dissimilarity among contrasted stations. The significances of the differences in density of these taxa were tested with t tests.

Observed differences between contrasted stations can be due to short-term and long-term impacts of mussel dredging or may be caused by environmental gradients in the study area. It was assumed that if an environmental gradient affected the similarity between stations there would be a positive relationship between spatial distance and similarity between stations. On a MDS plot, the stations would then form a unidirectional track with the spatial and similar close stations close to each other and most spatial distant and dissimilar station at the longest distance. To test whether the similarity among stations was related to their spatial relation, the MDS plots of the stations, including an indication of the stations spatial relationships, was analyzed.

Impact on Substratum

The data on weights of shell debris and gravel at each of the short-term impact study stations were tested separately for the Løgstør and the Agerø Area in nested two-way ANOVAs with fishery/closed status as the first factor and the stations as the second factor—nested to the fishery/closed factor. Before the tests, the data were *ln*-transformed and tested for homoscedasticity (F_{max}-test). To test the relationship between diversity and substratum composition, a Shannon–Wiener index was calculated for each sample from the short-term study stations. The relationships between this index and the amounts of shell debris and pebbles were analyzed by linear regression. To test the role of mussels forming a biogenic substratum important to the associated fauna, the relation between the Shannon–Wiener index and the density of mussels in each sample was analyzed by linear regressions at station 7 and 8 and 9 and 10 in Løgstør Broad.

RESULTS

During the investigation, a total of 11 and 20 different epifauna species were recorded in Løgstør Broad and the Agerø area, respectively. MDS ordinations of the stations in Løgstør Broad and the Agerø area showed that the stations in the short-term study areas were separated into two clusters, including stations from the fished and the closed part of the study areas, respectively (Fig. 2). The separation of the stations from the long-term study areas was not that clear-cut. Low oxygen concentrations (<2 mg O_2 l^{-1}) were recorded during two weeks in August 1999 in parts of Løgstør Broad. Some mortality of blue mussels was observed after this oxygen deficiency, but also other species may have been affected. Because the distribution of areas with oxygen deficiency did not overlap with the distribution of the fished areas, the low oxygen concentration did not interfere with the results of this study.

Short-Term Impact

In the two short-term study areas, no differences were observed among stations within each treatment area and the difference between dredged and closed stations was tested on complete sets of data. The ANOSIM analysis showed that there were significant differences between the two dredged stations and the two control stations in both Løgstør Broad ($P = 0.001$) and the Agerø area ($P = 0.001$). The SIMPER procedure and *t* tests indicated that a large number of species disappeared or had a reduced density in the two dredged areas, including poriferans, echinoderms, anthozoans, gastropods, crustaceans, and ascidians (Tables 1 and 2). Splitting the data into two functional groups, mobile and sessile species, indicated that the sessile species contributed to a larger part of the dissimilarity between the control and the dredged stations (51–61%) than the mobile species, although these species still contributed significantly to the dissimilarity (30–42%). ANOSIM analyses on data split into functional groups showed a significant dredging impact on sessile fauna both in Løgstør Broad and in the Agerø area (Table 3).

Long-Term Impact

In both Løgstør Broad and the Agerø area, significant differences among stations within each treatment area (fished contra closed) were observed (Table 3). Consequently, the analysis of the long-term fishery impact was conducted on average rankings and re-ranked data. The ANOSIM analyses testing the species composition at the stations in the fished area in the southern part of Løgstør Broad and in the closed northern part showed that there was no difference ($P = 0.771$) between stations indicating that no long-term effects could be observed. In the Agerø area, a significant difference ($P = 0.024$) between the species composition in the central closed area and the southern area where mussel dredging is legal may indicate a long-term impact. The SIMPER procedure and *t* tests on data from the Agerø area indicated that a number of species disappeared or had a reduced density in the area open to mussel dredging (Table 4).

The MDS of the similarity between stations and their spatial relationships (Fig. 3a and b) show no relationship between similarity and spatial distance in the two short-term study areas. This indicates that differences in species composition between stations are not due to an environmental gradient in the area. In the long-term study areas, some of the stations with the longest distance in-between showed a relationship between similarity and distance

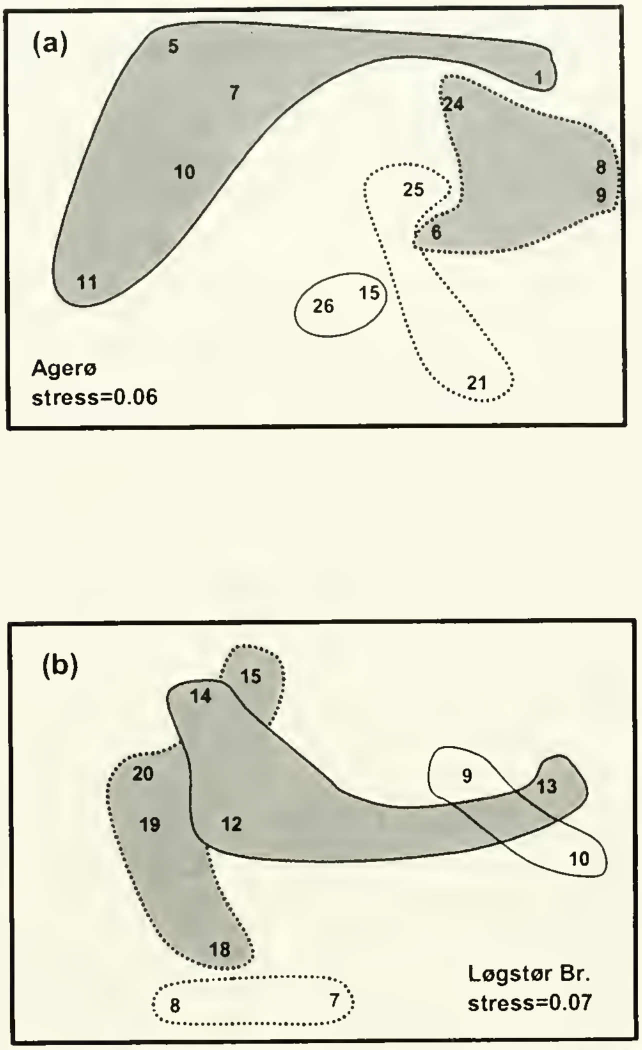

Figure 2. MDS plot of station means in the Agerø area (a) and Løgstør Broad (b). The control area stations are delineated with a solid line and stations from fished areas with a dashed line. The gray background indicates long-term study stations and the white background short-term stations.

(stations 11-10-7 in the Agero area and stations18-19-20-15 in Løgstor Broad). The spatial distribution of the dredged part of the long-term study area in Agerø, with a central station (station 6) and three stations in a semicircle, make an analysis of environmental gradients impossible because no relation between similarity and distance can be established because the distances from station 6 to all three stations in the semicircle are equivalent.

Impact on Substratum

Amounts of shell debris and gravel differed significantly between dredged and control stations in both areas (Fig. 4a and b). In Løgstør Broad, the weights of shell debris ranged from 0.5 kg m^{-2} at station 7 and 8 in the fished area to approximately 2 kg m^{-2} at station 9 and 10 in the control area. In the Agerø area, the weights of shell debris ranged from 0 kg m^{-2} at station 21 and 25 in the fished area to 1 kg m^{-2} at station 15 in the control station. Gravel was only found at the stations in Løgstør Broad ranging from 0 kg m^{-2} at station 7 in the fished area to 0.7 kg m^{-2} at station 9 in the closed area. The nested ANOVA analyses for both areas showed a significant effect of dredging on the weights of shell debris (P = 0.000) whereas no significant differences were observed among stations when nested to the fished/control areas (Løgstør B: P = 0.467, Agerø: P = 0.215). The weights of gravel were not only significantly affected by the dredging (P = 0.000), but also by the stations (P = 0.001). Plotting the Shannon–Wiener diversity (H′) index in the samples from Løgstør Broad as a function of the amount of shell debris a significant ($P < 0.01$) positively correlation was obtained for the data from the fished area. The similar relationship hold constant in the permanently closed area (Fig. 5). As the amount of shell debris in the Agerø and the weight of pebbles in Løgstør broad are low at the fished stations, similar relations cannot be plotted for these data. The relationship between H′ and the mussel density (Fig. 6) showed a similar pattern. A significant positively correlation ($P < 0.01$) was observed in the area open to mussel dredging whereas in the permanently closed area only a trend can be detected (P = 0.09)

DISCUSSION

To achieve a holistic sustainable management of fisheries, knowledge of the fishery impacts on the target populations and the ecosystem is required. In respect to fishery impact on ecosystems, such management has to consider both the short-term and long-term impact and the recovery time for the ecosystem to re-establish a habitat. This study describes significant differences in the species composition among stations in areas fished four months before sampling and control stations. A number of taxa (poriferans, echinoderms, anthozoans, molluscs, crustaceans, and ascidians) had a reduced density or were not observed in fished areas four months after the fishing was ended. These differences included reductions in both mobile and sessile species. Differences in the species composition were also observed between an area that had not been fished for more than four years and a closed control area. A significant reduction in shell debris and gravel was observed in dredged areas.

The observed differences between stations in fished and closed areas may be due to mussel dredging but could also be caused by environmental gradients in the study area (e.g., wind exposure, water current, sediment composition, primary production). A MDS plot relating similarity and spatial distance between stations, however, did not indicate any relation in the short-term areas, suggesting that the observed differences in species compositions are due to dredging activity and not due to environmental gradients. As the distance between the closest dredged and control stations in the short-term in Agero and Løgstør Broad is 750 and 200 m, respectively and that the demarcation line between fished and closed area was drawn without any reference to physical conditions in the area, it is reasonable to conclude that no environmental gradients were involved. The stations used in the long-term impact analysis had a more extensive distribution, with <10 km between the most distant stations. In Løgstør Broad, the dissimilarity was related to the distance between stations, indicating that a gradient probably influenced the area. This environmental gradient may increase the variability in species composition, making it more difficult to detect long-term impact of the fishery. In the Agerø area, the way the stations were distributed made the detection of an environmental gradient difficult. Consequently, it is difficult to judge whether the observed differences in species compositions were due to long-term impact of mussel dredging or to a gradient.

The design of fishery impact studies is a trade-off between a robust experimental design and the amount of effort that can be invested in the study. An often-used statistical design is a BACI

TABLE 1.

Short-term impact: species contributing to the dissimilarity (%) between fished stations 7–8 and control stations 9–10 in a closed area in Løgstør Broad.

			St 7–8 Dredged		St 9–10 Control		
	Mobility	Dissimilarity (%)	Mean	2 SE	Mean	2 SE	*t* test *P*
Corella parallelogramma	S	37	0.00	0.00	16.00	8.25	0.00**
Macropodia rostrata	M	14	0.00	0.00	0.33	0.29	0.02**
Crangon crangon	M	10	0.07	0.13	0.60	0.46	0.03*
Mytilus edulis	S	9	15.40	9.85	15.00	7.70	—
Sagartia troglodytes	S	8	0.73	0.52	3.40	0.93	0.00**
Metridium senile	S	7	0.13	0.19	0.07	0.13	0.56
Carcinus maenas	M	6	0.00	0.00	0.13	0.19	0.16
Mobile species		30	0.07	—	1.06	—	
Sessile species		61	16.26	—	34.47	—	

The species are separated into mobile species (M) and sessile species (S). The pooled mean density (individuals m^{-2}) and 2 SE are given and the differences in densities between dredged and undredged areas are compared with *t* tests (**$P < 0.025$, *$P < 0.05$). Pooled dissimilarity contribution and mean-densities are given for two functional groups. Only species contributing more than 4% to the dissimilarity are included in the table.

TABLE 2.

Short-term impact: species contributing to the dissimilarity (%) between fished stations 21–25 and control stations 15–26 in a closed area in the Agero area.

			St 21–25 Dredged		St 15–26 Control		
	Mobility	Dissimilarity %	Mean	2 SE	Mean	2 SE	*t* test *P*
Halichondria panicea	S	16	0.00	0.00	0.40	0.37	0.03*
Asterias rubens	M	12	0.27	0.26	1.00	0.49	0.01**
Sagartia troglodytes	S	11	0.27	0.32	2.27	1.06	0.00**
Tealia felina	S	10	0.07	0.13	0.40	0.31	0.05*
Metridium senile	S	8	0.93	0.98	2.33	1.89	0.19
Carcinus maenas	M	7	0.60	0.37	1.80	0.67	0.00**
Macropodia rostrata	M	6	0.07	0.13	0.00	0.00	0.32
Mytilus edulis	S	6	0.00	0.00	0.07	0.13	0.32
Buccinum undatum	M	6	0.07	0.13	0.00	0.00	0.32
Hinia reticulata	M	6	3.87	0.95	7.20	1.72	0.00**
Crangon crangon	M	5	1.67	0.72	1.27	0.62	0.40
Mobile species		42	6.55	—	11.27	—	
Sessile species		51	1.27	—	5.47	—	

The species are separated into mobile species (M) and sessile species (S). The pooled mean density (individuals m^{-2}) and 2 SE are given and the differences in densities are tested with *t* tests ($^{**}P < 0.025$, $^{*}P < 0.05$). Pooled dissimilarity contribution and mean-densities are given for two functional groups. Only species contributing more than 4% to the dissimilarity are included in the table.

TABLE 3.

ANOSIM analyses of epifauna species composition and density.

	Similarity of Treatment Stations (%)	Data for Analysis of Fishery Impact	Fishery Impact Global R	Fishery Impact *P* (%)
Test area				
Short-term impact Logstor Br.	0.1–2	Complete set of data	0.224	0.1
Short-term impact Agero	11–41	Complete set of data	0.066	0.2
Long-term impact Løgstør Br.	0.1	Average ranking	−0.093	77.1
Long-term impact Agero	0.1	Average ranking	0.363	2.4
Test on functional groups				
Mobile species Løgstør Br.	4–38	Complete set of data	−0.082	94
Sessile species Løgstør Br.	19–25	Complete set of data	0.3	0
Mobile species Agero	0.1–2	Complete set of data	0.016	36
Sessile species Agerø	0.1–91	Complete set of data	0.039	4

The data in each area was analysed in two steps. First it was tested if there was difference among stations within each treatment area (fished contra closed), and if there were differences ($P < 1\%$) then the analysis of the fishery impact was conducted on average rankings. If no difference was observed, then the impact analysis was conducted on complete sets of data.

TABLE 4.

Long-term impact: species contributing to the dissimilarity (%) between fished stations (6, 8, 9, 24) and control stations (1, 5, 7, 10, 11) in a closed area in the Agero area.

		Dredged		Control		
	Dissimilarity (%)	Mean	2 SE	Mean	2 SE	*t* test *P*
Hinia reticulata	28	3.90	0.66	13.20	1.71	0.00**
Crepidula fornicata	22	0.00	0.00	62.40	20.98	
Crangon crangon	11	1.53	0.41	3.79	0.79	0.00**
Ascidiella aspersa	7	0.00	0.00	5.28	2.50	0.01**
Metridium senile	6	0.10	0.11	2.32	0.70	0.00**
Carcinus maenas	5	0.67	0.27	2.51	0.58	0.29

The mean density (individuals m^{-2}) and 2 SE are given and the differences in densities are tested with *t* tests ($^{**}P < 0.025$, $^{*}P < 0.05$). Only species contributing more than 4% to the dissimilarity are included in the table.

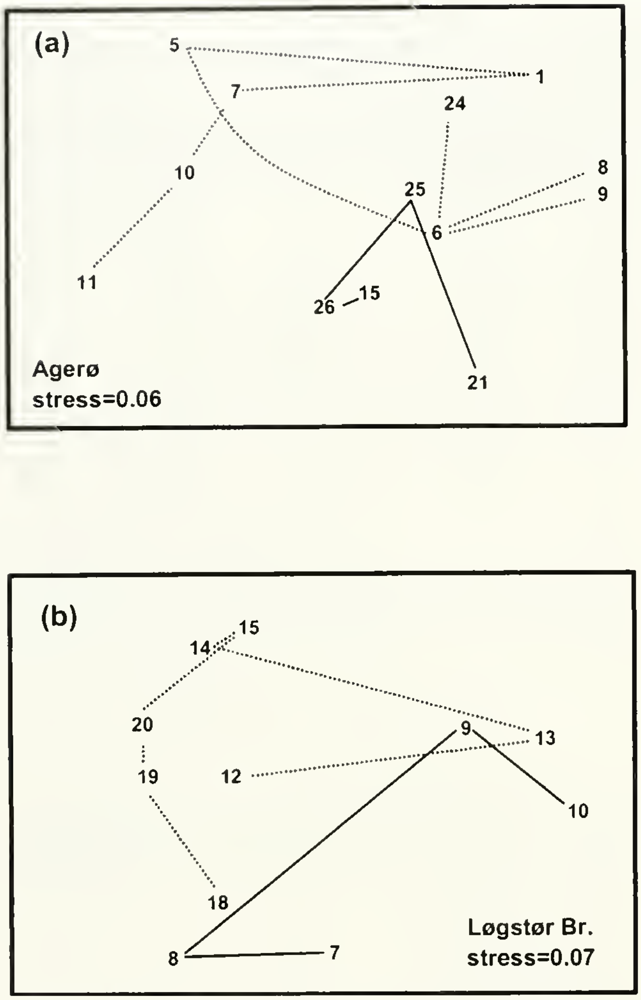

Figure 3. MDS plot of station means in the Agero area (a) and Løgstør Broad (b). The spatial relationship between stations is indicated with a dashed line between the nearest stations in the long-term study areas, and with a solid line in the short-term study areas.

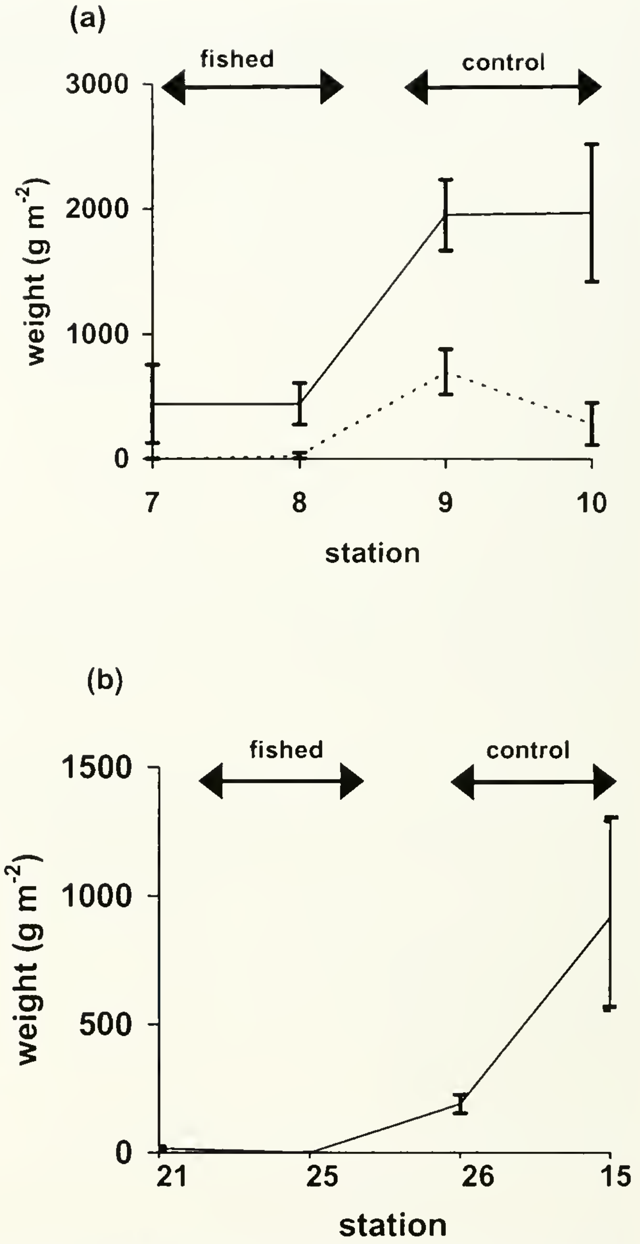

Figure 4. The weights of shell debris (solid line) and gravel (dashed line) at two fished stations and two stations in a closed area in Løgstør Broad (a) and the Agero area (b). The weights are given as mean ± SE.

design (Before-After-Control-Impact) that also tests for temporal and spatial variability in a study area. It is important for the results of experimental impact studies of trawling and dredging that impacted areas are large (Thrush et al. 1995). A meta-analysis of the impact of demersal fishing gear on benthos (Collie et al. 2000) demonstrated that the recovery time is shorter in small impacted patches because of larger edge effects. The results of experimental studies may then be strongly dependent on the experimental design. This study investigated the impact of an unplanned mussel fishery, in which the scale of the study area is very large compared with the experimental studies. This ensures that the results are not affected by the experimental design. Contrary, the character of the study implies that a causal relation between the observed variations in species and substrate compositions and fishery impact not can be established, although the study represents strong indices of the impact of dredging.

Fishery impacts may significantly affect ecosystem function. Some of the species that were partly or fully eliminated from fished areas were sessile filter feeders (poriferans, anthozoans, molluscs, and ascidians). In shallow-water bodies such as Limfjorden, these filter feeders exert an important control mechanism over the pelagic primary producers (Cloern 1982, 1991, Petersen & Riisgård 1992, Dolmer 2000) and reduction of a benthic filtration capacity may weaken bentho-pelagic coupling. Apart from the filtration capacity, the coupling between the pelagia and benthos depends on the mixing rates of the water column and the transport rates of material and energy from the water column to the benthos. Seabed roughness is a determinant of near-bed mixing (Herman et al. 1999), so smoothing of the seabed by removing mussel beds and larger particles reduces the transport of particles to the seabed and reduces the population filtration rates. Consequently, the fishery interferes with benthic filtration both by removing filter feeders and by changing near-bed hydrodynamics.

The distribution of blue mussels was patchy, and in Løgstør Broad the density of mussels in the fished area was slightly higher than in the permanently closed area. This suggests that the density

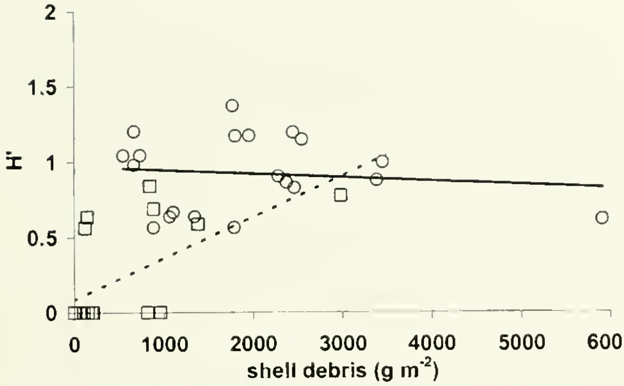

Figure 5. Relationship between the amount of shell debris and Shannon-Wiener indices for each sample at stations 7–10 in Løgstør Broad. (□) indicates samples from the area open to mussel dredging and (○) indicates closed areas. Data were analyzed with linear regression. The dotted line shows the regression on data from the fished area (R^2 = 0.385; $P < 0.01$), and the solid line that on data for the closed area (R^2 = 0.017; $P > 0.05$).

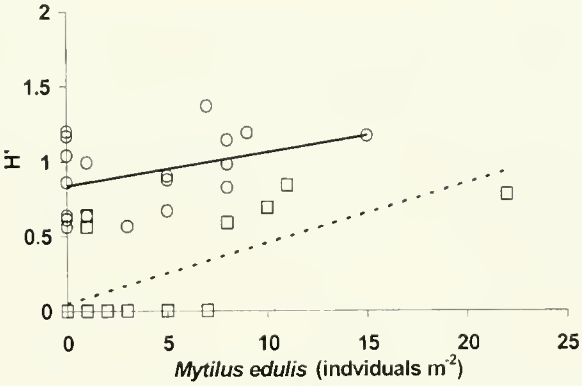

Figure 6. Relationship between density of blue mussels and Shannon–Wiener indices for each sample at stations 7–10 in Løgstør Broad. (□) indicates samples from the area open to mussel dredging and (○) indicates closed areas. Data were analyzed with linear regression. The dotted line shows the regression on data from the fished area (R^2 = 0.470; $P < 0.01$), and the solid line that on data for the closed area (R^2 = 0.152; P = 0.09).

of mussels was much higher in the fished area than in the closed area before the fishery was initiated. As the mussels form a biogenic substratum important for associated fauna, mussel density may influence the distribution of other species. A higher initial mussel density in the area open to mussel dredging, as in the short-term study area in Løgstør Broad, would result in an underestimate of the impact of mussel dredging, as a more diverse associated fauna probably characterized the fished area before the fishery was initiated.

In Løgstør Broad, no long-term impact of mussel dredging was apparent. A previous study also failed to show long-term effects (Hoffmann & Dolmer 2000). In the present study, the multivariate analyses of long-term impacts was conducted on average ranked data resulting from differences among stations within each treatment area. The power of this analysis is much lower than an analysis of the complete data set, which can be used when no difference is observed among stations. To detect an impact of mussel dredging fishery impact must be separated from the noise of other factors affecting the ecosystem (Jennings & Kaiser 1998). Limfjorden is almost bi-annually perturbed by extensive oxygen depletion lasting for several weeks resulting in mass mortality of mussels and other benthic invertebrates. In 1994 and 1997 oxygen depletion caused mortalities of 25–50% of the mussel populations. The scale of these events has to be considered when evaluating the impact of the mussel fishery.

Seabed heterogeneity and complexity is an important feature when discussing the character of benthic habitats. This study has shown that mussel dredging removes larger sessile animals, shell debris, and gravel from fished areas. Furthermore, dredging removes mussels forming an important biogenic substratum for associated fauna. The investigations measured the largest impact on sessile species, but the fishery also affected mobile species after 4 mo. Mussel and shell beds are reported to reduce predation by creation of spatial refuges (Thiel & Dernedde 1994, Lee & Kneib 1994) and Kraeuter and Castagna (1977) reported that *Mercenaria mercenaria* had a 75% better survival when reseeded on shell and gravel substrata than on sand. The impact on epibenthic species is 2-fold: a direct impact of mussel dredging by killing and injuring individuals by direct contact with the mussel dredge and an effect from the changed habitat heterogeneity and complexity. The recovery of the fished habitats is a function of immigration of mobile species and recruitment of sessile and mobile species. Recruitment may also be affected by the changed seabed structure. In many invertebrates larval settlement depends on solid substrata (Young 1983, 1985, Witman & Suchanek 1984, Baker 1997, Lapointe & Bourget 1999) but also changed near-bed hydrodynamic forces may modify larval distribution (Butman 1987, Jonson et al. 1991).

Changing the handling of benthic animals and materials in the fishery by-catch can accelerate the recovery process of ecosystems following mussel extraction. Today mussels below the legal minimum size (<4.5 cm shell length) and waste material (other animals and shell debris) are relayed in certain areas (Kristensen & Lassen 1997). Mussel shells from the cooking process must not be recycled to the seabed and are deposited or used on land. As a consequence of this procedure, important materials are transported away from the fishing grounds. These important materials should be brought back to fished areas. Habitat restoration by relaying small mussels and other invertebrates, shell, pebbles, and larger stones after mussel dredging would reduce the recovery-time both in respect of the form and the function of the ecosystem and would improve the sustainability of the mussel fishery.

The conclusion of this study is that the mussel dredging exerts a significant short-term impact on the benthic fauna. No unequivocal long-term impact could be demonstrated. The fishery changes the seabed structure by removing solid particles and biogenic particles. This impact may be long lasting or even irreversible and may significantly change the function of the ecosystem.

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FOULING IN SCALLOP CULTIVATION: HELP OR HINDRANCE?

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ABSTRACT We examined the effects of fouling on physiochemical and food conditions inside nets used for suspended great scallop (*Pecten maximus*) cultivation. Conditions in clean nets and sites with no nets were similar for the parameters studied and differed from those in fouled nets. Fouling of nets reduced water movement and, contrary to common assumptions, was associated with high levels of plankton and detritus. Obvious negative effects of fouling (e.g., accumulation of inorganic matter or nitrate and ammonia) were absent. It was concluded that in some areas negative effects of fouling may be caused by foulers parasitising or mechanically interfering with scallops rather than creating an unfavorable environment. Our results have important consequences for scallop growers, researchers modelling cultivation in an ecosystem context, and those investigating relationships between the growth of cultivated scallops and environmental conditions.

KEY WORDS: scallop, aquaculture, biofouling, *Pecten maximus*, suspended culture, pearl nets, environmental conditions, particulate matter, Irish Sea

INTRODUCTION

Immersion in plankton-rich water generally enables scallops in suspended cultivation to grow faster than on the seabed, under natural conditions (MacDonald & Thompson 1985, Wallace & Reinsnes 1985, Hardy 1991). Unfortunately, the conditions that promote scallop growth also encourage fouling of cultivation nets and scallop shells. This is costly because it increases the weight and drag of cultivation equipment and is difficult to remove (Hardy 1991). Fouling can also affect scallop growth and appearance.

Fouling of scallop shells and cultivation nets has been shown to reduce the growth of immature scallops (Claereboudt et al. 1994a, Lodeiros & Himmelman 1996, 2000). It is assumed that fouling organisms reduce scallop growth by competing for food and space or by reducing water flow through nets, and hence the supply of food and oxygen and removal of waste products (Duggan 1973, Leighton 1979, Huguenin & Huguenin 1982, Côté et al. 1993, Enright 1993, Claereboudt et al. 1994b, Lodeiros & Himmelman 1996, Lu & Blake 1997). Fouling of scallop shells increases the weight of upper valves and can bind upper and lower valves together. This increases mortality or reduces growth, probably because scallop feeding and respiration is inhibited (Paul & Davies 1986, Minchin & Duggan 1989, Roman 1991, Lu & Blake 1997, Lodeiros & Himmelman 2000). Other potentially negative effects of fouling include irritation of the scallop mantle (Getchell 1991, Mortensen et al. 2000) and parasitism by species, including polychaete worms and amphipods (Leibovitz et al. 1984, Mortensen et al. 2000). However, the influence of fouling varies. Some researchers have concluded that fouling does not affect bivalve growth (Wallace & Reinsnes 1985, Widman & Rhodes 1991, Lesser et al. 1992, Lodeiros et al. 1993, Lodeiros et al. 1999), whereas other work suggests that fouling can have beneficial effects (Ross 2002). Potentially beneficial effects include a positive influence on plankton abundance (invertebrate assemblages release nutrients, promoting primary production, Dame & Dankers 1988, Asmus & Asmus 1991, Peterson & Heck 1999, Arzul et al. 2001, Mazouni et al. 2001) and, in areas of high flow, reduction of fast water currents that might otherwise inhibit scallop feeding (Côté et al. 1993, Devaraj & Parsons 1997).

The present study aimed to determine how fouling on pearl nets alters the environment for great scallops (*Pecten maximus* (L.)). Divers collected water samples from clean and fouled nets (both containing scallops) and from sites with no nets (i.e., the water column nearby), enabling us to isolate the influence of fouling on physiochemical and food conditions. This is the first investigation to examine environmental conditions inside bivalve cultivation nets and thus provides a unique description of how fouling could affect scallop growth. Fouling is typically countered by frequent net cleaning with high-pressure water hoses or regular net changes (Hardy 1991, Laing & Spencer 1997). Both methods are labor intensive, increase equipment requirements, and are thought to stress scallops, reducing their growth rates (Wildish & Kristmanson 1988, Parsons & Dadswell 1992, Enright 1993, McDonough 1998, Ross 2002). The results of this experiment should help growers to tackle fouling efficiently, thereby saving time and money. The study was conducted in an exposed Irish Sea location off the Isle of Man. However, the major foulers (hydroids and amphipods) are ubiquitous (see e.g., Hidu et al. 1981, Arakawa 1990, Enright 1993, Claereboudt et al. 1994a) and thus the results are likely to be relevant where scallop cultivation is performed in other areas with high water flow.

MATERIALS AND METHODS

Field Work

Two longline systems (subsequently referred to as the north and south systems) were positioned off the Southwest coast of the Isle of Man in approximately 23 m of water (Fig. 1). Longline head-ropes were approximately 10 m below the sea surface. The tidal range in this area is 6 m and peak flows are approximately 1 m/s. Experiments were performed in June 2000 during spring tides. Gross tidal flow runs parallel to the systems, but nearby rocky outcrops cause erratic local flow patterns that sometimes extended to the south system. Salinity is 34 ppt, and water temperatures reach a summer maximum of about 15°C and winter minimum of 6°C (T. Shammon, personal communication 2001). To the north east of the longlines untreated sewage from Port Erin (population ca. 2,800) is discharged in the lower intertidal (Fig. 1).

Pearl nets were hung in strings of three, with a 2-kg weight attached below the lowest net. The nets had a plastic covered square wire frame base with sides of 34 cm and black, monofilament mesh with 16 mm^2 spacing. The mesh is the same as that

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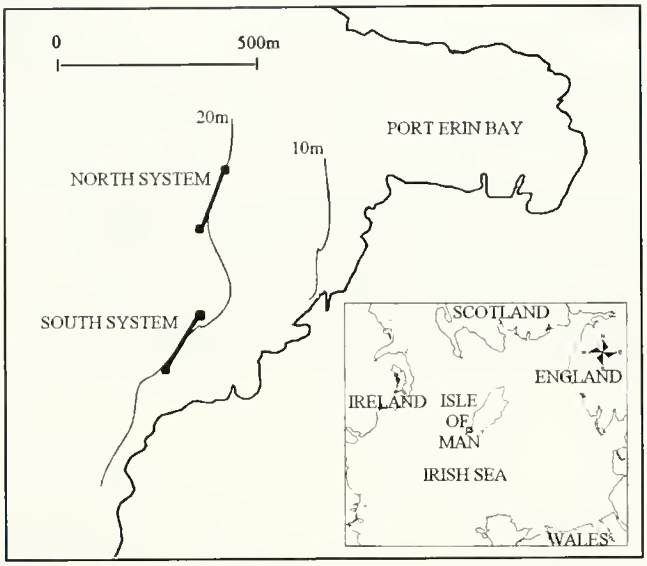

Figure 1. Location of longline systems off the Isle of Man, Irish Sea.

commonly used for lantern nets, and thus the results of these investigations are probably relevant for on-growth in both pearl and lantern nets. For logistical reasons only the top nets, hung about 0.15 m below the head-rope, were sampled. The nets used for nutrient samples contained ten 2–3 yr old *P. maximus* with a shell length of 65–85 mm. For water flow experiments, nets contained 10 flat pebbles whose combined weight equalled that of 10 scallops. Pebbles were used instead of scallops because scallop movement may have abraded the plaster balls, leading to inaccurate estimates of water motion. Scallop-sized pebbles were chosen so that water flow and net movements matched those of nets containing scallops. Nets for flow measurements had loops in the central, supporting rope, and a door so that plaster balls could be inserted and fixed centrally. Door fastenings and support were on net seams so that they did not alter water flow.

Ammonia, nitrate, particulate matter, plankton, and water motion were measured in clean nets, fouled nets, and open water sites. Fouled nets had been immersed for 16 weeks before the sampling whereas "clean" nets had been deployed for only 2 wk. Open-water sites were positions under the head-rope of the longline, at the same depth as experimental nets. Treatments were arranged randomly, at 1-min intervals, along the two systems. On each system, treatments were replicated five times for nutrient experiments and four times for water flow experiments. Nutrient and water motion experiments were performed side by side on the longlines. Water motion was measured for 48 h, during which time water samples were collected.

Diving was performed from the R.V. Sula. Syringes with 120-mm Teflon tubing tips (3-mm diameter) were used to collect samples in preference to permanent sampling tubes (which would have become fouled) or electronic probes, which are difficult to use accurately *in situ*. Divers collected a complete set of 15 or 20 nutrient samples (for example, all of the ammonia samples from one longline) using labeled syringes. To prevent disturbance, nets were not touched or moved either before or during sampling. To remove any trapped debris from the syringe tip, 5 mL of water was taken up outside the nets and expelled once the tip was in position. Water samples were then collected slowly to minimize disturbance and to avoid sampling water from outside the net. Dives lasted a maximum of 20 min, after which samples were returned to the boat. Samples were collected from nets by two pairs of divers deployed at 10-min intervals. Sampling was alternated between longlines so that no more than two samples, totalling 160 mL of water (1% of the net volume), were taken from a net in 4 h. Before sampling, all bottles and syringes were washed in dilute acid and rinsed in distilled water.

Physiochemical Conditions

Ammonia and nitrate were measured in 100-mL water samples, which were kept in the dark on ice during the short boat journey back to the laboratory. In the laboratory ammonia and nitrate samples were filtered, through GF/F papers, into bottles and frozen. An Alpkem autoanalyser (RFA 2) was later used to determine nutrient concentrations.

Plaster of Paris spheres can be used to accurately measure time integrated water motion (Thompson et al. 1994); in these experiments, they proved to be a reliable alternative to expensive micro-flow meters. The spheres were made by combining 100 g of Plaster of Paris ($CaSO_4$) with 90 mL of distilled water. The plaster was mixed to a smooth paste, tapped to remove air bubbles, and then poured into moulds. Moulds were plastic spheres (70-mm diameter) with a central wire. Filled moulds were vibrated for 10 min to remove trapped air. Plaster spheres were removed from their moulds after approximately 12 h and placed in a well-ventilated area to dry. After at least 4 wk, spheres were dried at 30°C to a constant mass (accelerated drying at high temperatures can affect the crystalline structure of $CaSO_4$ (Muus 1968).

Before immersion, plaster spheres were wrapped in soft cloths to prevent chipping and to minimize dissolution. Divers opened nets and fixed spheres centrally so that they were not abraded by contact with fouling organisms or nets. At open water sites, wire was used to suspend spheres below the head-rope, at the same level as spheres inside nets. Once in position, the cloths were removed and the nets were resealed. After 48 h, divers retrieved spheres and wrapped them in soft cloths before returning to the boat. In the laboratory, the spheres were dried to a constant mass and their final surface area was calculated from volume measurements obtained by fluid displacement.

Plaster dissolution rates (V_d) provide an indication of relative water motion:

$$V_d = \frac{(W_1 - W_2)}{AT}$$

Where W_1 and W_2 are the weight of the sphere at the beginning and end of the experiment respectively, A is the mean surface areas of the sphere, calculated from start and end values, and T is the time over which spheres were immersed (Thompson et al. 1994).

Food Conditions

Water samples (100 mL) for particulate matter analysis were kept in the dark on ice during the boat journey back to the laboratory. Particulate matter was filtered onto preashed papers immediately on return to the laboratory. The papers were rinsed with isotonic ammonium formate and then dried at 40°C to a constant weight and ashed overnight at 450°C. The GF/F filter paper used to collect particulate matter had a pore size of 0.7 μm. Particulate organic matter (POM), particulate inorganic matter (PIM), and total particulate matter (TPM) were calculated as follows:

$$\begin{aligned}\text{POM} = {} & \text{dry weight of filter paper and sample} \\ & - \text{ashed weight of filter paper and sample}\end{aligned}$$

TPM = dry weight of filter paper and sample
– filter paper ashed weight
PIM = ashed weight of filter paper and sample
– filter paper ashed weight

As soon as the water samples were taken up to the boat, 60-mL samples were transferred to bottles with 1.2 mL of neutral Lugol's iodine. The bottles were stored in the dark until plankton were counted, measured, and categorized using inverted microscopy and the computer programme SCION image analysis for Windows. Samples were settled in a counting chamber following the methods of Utermöhl (Hasle 1978). Dense samples were diluted with filtered seawater so that all of the plankton in the chamber could be counted. To ensure that the precision of plankton counts was greater than 20% of the total count, the volume of sample enumerated always contained more than 150 individuals of the most abundant organisms (Postel et al. 2000). Organisms were recorded according to type (small plankton, centric diatoms, pennate diatoms, diatom chains, solitary chain-forming diatoms, pelagic ciliates, benthic ciliates, dinoflagellates, flagellates, crustaceans, nematodes, invertebrate larvae, and eggs and spores) and maximum length (5–10, 11–20, 21–50, 51–100, and >100 μm). Small plankton were all organisms of 50–10 μm; generally these were flagellates and diatoms.

Statistical Analyses

The experimental design was balanced; location was a random factor with two levels, north and south systems, and treatment was a fixed factor with three levels, open-water sites, clean nets, and fouled nets (Underwood 1997). Physiochemical conditions were measured in five replicates per treatment for each system, but time constraints meant that plankton data were obtained only for three replicates per treatment-longline combination. Concentrations of plankton and nutrients and rates of plaster erosion for each treatment were examined using two-way analysis of variance (ANOVA). Heterogeneity of variance was tested for using Cochran's test (Winer 1971) and where necessary data were transformed. Some data were heterogeneous even after transformation, but ANOVA was still applied because the experimental design was balanced and large (Underwood 1997). However, such analyses increase the probability of a type I error, and therefore significant results should be interpreted with caution. When ANOVA showed that the probability of a treatment effect exceeded 0.05 and there was no interaction between location and treatment ($P > 0.25$), data for the two systems were pooled, thus increasing the power of ANOVA to detect treatment effects (Underwood 1997). When ANOVA indicated significant factors or interactions between factors, post-hoc Student–Newman–Keuls tests were performed to determine which means differed. All analyses were performed using GMAV5 (Underwood et al. 1998). Ammonia measurements were analyzed by ANOVA and concentrations below the limit of detection were included as 5 μg/L, the highest undetectable value. This conservative approach increased the probability of type II error.

As described above, plankton were classified as one of 45 groups according to their size and type. The data set was then analyzed using nonparametric, multivariate techniques included in the PRIMER (Plymouth Routines in Multivariate Research) software package (Clarke & Warwick 1994). Bray-Curtis similarity indices (Bray & Curtis 1957) were calculated between all pairs of samples to produce a data matrix (after a square-root transformation was used to slightly reduce the contributions to similarity of the most abundant species). The similarity matrices were ordinated and clustered using non-metric multi-dimensional scaling (MDS) and hierarchical agglomerative clustering (on group-average linkage), respectively (Clarke & Warwick 1994). The two-dimensional MDS plot had a low stress value and hence the dendrogram from CLUSTER analysis is not presented here. Instead, levels of similarity from cluster analysis are indicated on the MDS plot (Fig. 4). *A priori* tests of the differences between locations and treatments were performed using a two-way, crossed ANOSIM (analysis of similarity), and the plankton groups contributing most to any differences found between the groups were determined using

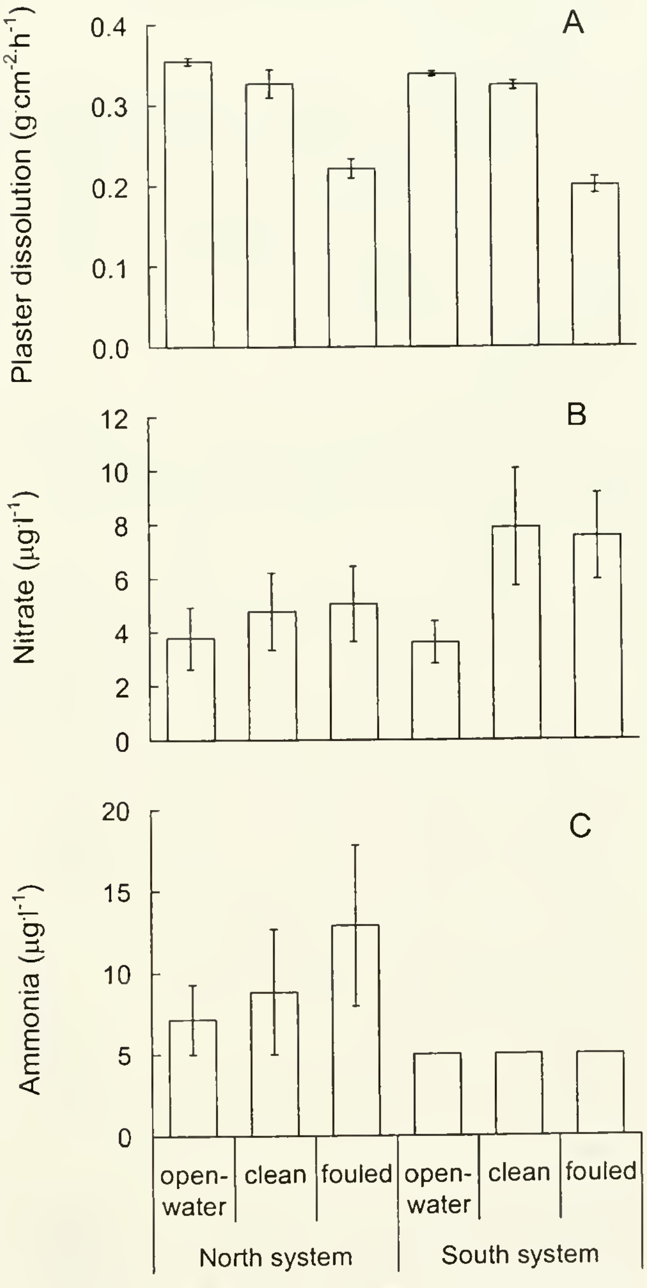

Figure 2. Physiochemical conditions. A, Water motion; B, nitrate concentrations; C, ammonia concentrations (mean ± SE) in clean and fouled pearl nets and open-water sites.

SIMPER (similarity percentages analysis, Clarke & Warwick 1994).

RESULTS

Macrofouling Communities

Fouled nets were almost entirely covered (>90%) in the hydroids *Tubularia indivisa* L. and *T. larynx* Ellis & Solander; the amphipod *Jassa falcata* (Montagu) and its silt tubes were common as were the nudibranchs *Dendronotus frondosus* (Ascanius), *Coryphella lineata* (Lovén), and *Facelina bostoniensis* (Couthouy). Small hydroids (e.g., *Obelia* sp. and *Clytia hemispherica* (L.)) were present but occupied little space compared with the large *Tubularia* spp. "Clean" nets were sparsely colonized (<5%) by small hydroids.

Physiochemical Conditions

The plaster spheres used to measure water motion remained spherical throughout their deployment. Water motion was similar on both longline systems (Fig. 2) and was only significantly reduced by fouled nets (Table 1). Nitrate and ammonia concentrations were not affected by treatment (Fig. 2). However, only four ammonia measurements exceeded the minimum detection level of the autoanalyser (5 μg/L); these were all on the north system, which therefore had significantly higher rates of dissolution than the south system (Table 1).

Food Conditions

Total particulate matter was most abundant on the south system and here there was significantly more in fouled nets compared with clean nets and open-water sites (Table 2). Although this trend was apparent on the north system (Fig. 3), differences were smaller and not significant. The ratio of PIM to POM was lowest in fouled nets, for both north and south systems (Fig. 3, Table 2). Microscopy revealed that particulate matter included detritus and invertebrate faeces, in addition to plankton.

Plankton larger than 21 μm were rare and thus numbers in the three largest size classes were pooled for univariate analysis. Total plankton and plankton in size classes 5–10 μm and 11–20 μm were most abundant in fouled nets (Table 2). Multivariate analysis also distinguished fouled nets from clean nets and open water sites (which clustered together on the MDS, Fig. 4). Interestingly fouled nets from the two systems were also distinct. ANOSIM revealed significant differences between both locations and treatments ($R = 0.42$, $P < 0.01$ and $R = 0.57$, $P < 0.01$, respectively). Pairwise comparisons found significant differences between fouled nets, open-water sites ($R = 0.78$, $P = 0.01$), and clean nets ($R = 0.89$, $P = 0.01$), but, as indicated by the MDS plot, open-water sites, and clean nets, contained similar communities ($R = 0.278$, $P = 0.08$). SIMPER analysis showed that high abundances of centric diatoms, flagellates, pennate diatoms (all 5–10 μm), and small plankton primarily distinguished fouled nets from clean nets and open water sites. High abundances of these organisms also distinguished samples from north and south systems. Although less common, eggs and spores, nematodes, pennate diatoms, and invertebrate larvae also appeared most frequently or uniquely in fouled nets. Consistently low plankton abundance explains the atypical position of one sample from fouled nets on the MDS plot (Fig. 4): despite its low content, this replicate contained most of the plankton that characterized other samples from fouled nets.

DISCUSSION

Fouling of cultivation nets created a unique environment for the scallops inside; physiochemical and food conditions differed from those both in clean nets with scallops and those in the water

TABLE 1.

Two-way ANOVA and Student–Newman–Keuls multiple comparisons testing for the effect of location and treatment (clean nets, fouled nets, and open-water sites) on physiochemical conditions.

Source of Variation	df	MS	F	P	F Ratio Versus
Water motion					
$C = 0.4799$, $P > 0.05$					
Location	1	0.0012	3.18	0.091	Residual
Treatment	2	0.0425	196	**0.005**	Location × treatment
Location × treatment	2	0.0002	0.57	0.575	Residual
Residual	18	0.0004			
Total	23				
SNK multiple comparison of treatment results:			North system: open-water = clean > fouled		
Nitrate					
$C = 0.3539$, $P > 0.05$					
Location	1	0.1242	2.22	0.149	Residual
Treatment	2	0.1141	2.91	0.256	Location × treatment
Location × treatment	2	0.0392	0.70	0.506	Residual
Residual	24	0.0558			
Total	29				
Ammonia					
$C = 5574$, $\mathbf{P < 0.05}$					
Location	1	160.5453	4.41	**0.046**	Residual
Treatment	2	21.8963	1.00	0.500	Location × treatment
Location × treatment	2	21.8963	0.60	0.556	Residual
Residual	24	36.3827			
Total	29				

Cochran's test results are given. (Bold type indicates a significant result, $P < 0.05$).

TABLE 2.

Two-way ANOVA and Student–Newman–Keuls multiple comparisons effect of location and treatment (clean nets, fouled nets, and open-water sites) on particulate matter and plankton.

Source of Variation	df	MS	*F*	*P*	*F* Ratio Versus
TPM					
$C = 0.3013$, $P > 0.05$					
Location	1	1040	7.08	**0.014**	Residual
Treatment	2	2378	4.35	0.187	Location × treatment
Location × treatment	2	547	3.73	**0.039**	Residual
Residual	24	147			
Total	29				
SNK multiple comparison of interaction:			Open-water: North system = South system		
North system: open-water = clean = fouled			Clean: North system = South system		
South system: open-water = clean < fouled			Fouled: North system < South system		
PIM:POM					
Transformation = Ln (X + 1), $C = 0.4331$, $P > 0.05$					
Location	1	0.0003	0.00	0.944	Pooled data
Treatment	2	0.3369	5.61	**0.001**	Pooled data
Location × treatment	2	0.0293	0.49	0.620	Pooled data
Residual	24	0.0627			
Total	29				
Pooled data	26	0.0601			
SNK multiple comparison of treatment results:			Open-water = clean > fouled		
Plankton 5–10 μm					
Transformation = Ln (X), $C = 0.7020$, $P <$ **0.05**					
Location	1	0.15	0.17	0.686	Residual
Treatment	2	11.44	22.56	**0.042**	Location × treatment
Location × treatment	2	0.51	0.58	0.576	Residual
Residual	12	0.88			
Total	17				
SNK multiple comparison of treatment results:			Open-water = clean < fouled		
Plankton 11–20 μm					
Transformation = Ln (X + 1), $C = 0.7042$, $P <$ **0.05**					
Location	1	0.15	0.18	0.678	Pooled data
Treatment	2	11.34	13.88	**0.001**	Pooled data
Location × treatment	2	0.50	0.61	0.559	Pooled data
Residual	12	0.87			
Total	17				
Pooled data	14	0.82			
SNK multiple comparison of treatment results:			Open-water = clean < fouled		
Plankton 21+ μm					
Transformation = Ln (X + 1), $C = 0.5244$, $P > 0.05$					
Location	1	0.97	7.26	**0.020**	Residual
Treatment	2	1.85	5.24	0.160	Location × treatment
Location × treatment	2	0.35	2.66	0.111	Residual
Residual	12	0.13			
Total	17				
Total plankton (>5 μm)					
Transformation = Ln (X + 1), $C = 0.4092$, $P > 0.05$					
Location	1	279000	1.25	0.283	Pooled data
Treatment	2	16300000	7.29	**0.007**	Pooled data
Location × treatment	2	2890000	1.29	0.305	Pooled data
Residual	12	2130000			
Total	17				
SNK multiple comparison of treatment results:			Open water = clean < fouled		

The results of Cochran's test are given. (Bold type indicates a significant result, $P < 0.05$.)

nearby. Other work has indicated that sometimes scallops in fouled nets grow faster than scallops in clean nets (Ross 2002). High water flow can inhibit scallop feeding (Wildish & Saulnier 1993, Claereboudt et al. 1994b, Skjaeggestad 1997) and it could be that, in a high current area, heavy fouling aids scallop feeding by roughly halving water movement. Similarly, Skjaeggestad (1997) found that water-motion inside plastic cages was reduced by up to 68% by fouling. In addition, fouling might prevent seston depletion around scallops by creating turbulent flow and actively mixing the water (Fréchette et al. 1989, Larsen & Riisgård 1997).

Ammonia and nitrate concentrations were similar for all treatments, indicating that neither clean nor fouled nets caused a build

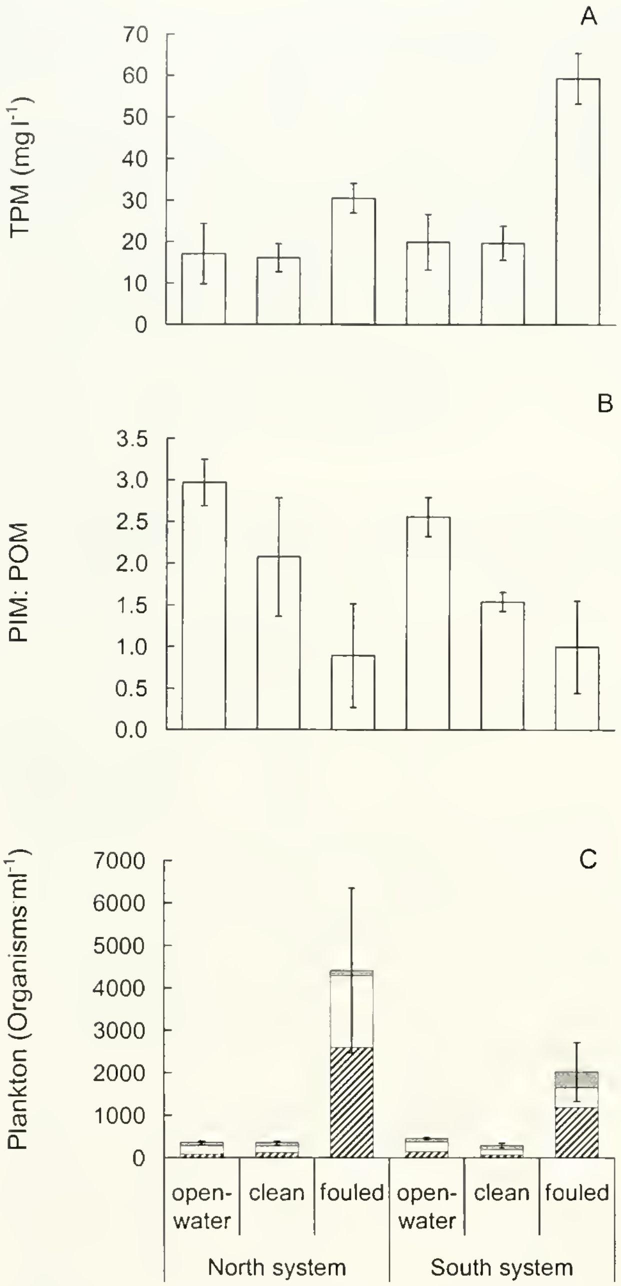

Figure 3. Food conditions. A, Concentrations of total particulate matter (TPM); B, ratios of inorganic to organic particles (PIM:POM); C, concentrations of 5–10 μm (hatched areas), 11–20 μm (white areas), and >21 μm (grey areas) plankton (mean ± SE), in clean and fouled pearl nets and open-water sites.

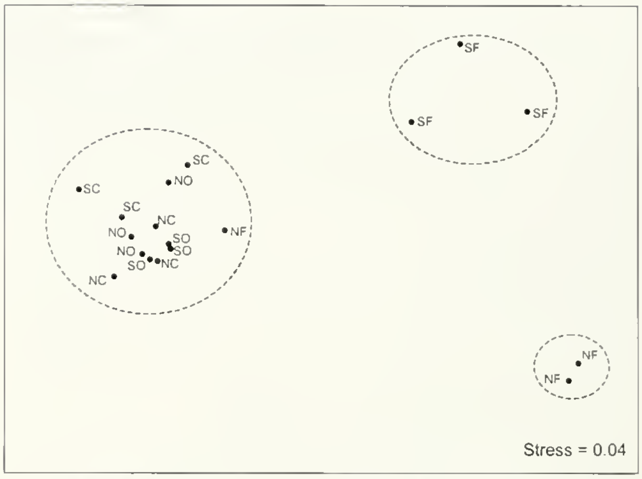

Figure 4. MDS ordination of Bray–Curtis similarity matrix for square-root transformed plankton-abundance-data. Samples are labelled with their location: north system (N), south system (S), and treatment: clean nets (C), fouled nets (F), and open-water sites (O). Samples are grouped at a 70% level of similarity from CLUSTER analysis.

up of decay or excretory products. A few apparently high ammonia measurements may have been caused by the capture of detritus in water samples (kelp detritus was abundant and the northern longline was close to a sewage outfall pipe). Inorganic nitrogen is absorbed by phytoplankton and it is possible that high numbers of phytoplankton reduced levels of nitrate and ammonia in fouled nets. In finfish cages, fouling can reduce water exchange, leading to oxygen depletion, fish mortality, or reduced growth (Cronin et al. 1999). Similarly, reduced oxygen levels have been invoked to explain reductions in scallop growth when fouling is heavy (Huguenin & Huguenin 1982, Enright 1993, Lu & Blake 1997). However, oxygen concentrations have been found to track the abundance of autotrophic plankton, peaking in fouled nets (Ross 2002). Thus, it seems unlikely that oxygen depletion is a consequence of fouling in scallop net culture in temperate locations with high water flow.

Contrary to previous suggestions (Duggan 1973, Leighton 1979, Huguenin and Huguenin 1982, Côté et al. 1993, Enright 1993, Claereboudt et al. 1994, Lodeiros and Himmelman 1996, Lu and Blake 1997), fouling did not reduce the quantity or quality of food particles available for scallops. Instead, net fouling was associated with abundant plankton and detritus and a favorable PIM/POM ratio. Trends in plankton abundance were strong enough to be identified, despite the low number of replicates and the notoriously patchy distribution of plankton (Hasle 1978).

Proximity to the shore and to a sewage outfall may explain why the study area was characterized by high seston loadings (ca. 20 mg/L); similar loadings were found by Cranford et al. (1998) and Lodeiros et al. (1998) in the sea off Canada and Venezuela, respectively. High levels of organic matter in fouled nets suggest that fouling may trap, produce, and perhaps support the production of organic matter—a potential energy source for scallops. Even at high seston concentrations (15 mg/L) scallop scope for growth can be reduced by dilution of POM by PIM (MacDonald et al. 1998). When seston concentrations are not limiting a low PIM/POM ratio (critical values are between 3.5 and 6; the exact number varies between authors) is required by scallops to maintain a positive energy balance and maximum scope for growth (Vahl 1980, Wallace & Reinsnes 1985, Cranford 1995, MacDonald et al. 1998). In this study, open-water sites had PIM/POM ratios of around three, close to the critical value, and significantly higher than in fouled nets. Enhanced levels of POM in fouled nets could thus prevent high ambient PIM concentrations from depressing scallop growth rates.

Plankton communities were dominated by autotrophs of 5–20 μm, reflecting Graziano's conclusion that 65% of primary production in the northeast Irish Sea is from phytoplankton of 5–20 μm (Graziano 1988). Fouling may have encouraged primary production by releasing nutrients, by retaining plankton in a favorable light environment or by providing a substrate for benthic autotrophs. Increased primary production seems, in turn, to have supported heterotrophic and mixotrophic organisms such as dinoflagellates and ciliates. Although this finding contradicts common assumptions of those interested in shellfish cultivation, ecologists recognize that beds of suspension feeders have the potential to induce the growth of more phytoplankton than they consume (e.g., Asmus & Asmus 1991). This is because suspension feeders increase local inorganic and organic phosphate and nitrate concentrations directly through excretion and indirectly via bacterial decay of their faeces (e.g., Dame & Dankers 1988, Asmus & Asmus 1991, Peterson & Heck 1999, Arzul et al. 2001, Mazouni et al. 2001). A possible mechanism by which suspension-feeding foulers and scallops could produce and retain nutrients is described in Figure 5. This is important because in most marine systems, including the Irish Sea, phytoplankton are likely to be nitrate limited at certain times of year (Allen et al. 1998, Kennington et al. 1999). Mazouni et al. (2001), for example, suggest that during summer months nutrient recycling by oyster culture units may drive primary production in a French lagoon.

Benthic plankton are often suspended by coastal turbulence and generally survive well in the water column (Newell & Newell 1979); thus, the prevalence of benthic ciliates and pennate diatoms in our open-water samples. However, many benthic species were most common in fouled nets where they may have proliferated because of conditions described above or because of the presence of a solid surface onto which they could attach or settle. Benthic organisms might have been suspended by the passage of the sampling syringe, but movement of scallops and water currents are also likely to make them readily available as food for scallops. Increased plankton abundances in fouled nets could promote scallop growth because ambient levels (<600 cells/mL in this study) are unlikely ever to exceed maximum concentrations for scallop uptake or assimilation (ca. 15,000 cells/mL, Cahalan et al. 1989, Skjaeggestad 1997).

Proliferation of phytoplankton in fouled nets indicates that light levels were not reduced below their compensation point, even by thick *Tubularia* fouling. Perhaps strong sunlight in June penetrated the translucent stalks of this hydroid. Fouled nets may also have encouraged plankton growth and reproduction by preventing cells from sinking below the euphotic zone. Future studies might try to measure light attenuation by different fouling communities. Invertebrate larvae (including decapods and echinoderms) and nematodes were only found in fouled nets; although relatively rare, these potential predators and parasites could have deleterious effects on scallop growth and survival (O'Connor et al. 1999, Freites et al. 2000). Net fouling could also be problematic if it promoted the growth of plankton responsible for shellfish poisoning.

This is the first description of how fouling influences the environment inside nets used for shellfish cultivation. The data contradict the common assumption that fouling reduces food levels. Instead, fouling can be associated with increased food availability and does not necessarily encourage a build up of decay products or inorganic matter, even when scallop densities are high. These findings may help to explain why in high current areas scallops inside pearl nets grow faster than scallops outside (Claereboudt et al. 1994b). They also support anecdotal evidence that a degree of fouling promotes the growth of cultivated oysters (Arakawa 1990, Mazouni et al. 2001). It might be inferred that in some areas negative effects of fouling on scallop growth are caused by fouling organisms mechanically interfering with scallops (e.g., binding them in unfavorable positions, inhibiting shell opening or disrupting feeding behavior), or by harboring predators and parasites, rather than altering the environment. Growers of scallops in high current or oligotrophic offshore locations should perhaps strive to reduce the mechanical interference of foulers rather than trying to prevent fouling altogether. Strategies might include biological control, which can keep bivalves clean and free to move, but does not completely remove biofouling (Hidu et al. 1981, Enright et al. 1983, Cigarria et al. 1998, Ross 2002). This approach could benefit scallop growth by reducing mechanical interference whilst retaining the potential food enhancing properties of fouling.

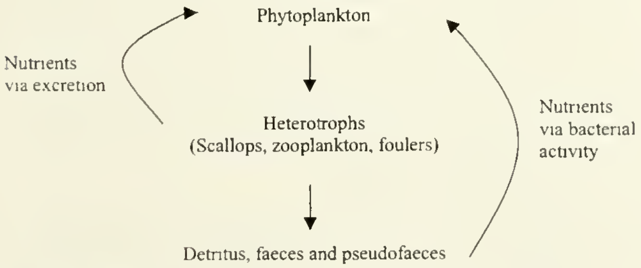
Phytoplankton
Nutrients via excretion
Heterotrophs (Scallops, zooplankton, foulers)
Nutrients via bacterial activity
Detritus, faeces and pseudofaeces

Figure 5. Mechanism by which nutrients might be retained and recycled in fouled pearl nets.

That the environment inside scallop cultivation nets can differ significantly from the water-column also has important consequences for two areas of research. First, studies often relate patterns of scallop growth in suspended culture to environmental conditions (e.g., Wallace & Reinsnes 1985, Côté et al. 1993, Claereboudt et al. 1994a, Emerson et al. 1994, Lodeiros & Himmelman 1994, Thorarinsdóttir 1994, Vélez et al. 1995, Kleinman et al. 1996, Lodeiros et al. 1998, Lodeiros & Himmelman, 2000). This relationship may be better understood if future studies consider the influence of net fouling, or measure conditions inside nets. On a wider scale, water column data have recently been used by researchers assessing the affects of bivalve cultivation on nutrient and seston dynamics of bays, or to predict the capacities of areas for shellfish cultivation (e.g., Penney et al. 2001, Pilditch et al. 2001). Because of its potential to uncouple scallop processes from water-column seston conditions, the influence of net fouling should also be included in such models.

Here we have provided a snapshot examination of how fouling can alter environmental conditions. There is evidence that effects vary with season and the age or composition of the fouling community (Ross 2002). Though difficult, simultaneous assessment of fouling communities, the environment inside nets and scallop growth would enable the influence of fouling to be better understood. We used wide-mesh pearl nets containing intermediate sized scallops in a fast current area. Future work might examine the effects of fouling in low current areas or with the fine mesh nets used for growing spat. Studies to determine how common fouling assemblages (e.g., hydroid, tunicate and bivalve dominated communities) affect the net environment could help growers to choose cultivation sites, depths or methods of fouling control.

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ANNUAL FEEDING CYCLE OF THE PATAGONIAN SCALLOP *ZYGOCHLAMYS PATAGONICA* (KING AND BRODERIP, 1832) IN RECLUTAS BED (39°S–55°W), ARGENTINE SEA

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ABSTRACT This article deals with the diet of the Patagonian scallop, *Zygochlamys patagonica*, during a yearly period at Reclutas bed (39°S–55°W), Argentine Sea, and relates the results with the oceanographic conditions and the growth pattern known for this species. Scallops (n = 180) from six samples were dissected and the gut contents were identified and counted. Results showed a predominance of diatoms in the diet and maximum food ingestion in spring. Maximum somatic (muscle) growth for this species at the same study site was also found during spring. It is suggested that after the spring phytoplanktonic bloom, oceanographic conditions (thermocline in development) allow the sedimentation of food particles to the bottom; this input of energy could produce the somatic growth documented for this filter-feeding species.

KEY WORDS: diet, annual cycle, Patagonian scallop gut contents, *Zygochlamys patagonica*, Argentine Sea

INTRODUCTION

Scallops are suspension-feeding organisms, feeding on detritus and phytoplankton (Bricelj & Shumway 1991). Particles are mainly captured by cilia and mucus on the dorsal infrabranchial surface of the gill and the gill arch thus provides the major capture site (Beninger & Le Pennec 1991). Ciocco (1995) documented the importance of the labial palps in food particle transport to the mouth and also their contribution in preventing reflux and favoring agglutination and particle selection in *Aequipecten tehuelchus* (d'Orbigny). It is known that particle selection may occur at the labial palps and/or gills (Jørgensen 1990; Ward et al. 1997) and also that selection is not only based on particle size but on other important characteristics (Shumway 1985, Shumway et al. 1997). The effective lower limit of particle retention in studied pectinids ranges between 5 and 7 μm, and therefore bacterioplankton (typically between 0.3 and 1 μm; Bricelj & Shumway 1991) and picoplankton are not available as food. Some gut content studies in pectinids showed the importance of benthic and/or tychopelagic algae in the diet of scallops (Vernet de Hall 1977, Shumway et al. 1987, Bricelj & Shumway 1991).

Seasonal growth in bivalves, including scallops, is influenced by environmental changes, especially food supply (Bricelj & Shumway 1991). It is known that food availability is correlated with growth in scallops (Griffiths & Griffiths 1987, Barber & Blake 1991), and that it is possible to find abnormalities in growth during diatom blooms (Lorrain et al. 2000). In addition, Ciocco (1992) concluded that differences in growth between populations of *Aequipecten tehuelchus* were related with environmental factors, such as temperature, depth, and food availability, and not with genetic factors. Valero et al. (2000) studied growth in *Zygochlamys patagonica* (King and Broderip) using samples taken from the Reclutas bed of the Argentine Sea (Fig. 1). They found that maximum growth in shell, muscle and gonad occurred during different months of the year.

The Patagonian scallop, *Zygochlamys patagonica*, is distributed in the Magellanic Biogeographic Province, in the Atlantic Ocean, from 35°S to Tierra del Fuego, and in the Pacific Ocean, up to 42°S, between 40 and 200 m depth (Waloszek & Waloszek 1986, Ciocco et al. 1998). *Zygochlamys patagonica* and *Aequipecten tehuelchus* are the two commercial pectinid species in the Argentine Sea. There are nine Patagonian scallop beds in the Argentine Sea: Two are located in the intermediate shelf between 60 and 70 m depth and seven in the shelf break front area along the 100 m depth isobath (Lasta & Bremec 1995, 1998, 1999; Fig. 1), which is characterized by high productivity (Brandhorst & Castello 1971, Carreto et al. 1981, 1995, Podestá & Esaías 1988). The Reclutas bed is located in the northern zone of this area, where oceanographic seasonal changes occur. During summer, the water column is stratified, and the surface and the bottom layers are separated by a pronounced pycno and thermocline between 30 and 40 m depth. During autumn and winter, the water column is vertically mixed by convective circulation patterns, which result in breakdown of stratification (Guerrero & Piola 1997). Baldoni and Guerrero (2000) provided a more detailed study of the evolution of the temperature vertical structure of the water column in Reclutas bed area during the year.

The objectives of this work were to study the diet of the Patagonian scallop, *Zygochlamys patagonica*, from samples taken at Reclutas bed during an annual period, to detect possible seasonal changes in diet, and to relate these results with the oceanographic conditions in the study area and with the growth pattern of the species.

MATERIALS AND METHODS

Gut contents of 180 scallops (35–50 mm shell height) were analyzed from samples (n = 30) preserved in formaldehyde (10%), taken in June, August, October, and November 1996 and February and March 1997 in Reclutas bed (39°24′S–55°56′W, 100-m depth). The protocol used involved the dissection of gut + digestive gland complex in all sampled scallops. The gut was cut open and the contents were washed in 2 mL of fresh water. A 1-mL subsample of the gut content washed suspension was analyzed in a Sedgwick–Rafter counting chamber that was divided into 7 rows and 17 columns. All particles within six columns were counted and identified under the microscope (100–250×). This quantity repre-

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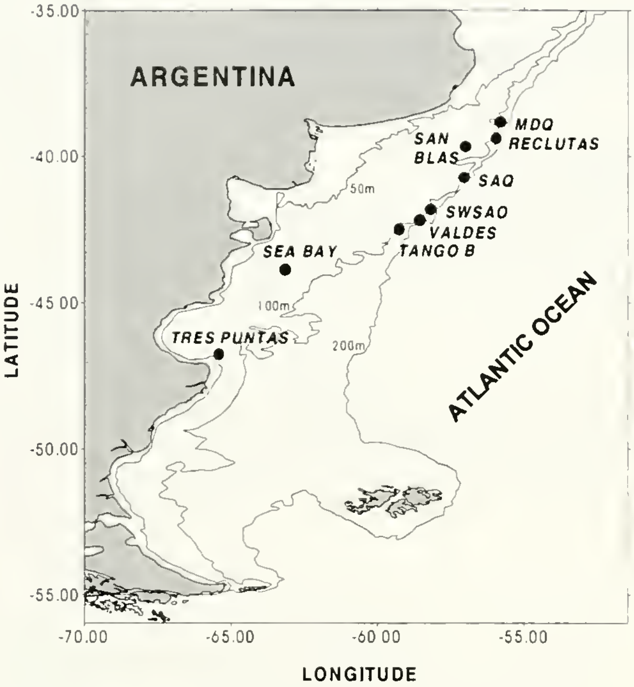

Figure 1. *Zygochlamys patagonica* **beds in the Argentine Sea.**

sents approximately 1/6 of the total gut content. The number of samples and columns studied were statistically tested and the estimated error of the procedure was <16% (Schejter 2000).

Multivariate MANOVA (Morrison 1976), univariate ANOVA, and the Tukey test (Sokal & Rohlf 1979) were used to establish the significance in the observed differences in food groups or species found in gut contents during the study. The significance level was $P = 0.05$ in all cases, but a Bonferroni correction (Morrison 1976) was used when necessary. Data were normalized using the square root transformation; the variance was found to be heterogenous. However, as MANOVA and ANOVA are robust tests when there is a balanced design (Ito & Schull 1964, Ito 1988), it seemed correct to use them in this case in which sample sizes were the same in all months.

The IR index (an index of relative importance and abundance; Bucher & Herrera 1981) was calculated for the main groups and species in gut contents. This index [IR = (Ni/Nt) × (Mi/Mt) × 100] considers not only the total abundance of an item (or group) per month in all guts (Ni/Nt), but also whether the item (group) was present in all guts or just in a few (Mi/Mt).

RESULTS

Twelve species and resting stages of diatoms, five species of dinoflagellates and cysts, two species of silicoflagellates, prasinophytes, and a number of other organisms and remains grouped as miscellanea (tintinnids, foraminifers, nematodes, etc.) were found in gut contents of the Patagonian scallop (Table 1). Particle sizes varied between 19 and 170 μm (mainly 25 to 60 μm), although some crustacean remains (~250 μm) and some small diatoms (<19 μm) were found (Table 2). The majority of items found in gut contents had a planktonic origin. This is the case of dinoflagellates, silicoflagellates, prasinophytes and tintinnids, and most diatom species. Nevertheless, some of them, namely *Paralia sulcata*, are tychopelagic. Dinoflagellate cysts and resting diatom stages are typically benthic.

TABLE 1.

Food items found in ***Zygachlamys patagonica*** **gut contents during the study period at Reclutas bed, Argentine Sea.**

- Diatoms
    - *Paralia sulcata* (Ehrenberg) Cleve
    - *Nitzschia coarctata* Grunow
    - *Nitzschia* sp.
    - *Thalassiosira* spp.
    - *Pleurosigma normanii* Ralfs *in* Pritchard
    - *Thalassionema nitzschioides* (Grunow) Meereschkowsky
    - *Stephanopyxis turris* (Arnott in Greville) Ralfs *in* Pritchard
    - *Biddulphia* sp.
    - *Actinoptychus* sp.
    - Pennate 1
    - Centric 1
    - Centric 2
    - Resting stages
- Dinoflagellates
    - *Dinophysis mawsoni* (Wood) Balech
    - *Dinophysis rotundata* (Claparède *et* Lachmann)
    - *Dinophysis contracta* (Kofoid *et* Skogsberg) Balech
    - *Dinophysis acuminata* (Claparède *et* Lachmann)
    - *Protoperidinium metananum* (Balech) Balech
    - *Gonyaulax digitalis* (Pouchet) Kofoid (cysts)
    - Resting cysts
- Silicoflagellates
    - *Dictyocha speculum* Ehrenberg
    - *Dictyocha fibula* Ehrenberg
- Prasinophytes
    - *Pterosperma* spp.
- Tintinnids
    - *Dictyocysta elegans* var. *lepida* (Ehrenberg) Kofoid *et* Campbell
    - *Dictyocysta elegans* var. *speciosa* Jörgensen
    - *Acanthostomella* sp.
    - Others
- Miscellanea
    - Foraminifera
    - Rotifers
    - Nematodes
    - Nauplii larval stages
    - Invertebrate eggs
    - Copepod spermatophores
    - Radiolarian remains
    - Unidentified remains
    - Polychaete chaetae
    - Sponge spicules
    - Detritus
    - Pollen

The total number of ingested particles varied between 300 and 8,000 per gut and their mean number differed significantly among months ($F_{[5,174]} = 55.859$; $P < 0.001$). Maximum food ingestion was registered in November, and minimum food ingestion periods were October and March (Fig. 2). Total abundance of diatoms, dinoflagellates, silicoflagellates, prasinophytes, foraminifera, and tintinnids differed significantly between months (MANOVA: $F_{[30,678]} = 46.86597$; $P < 0.001$; Fig. 3).

Diatoms were always the most abundant food item (IR = 45–90%, with mean values between 320 and 1450 diatoms per gut) and were present in all guts during the entire sampled period (Fig. 4). The most abundant species was *Paralia sulcata* (maximum values in November: IR = 66%; Fig. 5). This species was present throughout all months and in all guts and was the dominant spe-

TABLE 2.

Size ranges of main food items found in *Zygochlamys patagonica* gut contents from Reclutas bed.

Food Item	Size (μm)
Paralia sulcata	19–180[a]
Thalassiosira spp.	30–55
Nitzschia spp.	34–64
Pleurosigma normanii	100–121
Pennate 1	30–42
Dinophysis spp.	36–62
Dictyocha fibula	50–55
Dictyocha speculum	38–50
Pterosperma spp.	19–75
Dictyocysta elegans	64–70
Acanthostomella sp.	31–37
Foraminifera	30–170
Rotifers	59–66
Invertebrate eggs	146–162
Sponge spicules	39–190
Pollen	19–23

[a] Minimum individual frustule diameter and maximum chain length, respectively.

cies, except in February. In this month, the typical planktonic diatom genus *Thalassiosira* was almost the only food item found in gut contents (IR = 80%). In March, this species was still an important component of the diet (IR = 46%) but decreased in importance during the remaining months (IR = 7–16%; Fig. 6). *Nitzschia* spp. and *Pleurosigma normanii* made a minor contribution to total gut content (IR < 19% and IR < 4.5%, respectively; mean = up to 140 items per gut and up to 23 items per gut, respectively).

Dinoflagellates followed diatoms in importance (up to 1,000 items per gut, maximum IR = 25%, in November) in four of the six sampled months (Fig. 4). *Dinophysis rotundata* was present most of the time. *Dinophysis mawsonii* was present only in the spring, *Gonyaulax digitalis* cysts were found by the end of summer (February and March) and only a few in June. Unidentified dinoflagellates cysts were found in February, March, June, and August.

Silicoflagellates (two species of *Dictyocha*) were always observed, and they were the second most abundant item in August (IR = 0.02–12%; mean between 1 and 156 items per gut). Prasinophytes were relatively important in gut contents in June and March (IR = 9.6 and 5%, respectively, mean = 110 and 32 items per gut, respectively). Foraminifers were present in all months and in most guts (except in March) but with low IR and abundance values (IR = 0.7–6.2, mean between 8 and 79 items per gut). Tintinnids were frequent in spring (October and November) but even in these months had low IR and abundance values (IR < 1.7; mean <20 items per gut). Invertebrate eggs and copepod spermatophores were abundant in February and March.

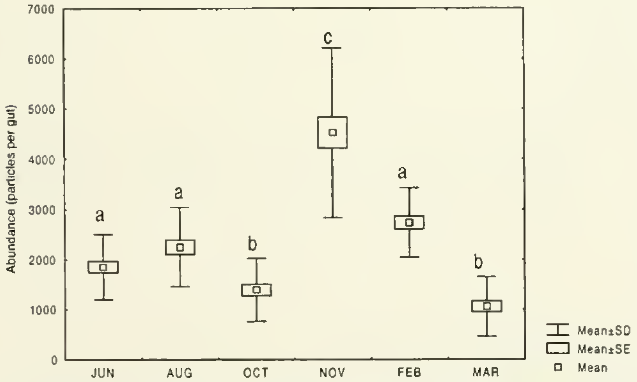

Figure 2. Mean abundance of particles per gut of *Zygochlamys patagonica* in the Reclutas bed during the study period. Different letters (a, b, c) indicate significant differences ($P < 0.05$).

DISCUSSION

The diet of *Zygochlamys patagonica* is mainly composed of diatoms. These results agree with similar studies on bivalve gut contents, which also showed a predominance of diatoms (Vernet de Hall 1977, Pollovero 1984, Newell et al. 1989, Leonardi et al. 1996). In addition, it is remarkable that most of the food items found had a planktonic origin. *Paralia sulcata*, one of the more important diatoms recorded in the diet of *Zygochlamys patagonica* and known as a tychopelagic species, is frequent in the shelf and the shelf break area during most of the year (Lange 1985). Reclutas bed, approximately 110 nautical miles offshore and 100-m deep, is undoubtedly located in the pelagic zone.

It must be pointed out that among the food items found in *Zygochalmys patagonica* gut contents, there were two potentially harmful dinoflagellate species present, *Dinophysis acuminata* and *Dinophysis rotundata*, which could produce diarrhetic shellfish toxins (Lee et al. 1989).

The maximum food contents were recorded in November (up to 8,000 food items per gut; mean = 2,800 items per gut), being *Paralia sulcata* the main food item. *Paralia sulcata* was also dominant in gut contents during the year, except in February when *Thalassiosira* spp. predominated and represented about 80% of total gut content. It should be mentioned that samples were preserved in formaldehyde, some food particles could have been destroyed because of the preservation, and also that highly digestible particles or naked cells were not recorded by this method.

The vertical movement of particles in the seawater column is very important for those animals that live far away from the photic zone (Valiela 1995). Algal cells settling during spring and fall blooms are one of the main inputs of particulate organic matter from the pelagic to the benthic system. Downward mixing of plankton during certain times of the year undoubtedly plays a large role in making food organisms available to deep-water scallops (Smetacek 1982, Shumway et al. 1987). The northern Argentine shelf shows an annual phytoplankton growth cycle with two peaks of which the most important one occurs in spring (Carreto et al. 1995, Akselman 1998). This peak was reported to occur by the end of September–October at the boundary of the coastal system/intermediate shelf (Akselman 1998), and maximum recorded chlorophyll *a* levels increase from the intermediate shelf to the shelf break (Carreto et al. 1981, 1995, Bertolotti et al. 1996). Thermocline formation begins in spring at the intermediate shelf and extends all along the shelf (Carreto et al. 1995). In October and November, the thermocline is already in development in the Reclutas bed area (Baldoni & Guerrero 2000), and oceanographic data for October and November 1996 were similar to mean estimated values for the study area by Baldoni and Guerrero (2000, Baldoni, personal communication).

Smetacek (1982) indicated that nutrients accumulated in a

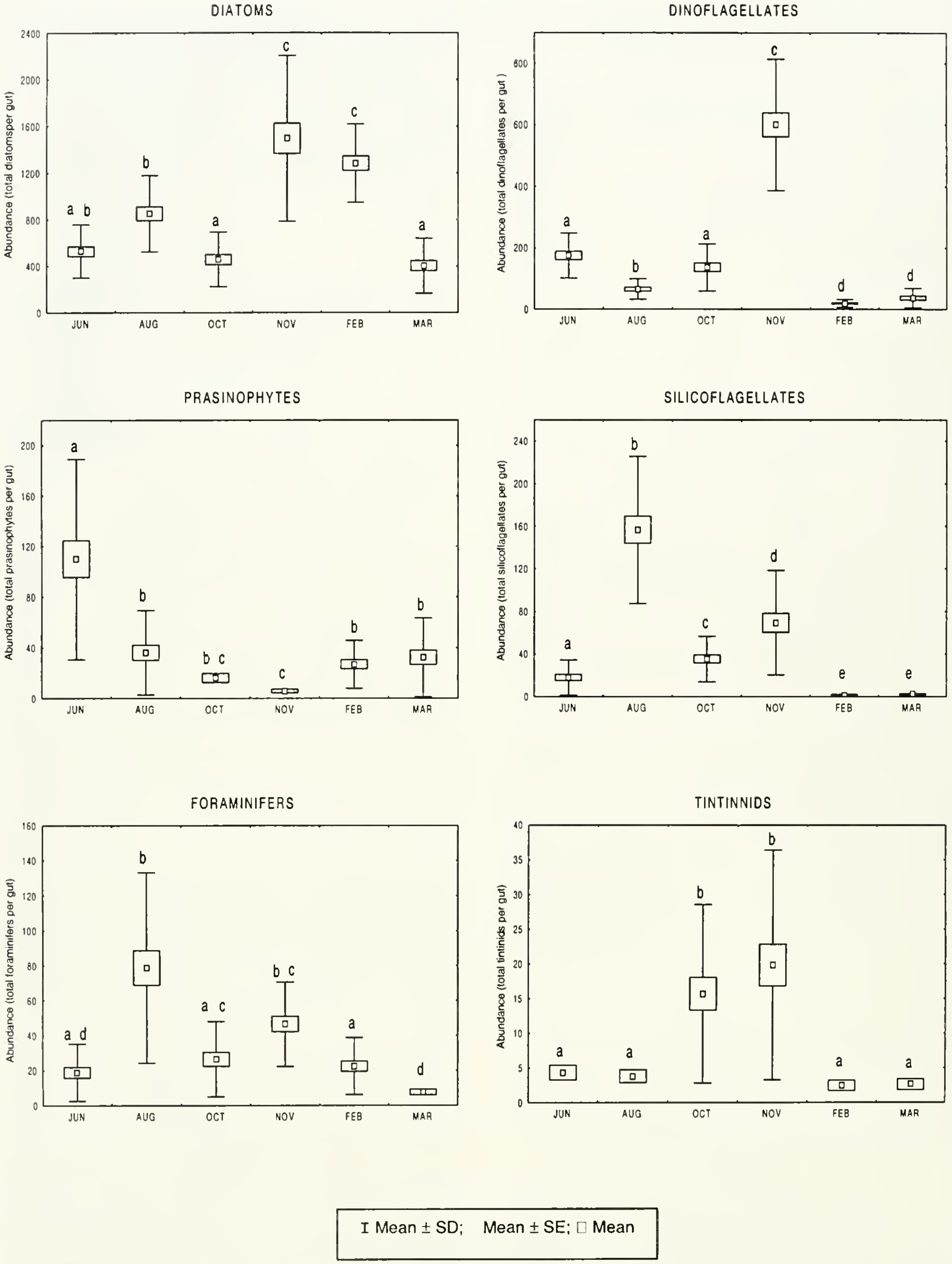

Figure 3. Mean abundance of principal food items per gut of *Zygochlamys patagonica* in the Reclutas bed during the study period. Different letters (a, b, c, d, e) indicate significant differences ($P < 0.05$).

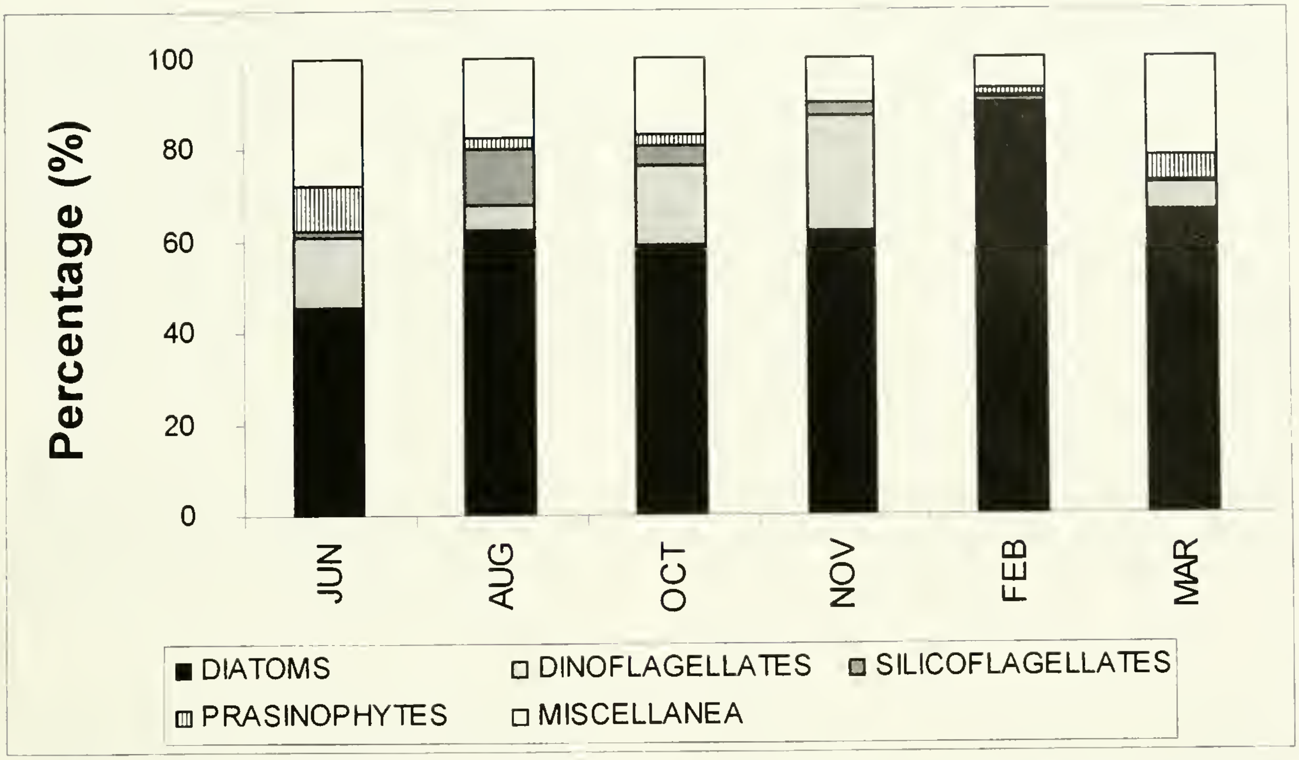

Figure 4. Percent composition of different food items groups in gut contents of *Zygochlamys patagonica* during the study period in the Reclutas bed. The group Miscellanea (see text) also includes tintinnids.

17-m water column at Kiel Bight over the winter were depleted within two weeks after phytoplankton bloom initiation. Sedimentation of phytoplankton took about one week and attained maximum values three or four days after bloom initiation. Sedimentation rates of diatoms and other phytoplankton components are related to their physiology, cell weight, and volume (Denman & Gargett 1983). As the organic matter sedimented was formed by a large number of living cells (and hence, was high in nutritional quality), benthic metabolic response to this input was rapid. For other deeper benthic systems a similar pattern is expected (Smetacek 1982).

It is thus possible that, given the oceanographic conditions described during spring at the Reclutas bed (thermocline in development), sedimentation processes or phytoplankton sinking downwards to the bottom have facilitated food availability to benthic organisms after the phytoplankton bloom started at the surface. This would explain the high abundance of cells observed in gut contents from scallops collected in November. It is probable that at the beginning of October (when samples were taken) increased primary production started in surface waters, as previously documented (Carreto et al. 1981), but availability of food at the bottom remained low because of the time requirement for sedimentation processes in a 100-m water column. During summer, the stratification of the water column is so pronounced that transport of phytoplankton cells to the bottom would probably be limited; under these circumstances we cannot explain the relatively higher abundance of *Thalassiosira* spp. during February. It is known that species of this genus are capable of producing blooms in other

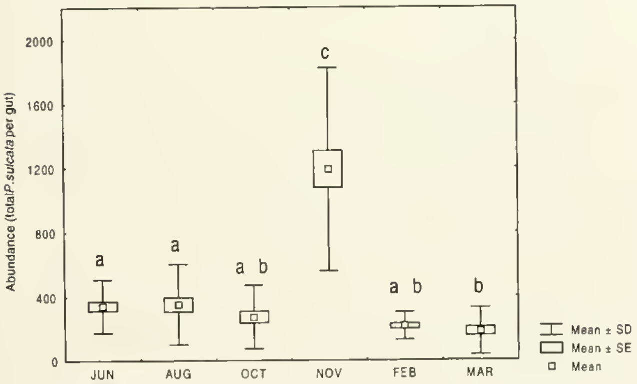

Figure 5. Mean abundance of *Paralia sulcata* per gut of *Zygochlamys patagonica* in the Reclutas bed during the study period. Different letters (a, b, c) indicate significant differences ($P < 0.05$).

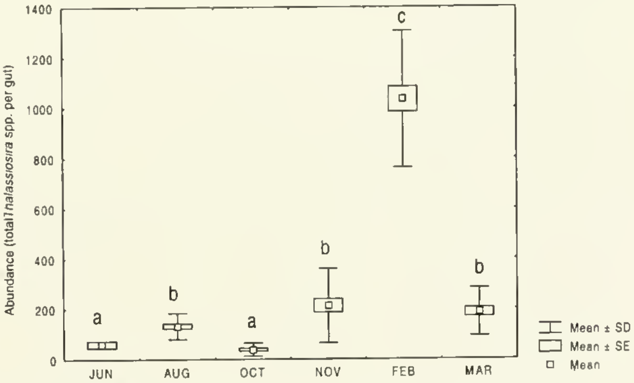

Figure 6. Mean abundance of *Thalassiosira* spp. per gut of *Zygochlamys patagonica* in the Reclutas bed during the study period. Different letters (a, b, c) indicate significant differences ($P < 0.05$).

areas of the Argentine Sea (Carreto et al. 1981), but oceanographic conditions at the Reclutas bed resulting in a strongly stratified water column would prevent cell sinking. However, occasional climatic events are able to disturb water column stratification, increasing sedimentation rates (Nielsen & Kiorbe 1991). On the other hand, Bode et al. (1998) conducted studies on the export of organic matter to the bottom, and found that most of it was pelagic in origin. Moreover, they determined that phytoplankton species found in sedimentation traps were not the same as those found at the surface, indicating that sedimentation traps were providing records of past production events. In summary, simultaneous seasonal information about gut contents and phytoplankton species composition in the study area is needed to establish the degree of particle selectivity in the feeding process, and whether the occurrence and abundance of food items are due to higher availability of potential food after phytoplankton growth.

The results of this study agree closely with recent findings of studies on biologic aspects of this species. Valero et al. (2000) studied the growth pattern of *Zygochlamys patagonica* at Reclutas bed. As was already found for several species of scallops from shelf and coastal areas (Barber & Blake 1991, Ciocco et al. unpubl.), growth of different body components of the Patagonian scallop are not simultaneous: maximum shell growth occurred in July, maximum gonadal growth was obtained in June, and maximum somatic (muscle) growth occurred in November. In view of this pattern, it is possible to link the period of maximum muscle growth with that in which scallops showed higher cell abundance in gut contents, both of which occur in November. Consequently, we hypothesize that somatic (muscle) growth of *Zygochlamys patagonica* located in the Reclutas bed occurs during the period of higher food availability in the bottom, which follows the period of spring phytoplankton growth in the upper layer of the water column and its subsequent sinking to the bottom before the development of the seasonal thermocline.

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COMPARISON OF THE PARASITES AND PATHOGENS PRESENT IN A CULTIVATED AND IN A WILD POPULATION OF SCALLOPS (*ARGOPECTEN PURPURATUS* LAMARCK, 1819) IN TONGOY BAY, CHILE

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ABSTRACT Cultivation of the "ostión del norte", *Argopecten purpuratus*, is an important economic activity in the 3rd and 4th Regions of Chile. Studies of disease were undertaken on wild scallops to gather baseline information on healthy populations. However, as cultivated scallops are kept at higher densities than the wild populations, the occurrence and prevalence of parasites and tissue pathology differed as indicated in this study that compares the types of parasites and their prevalence in wild and cultivated scallops from Tongoy Bay, a major center for scallop cultivation. In January 1999, 151 cultivated and 154 wild scallops were examined histologically and via scanning electron microscopy (SEM). The same parasite taxa, as well as granulomas, were found in both populations, but their prevalence differed. The granulomas were small, with no apparent etiology, but the cultivated population harbored significantly more lesions than the wild population. The only parasites found were a prokaryote in the digestive gland and the ciliate protozoan *Trichodina* sp. on the gills of the scallops. *Trichodina* did not seem to elicit a host response and its prevalence was significantly higher in the cultivated scallops. The prokaryote, a rickettsiales-like organism (RLO) was observed as basophilic inclusions in digestive gland tubule epithelial cells. The intensity of infection was low, but significantly higher in the wild stock. No host reaction was seen, and the prevalence showed no significant difference between the two populations. The infection with RLOs was therefore independent of the source of the scallops, unlike the *Trichodina* or the granulomas, which were significantly more prevalent in the cultivated population.

KEY WORDS: scallop, parasite, long-line culture, rickettsiales, *Trichodina*

INTRODUCTION

Argopecten purpuratus, the "ostión del norte" (northern scallop) is distributed on the Eastern Pacific coast from Sechura, Perú (6°S), to Tongoy Bay, Chile (31°S) (von Brand et al. 2002), and is harvested and cultivated in both countries. In Chile, the most abundant beds are in the 4th Region, in the bays of Tongoy, Guanaqueros, and Coquimbo. These populations almost disappeared through overfishing, prompting a total harvesting ban for this species in 1986 and this ban is still in force for wild populations. In the early 1980s, *A. purpuratus* culture became established (DiSalvo et al. 1984, Illanes–Bücher 1987) and currently the 3rd and 4th Regions produce 97% of the cultivated scallop production of Chile (Aquanoticias 2000).

Tongoy Bay is the single most important bay for production of cultivated scallops. A serious disease outbreak here could have significant consequences for this industry, so a survey was undertaken to gather baseline information on parasites and tissue pathology present in healthy members of the cultivated population. There is no information available on any diseases of *A. purpuratus* from Tongoy Bay, but some metazoan parasites have been reported for this species in other bays in Chile and Perú. Mateo et al. (1975) described germ sacs and cercaria of a hemiuroidean in the gonad of *A. purpuratus* in Perú, causing castration of heavily infected scallops. In Chile, Oliva et al. (1986) described two larval cestodes located in the gonads of *A. purpuratus* in Antofagasta. One of the cestodes belonged to the family Phyllobothriidae, and the other possibly belonged to the family Oncobothriidae. In Coquimbo, Lohrmann et al. (1991) described a metacercaria of a fellodistomid trematode in the labial palps of *A. purpuratus* and an unidentified larval cestode in the intestine of a few animals of this species (Lohrmann & Smith 1993).

As there is still a small natural bed of *A.* purpuratus at the southern tip of Tongoy Bay, both wild and cultivated scallops are available for examination in this bay. The wild scallops from this natural bed live on the sea floor in depths of up to 15 m, at a density of 1 to 2 scallops m^{-2} (Stotz & González 1997). Cultivated scallops are kept in pearl nets at densities of about 166 individuals m^{-2} for scallops at a size below 50 mm, and 19 individuals m^{-2} in lantern nets at sizes above 50 mm (Illanes 1986). Since the cultivated scallops are kept at greater densities than the natural populations, it might be assumed that the occurrence and prevalence of parasites and other pathogens may differ. This investigation was carried out on wild and cultivated scallops, to test for differences in the prevalence of parasites and tissue pathology between the two populations of *A. purpuratus*.

MATERIALS AND METHODS

In January 1999, 151 cultivated, and 154 wild scallops were obtained from Tongoy Bay (Fig. 1). The 12-month-old cultivated scallops had been hatchery-produced, and transferred to the sea at age one month. They had been kept at a density of 19 scallops m^{-2} in lantern nets hanging from long lines at a depth of about 10 m from the surface. The natural scallops were obtained from a small bed located near the fishing community of Puerto Aldea, which is located at the southern tip of Tongoy Bay (Stotz & González 1997). These scallops were adults, but their ages were not known. They were collected by "hooka" diving (this consists in delivering air to the diver through a hose from a compressor in the boat), from a depth of 8 to 10 m. Each sample of scallops was transported to the central aquaculture laboratory of the Universidad Católica del Norte in Coquimbo, where they were placed in tanks with running seawater at ambient temperature. They were processed in batches of 50 scallops daily, starting on the day after their arrival.

The soft tissues of the scallops were taken out of the shells, and

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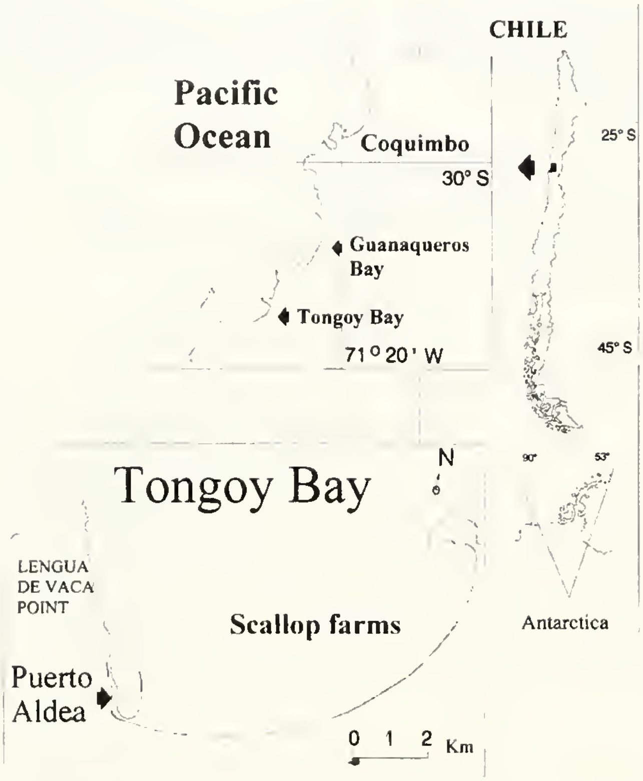

Figure 1. Map of Tongoy Bay showing the area dedicated to scallop farming (F) and the small natural bed (N) near Puerto Aldea.

were fixed for histology in Davidson's fluid (Shaw & Battle 1957), including the gills, digestive gland, gonad, kidney, mantle, and the adductor muscle. They were prepared for histology using standard methods and stained with haematoxylin and eosin (H & E). Giemsa and Ziehl-Neelson methods were used as appropriate to detect parasites and ceroid-like pigment respectively. Slides were analyzed and photographed using a Nikon E600 Eclipse photomicroscope. For assessing the intensity of infection of rickettsiales-like organisms (RLOs) and trichodinids, the most heavily infected area of the histologic section was selected, and the number of parasites present was counted at a magnification of 400 times, with a field diameter of 1,240 μm. Three categories of intensity were defined: grade I (very light) one parasite present, grade II (light), two to four parasites present, grade III (moderate), five or more parasites present.

For scanning electron microscopy (SEM), sections from selected wax blocks were cut at 12 μm, and mounted on coverslips. Sections were de-waxed in three changes of xylene, passed through three changes of 100% ethanol (modified from Toner et al. 1992), and critical point dried using CO_2. Samples were mounted on double-sided adhesive tape and ion sputtered with gold. The sections were viewed and photographed using a JEOL TS 300 microscope. Measurements of individual rickettsiales-like organisms and *Trichodina* were taken from SEM photographs.

For analyzing the prevalence of each parasite, a 2 × 2 contingency table was constructed with the frequency counts of each parasite in the wild and the cultivated scallops, and a χ^2 test for goodness of fit was applied. The intensities of infection were compared using the Mann Whitney rank sum test (Zar 1999).

RESULTS

From the total of 305 scallops analyzed, surprisingly few pathogens were detected. These included rickettsiales-like organisms (RLOs) and ciliates (*Trichodina* sp.). Small, granuloma-like tissue lesions were also detected. These are described in more detail later.

Rickettsiales-Like Organisms (RLOs)

Rickettsiales-like organisms (RLOs) were detected in digestive gland tubule epithelial cells in the form of spherical basophilic inclusions, with a diameter ranging between 7 and 14 μm. Occasionally, the inclusions were also seen in the lumina of the digestive tubules (Fig. 2A). In the interior of each inclusion there were darker staining bodies, which at the scanning electron microscope (SEM) level could be discerned as rod-shaped, and of fairly uniform size (Figs. 2B & C), ranging from 0.8 to 1.3 μm in length, and 0.38 to 0.46 μm in width. The inclusions seemed to be enclosed by a thin membrane, separating the RLOs from the cytoplasm of the host cell (Fig. 2B). The prevalence of these RLOs was 41% in hatchery-reared scallops and 37% for wild scallops (Table 1) but this difference was not statistically significant (P = 0.6247). The intensity of infection in both populations is shown in Table 1. A significant difference (P = 0.001) was detected in RLO intensity between populations, the natural population showing a higher proportion of scallops with degree II and III of infection.

Trichodina *sp.*

Trichodina sp., a ciliate protozoan, was found associated with the gills of the scallops (Fig. 3A). It was dome-shaped, with a horseshoe-shaped macronucleus (Figs. 3A & B). It measured 19 to 23 μm in height, and the basal disc was 14 to 18 μm in diameter. The basal disc was surrounded by a ciliary girdle (Figs. 3A & B). This trichodinid was always closely associated with the gill filaments, but no pathologic changes to the gill were detected. The prevalence was 56% for farmed scallops, but only 5.1% for wild scallops (Table 2), and this difference was highly significant ($P <$ 0.001). The intensity of infection is shown in Table 2 for both populations of scallops. The difference in intensity between the cultivated and the wild population was not statistically different.

Granulomas

In the base of the gills, small tissue lesions were found embedded in the connective tissue. These consisted of a central focus of pigmented material resembling ceroid that appeared to be contained within host cells and was surrounded by a thin capsule of fibroblast-like cells (Fig. 4). These lesions are hereafter referred to as granulomas. No evidence of infectious agents was detected in association with these granulomas. The prevalence was 12% in cultivated scallops, and 2.6% in the natural stock (Table 3) and this difference was highly significant (P = 0.0034).

DISCUSSION

In scallops, RLOs have been described as basophilic inclusion bodies in the gills of *Placopecten magellanicus* (Gulka et al. 1983), *Argopecten irradians* (Leibovitz et al. 1984, Elston 1986, Karlsson 1991), and *Pecten maximus* (Le Gall et al. 1988, Le Gall et al. 1991). They have also been found in the kidney of *Argopecten irradians* (Morrison & Shum 1983, Karlsson 1991, McGladdery et al. 1993), as well as in the digestive gland (McGladdery et al. 1993). The basophilic inclusions of *A. purpu-*

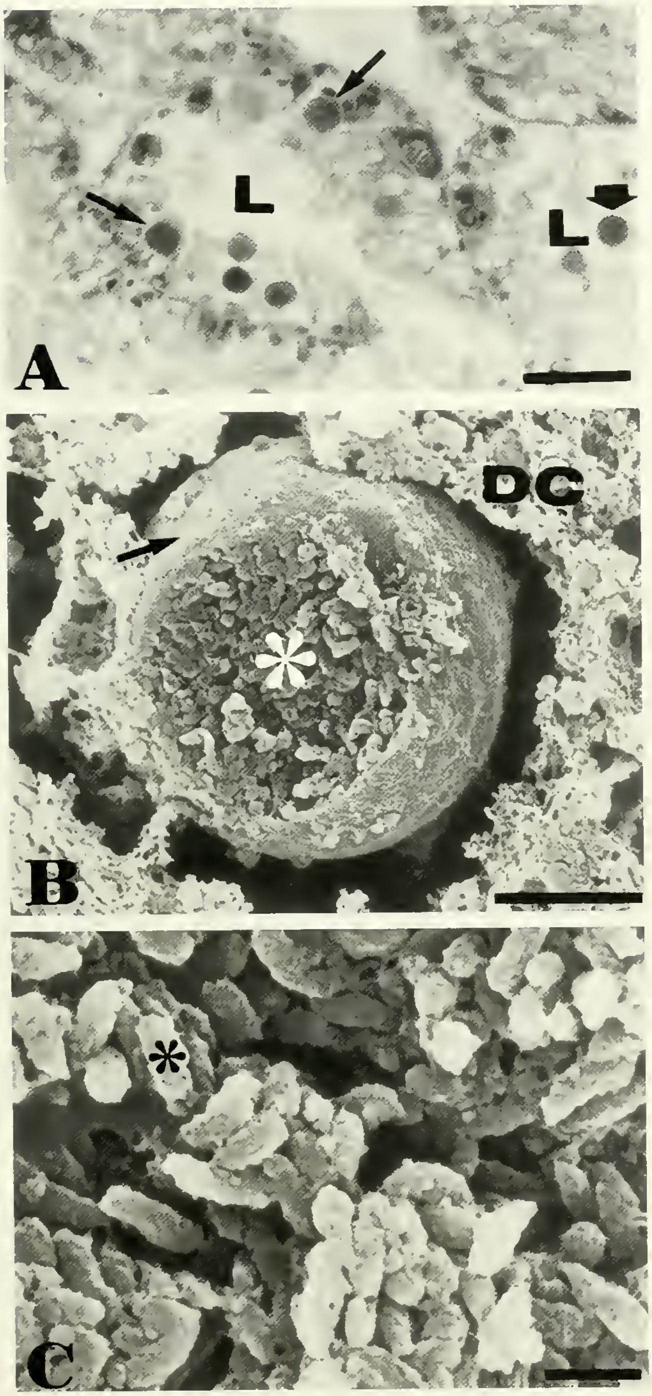

Figure 2. Rickettsiales-like organisms (RLOs) in the digestive gland of *A. purpuratus*. A: Light micrograph showing several basophilic inclusions (arrows) with RLOs in the cells of one tubule. One inclusion can be observed in the lumen (L) of another tubule (short arrow). Stain: H & E. Bar: 50 μm. B: Scanning electron microscopy (SEM) image of a histologic section of one basophilic inclusion. DC: digestive tubule cell. RLOs (*). Arrow: membrane separating the inclusion from the digestive cell. Bar: 5 μm. C: RLOs at higher magnification. One RLO (*). Bar: 1 μm.

TABLE 1.

Prevalence and intensity of infection with a rickettsiales-like organism in scallops from Tongoy Bay.

Scallop Group	No. +/No. Examined	Prevalence (%)	Intensity of Infection (%) Grade I	Grade II	Grade III
Cultivated	61/151	41	78	20	2
Natural	57/154	37	22	32	46

ratus were similar in size and structure to the rickettsial inclusions described by Morrison and Shum (1983) in the kidney of *A. irradians*, or in digestive gland tubules of clams by Elston and Peacock (1984). For the RLOs from *A. purpuratus* only one life cycle stage was found that indicates they are most likely rickettsiales and not chlamydiales-like organisms. Using SEM, the morphology and surface characteristics were discerned. However, as no other SEM images of RLOs were found in the literature, a comparison with other RLOs was not possible.

Since the prevalence of this organism showed no significant difference between the cultivated and the wild populations, host density does not seem to be an important factor in its transmission.

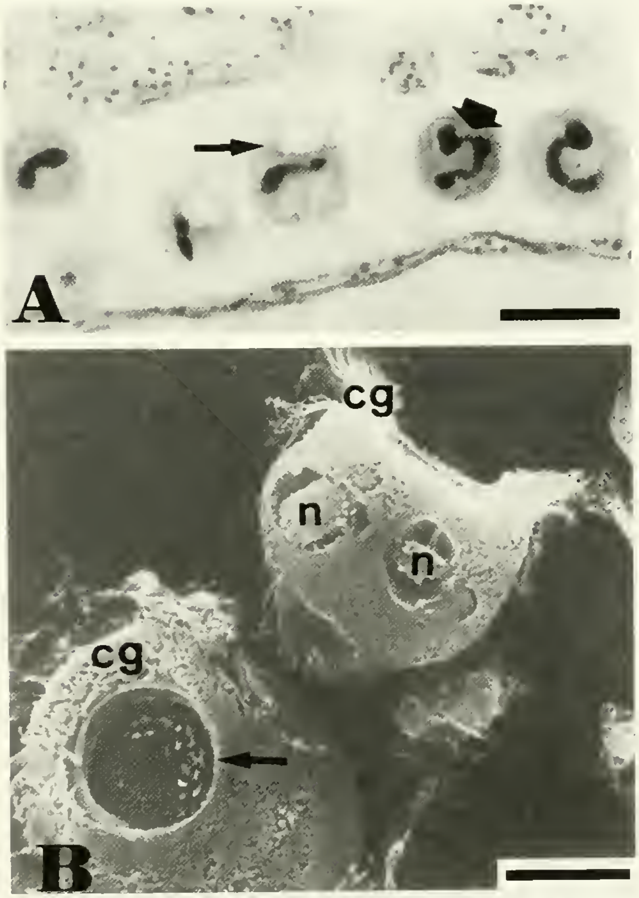

Figure 3. *Trichodina* sp on the gills of *A. purpuratus*. A: Light micrograph of a few individuals in different orientations. The horseshoe-shaped nucleus (short arrow) can be clearly seen, as well as the ciliary girdle (arrow). Stain: H & E. Bar: 50 μm. B: SEM image of a histologic section showing one complete, and part of another *Trichodina* sp. The whole individual appears longitudinally sectioned, showing the horseshoe-shaped nucleus (n), and the ciliary girdle (cg). The incomplete individual shows the basal disc (arrow) surrounded by the ciliary girdle (cg). Bar: 10 μm.

TABLE 2.

Prevalence and intensity of infection with *Trichodina* in scallops from Tongoy Bay.

Scallop Group	No. +/No. Examined	Prevalence (%)	Intensity of Infection (%) Grade I	Grade II	Grade III
Cultivated	84/151	56.0	64.0	29.0	7.0
Natural	8/154	5.1	37.0	37.0	26.0

TABLE 3.

Prevalence of granulomas in scallops from Tongoy Bay.

Scallop Group	No. +/No. Examined	Prevalence (%)
Cultivated	18/151	12
Natural	4/154	2.6

The RLOs observed in *A. purpuratus* do not seem to cause any harm, because there is no host response. Infections with RLOs are common in bivalves, where they usually cause only mild effects, if any (Lauckner 1983, Comps & Tigé 1999). However, RLOs were reported to have caused a few serious diseases but the relationship between the presence of RLOs and the mortalities was not experimentally demonstrated. In 1983, a mass mortality of *Placopecten magellanicus* occurred in Rhode Island, USA and a rickettsia-like organism was found in the gills and other tissues of these scallops (Gulka et al. 1983). Mass mortality due to branchial RLOs was also reported for *Pecten maximus* in Brittany, France by Le Gall et al. (1988), for the giant clam *Hippopus hippopus* (Norton et al. 1993), and for the clam *Venerupis rhomboides* from Spain by Villalba et al. (1999). With such limited knowledge it is important to undertake further studies on RLOs transmission and their effect on scallops of different ages and culture conditions such as density, temperature and depth.

Trichodina sp. ciliates are very common in bivalves (Lauckner 1983, Bower et al. 1994). They have been described from the following scallop species: *Mizuhopecten yessoensis* (Stein 1974, in Lauckner 1983), *Chlamys farreri* (Kuidong et al. 1995) and *Placopecten magellanicus* (McGladdery et al. 1993). In invertebrates, trichodinids are considered to be harmless commensals, feeding on bacteria (Lauckner 1983). However, they are present in large numbers in weakened animals (Bower et al. 1994), and also in organisms from areas polluted with chemicals and bacteria (Boussaïd et al. 1999). Boussaïd et al. (1999) found that *Crassostrea gigas* heavily infected with *Trichodina* exhibited an inflammatory response of the gill, and numerous desquamated epithelial cells, haemocytes, and tissue debris of host origin was observed surrounding the parasites. They also stated that an excessive mucus production covered the gill lamellae. This could interfere with the respiratory function of the gill, and may result in the death of the oyster (Boussaïd et al. 1999). A significant difference in prevalence of *Trichodina* was found between farmed (56%) and wild *A. purpuratus* (5.2%). This may be a consequence of the crowding of the scallops, their proximity facilitating the transfer of this commensal.

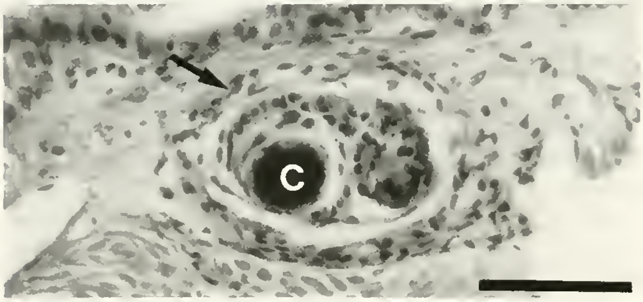

Figure 4. Light micrograph of one granuloma located in the base of the gills. Some degrading material and ceroid (c) can be observed, encircled by fibroblast-like cells (arrows) encapsulating it. Stain: H & E. Bar: 50 μm.

The granuloma-like tissue lesions found at the base of the gills are similar to lesions (that they called "swirl" encapsulation) observed by McGladdery et al. (1991) in bay scallops *Argopecten irradians* infected by what was thought to be a *Perkinsus* species. Goggin et al. (1996) made an assessment of these lesions, and concluded that they were not produced by a *Perkinsus* species, but were a general response to a foreign agent. In this study, the very low prevalence and intensity of the lesions precluded ultrastructural investigations that are needed to determine the etiology of the granulomas. However, Gonzalez et al. (1999) identified apparently identical lesions in *A. purpuratus* from Valparaíso, which were heavily infected with a protistan. Macroscopically, infected animals presented small dark brown pustules in the mantle, which was also retracted. Based on one electron microscopy image of the protistan they suggested that it could be an apicomplexan. However, details were indistinct and this finding needs to be confirmed. Since pathogen involvement cannot be ruled out as a cause for these granulomas and there was a significantly higher prevalence in cultivated stocks, further investigations are needed to identify their cause.

From the results of this study it can be concluded that both cultivated and natural *A. purpuratus* from Tongoy Bay barbored very few putative pathogens, and those that were present, were the same for the two scallop groups. The main difference between the two groups of scallops was the density at which they lived: 1 to 2 scallops m^{-2} for natural scallops in Puerto Aldea, and 19 scallops per m^{-2} for the cultivated scallops. They also differed in the location, with natural scallops living on the seabed and cultivated scallops in cages suspended in mid-water. The increased density can favor transmission of pathogens in two ways, either providing hosts that are in close vicinity, and/or increasing stress. Overcrowding can reduce food availability or increase the levels of toxic waste products, all of which contribute to stress (Newell & Barber 1988). Stressed organisms have less energy available to defend themselves from disease and this is recognized as an important factor that can trigger disease in otherwise healthy animals (Lauckner 1983, Newell & Barber 1988, Sindermann 1990).

It is surprising how few potential pathogens were harbored by these two populations of scallops, since some metazoan parasites have been previously found in *A. purpuratus* in other northern Chilean bays. Although the scallops examined during this study were from healthy populations, the threat of disease is always present. Any pathogens that are new to *A. purpuratus* could have a devastating effect on both cultivated and natural stocks. Pathogens may be involuntarily carried by fouling organisms on boats, in the ballast water of big ships, or by transporting scallops or other bivalves from other regions to Tongoy Bay. Newly introduced parasites can cause epidemics if the host's innate defense mechanisms are not able to destroy it, or if the host is not able to defend against a novel parasite strategy (Figueras & Fisher 1988). It is therefore recommended that *A. purpuratus* stocks be regularly assessed for disease agents, so as to identify any different pathogen from those known to be present in these populations.

ACKNOWLEDGMENTS

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BYSSAL ATTACHMENT OF *AMUSIUM BALLOTI* (BERNARDI, 1861) (BIVALVIA: PECTINIDAE) SPAT

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ABSTRACT It has been previously reported that the saucer scallop, *Amusium balloti*, either lacked the ability to produce byssal threads or could do so only briefly. This present study reports our main conclusion that in the early spat stages, *A. balloti* does indeed secrete byssus, albeit at a time different from most scallops. We found that *A. balloti* first attaches by a byssus only after metamorphosis (indicated by the presence of dissoconch shell), and we found no evidence of the byssal attachment before or during early metamorphosis. By the time spat reach a shell height of 1–2 mm they secrete two or three fine byssal filaments. Byssal attachment is maintained until the spat reaches 4–5 mm, around the stage where they develop the ability to swim. This pattern is unlike that found in most other scallops that initiate byssal production and attachment before metamorphosis. We also describe post-settlement behavior of *A. balloti*. The newly settled postlarvae (~200 μm shell height) crawl along the substratum by using their foot. During attachment, spat change their positions daily, moving an average of 17 mm per day. In culture, a gentle water jet and hypersaline bath (40‰) effectively detached spat, with the majority rapidly reattaching.

KEY WORDS: *Amusium balloti*, byssal attachment, aquaculture, scallop, captive breeding

INTRODUCTION

The broad stages whereby free swimming planktonic molluscan larvae undergo transition into spat is similar in most scallops species, and commences when competent larvae contact and attach to a substrate by secreted byssal threads (Bourne et al. 1989, Beninger & Le Pennec 1991). Metamorphosis and postlarval life follows, when the larvae lose their velum (swimming organ) and produce functional gills. Many species, such as *Chamys asperrimus*, retain the ability to form byssus throughout life, while other scallops, like *Pecten maximus*, cease to form byssus at around 15 mm shell height and live free of attachment on the sea bed (Brand 1991). *Amusium balloti* (Bernardi 1861) is one of these latter, free-living scallops, but one that was also previously thought to either lack the ability to produce byssal threads or to attach for any significant time, even as spat (Rose et al. 1988, Cropp 1992, Sumpton et al. 1990, Robins–Troeger & Dredge 1993).

Amusium balloti, commonly known as the saucer scallop or swimming scallop is a fast growing scallop that inhabits subtropical and tropical waters off the east and west coasts of Australia and is an important component of the multi-species trawl fishery in these areas. Some individuals recruit to the fishery (i.e., reach legal harvest size of 90-mm shell height) when 6 months old, although most attain this size in 9 to 11 months (Gwyther et al. 1991). *Amusium balloti* has been the subject of a number of studies on larval development (Rose et al. 1988), wild collection (Sumpton et al. 1990, Robins–Troeger & Dredge 1993) and hatchery production (Cropp 1992), with a view to developing the species for aquaculture or stock enhancement. An essential component of either of these latter activities is a reliable, relatively cheap and large source of spat. While most successful scallop culture operation worldwide utilize wild-sourced spat due to lower cost, hatchery production is an option, and offers some advantages, such as genetic selection. However, both wild and hatchery production of bivalve spat depend on attachment to some form of substrate for collection and handling (Bourne et al. 1989, Wang et al. 1993).

According to Rose et al. (1988), newly settled *A. balloti* spat crawl actively using their foot, but never appeared to attach permanently to substrata provided. Cropp (1992) determined that newly settled spat lacked a firm and long-term byssal attachment, but retained a strong and active foot that allowed them to crawl on the substratum and detach and swim in the water column at will. During hatchery production this apparent lack of permanent byssal attachment resulted in significant numbers of cultured spat accumulating on the tank floor after dropping from suspended collector bags. If left on the tank floor, associated fecal matter and algal detritus would kill the young spat (Cropp 1992). The conclusions drawn from these hatchery observations were further supported by data from spat collectors in the field. Attempts to obtain spat by deploying collectors during the spawning and settlement season of *A. balloti* resulted in very low numbers of captured spat (Sumpton et al. 1990, Robins–Troeger & Dredge 1993). The most likely explanation given for this lack of success was that *A. balloti* either had a brief or non-existent byssal attachment stage.

Here we present data demonstrating that *A. balloti* does produce byssal threads, but unusually for a scallop, does not do so until the dissoconch shell is produced, well after metamorphosis and postlarval stages. We also document, for the first time, the processes of byssal attachment and provide data on detachment and reattachment methods, and movement of *A. balloti* spat.

MATERIALS AND METHODS

General Specifications

Hatchery conditioned broodstock were induced to spawn by air-drying and heat shock using standard methods (Bourne et al. 1989). After fertilization the embryos were kept at 20°C in a 500-L tank until hatching. Larvae were reared at 20 ± 1°C until ready to metamorphose (i.e., competent). Competent larvae were allowed to settle in screen chambers, which were constructed by gluing nylon mesh (160 μm aperture) on to PVC pipe (250 mm diameter),

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and housed in a hatchery downweller system operated at 21 ± 1°C, pH 8.1–8.2 and 33–35‰ salinity. The rearing water was changed three times each week, followed by feeding with equal amounts of Tahitian *Isochrysis aff. galbana*, *Pavlova lutheri* and *Chaetoceros calcitrans* based on their dry weight at a density of approximately 15,000 cells ml^{-1}.

Qualitative Observations of Byssal Attachment and Movement

Three times per week postlarvae (i.e., after initiation of metamorphosis, but before evidence of a dissoconch shell) and spat (evident dissoconch shell) were placed into petri dishes and their movement was observed microscopically. After being left for approximately 30 min the attachment status of the immobile postlarvae and spat was determined by subjecting individuals to gentle water current produced by squeezing water from a Pasteur pipette. Postlarvae and spat that were moved by the gentle water current were considered to be unattached spat. Those that did not were considered to be byssally attached and were photodocumented (Sony, Mavica).

Spat attachment and movement was also qualitatively assessed by hanging small pieces of nylon mesh (tied to 2 × 3 cm PCV plates for support and weight) in three different ways in each of 3 replicate screen chambers (see General Specifications), each holding 1,000 to 2,000 spat. Specifically, mesh was: (1) suspended vertically 1cm from bottom of the screen chamber; (2) placed vertically in contact with bottom of the screen chamber; (3) placed in a Petri dish (5 cm in diameter with 1 cm side), that was suspended 1 cm from the bottom of screen chamber. In all cases, mesh or Petri dish was at least 1cm distant from the walls of the screen chambers. After 24 h mesh/PVC plates/petri dishes were inspected for settled spat and observed using a stereomicroscope. This experiment was performed twice when spat had a shell height between 1 and 2 mm.

Spat Detachment

Detachment of byssally-attached spat was investigated using two different treatments, high salinity water and a water jet. The high-salinity treatment consisted of exposing attached spat in a screen chamber to 40‰ seawater. After 5 min, detached animals were removed by very gentle rinsing, and counted. This procedure was repeated after 15, 30, 60, 90, and 120 min. This procedure was replicated using 3 screen chambers at the same time. The salinity was raised by adding rock salt (Olsson's) and measured using Horiba Water Checker (Model U–10, HORIBA Ltd., Japan). A control group using 33‰ salinity was also assessed using the same procedure. Spat detachment using a water jet was assessed using a small aquarium pump at a rate of 2,500 L/h (nozzle size: 0.8 cm). The jet was directed onto spat attached to the screen chambers suspended in water at 33‰ salinity. Detached spat were rinsed from the chamber and counted.

For all treatments, spat were hatchery reared at a water temperature of 21°C. Prior to the start of the experiment, any loose spat were gently rinsed from the screen chambers, leaving only attached spat at approximately 750–2,000 per chamber, or 1.6–4.4 spat/cm^2. Spat shell height was 1.2 mm (±0.05, SE). Following experimental procedures, 10 detached spat from each treatment were subsequently held in 400 ml-plastic dishes in normal seawater (33‰) to determine treatment effects on survival, which was assessed by inspection under a stereomicroscope 24 h after detachment.

Spat Reattachment

After being detached by water jet as described above, 10 spat were held in plastic dishes holding approximately 400-ml seawater at 21°C, 33‰, and pH 8.2. The size range of the spat was 1–1.5 mm. Spat were left to reattach for 5 min. after introduction to the dish and attachment status was determined by subjecting spat to a gentle water current from a Pasteur pipette. Scallops moved by the water current, were considered unattached. Reattachment was assessed after 5, 15, 30, 60, 120, 240, 360, and 720 min. using this procedure. Three replicate samples were used. Survival was also determined during this trial by microscopic inspection, immediately following reattachment assessment.

Quantitative Measurement of Spat Movement

Individual spat (shell height 2 mm ± 0.06, SE) were held in plastic dishes (32 in total) containing 400 ml seawater at 23°C, 34‰ and pH 8.2. Spat were left for 1 h (i.e., sufficient time to ensure reattachment as indicated by earlier results), and then the position of each spat was marked with permanent ink on the underside of the dish. Spat position was marked again every 24 h for seven days and the straight-line distance between the most recent and previous position recorded. At the same time the attachment status was checked as before and survival status of unattached spat was assessed by microscopic inspection. Water was exchanged daily to enhance water quality, and to minimize the effects of water exchange on spat attachment and movement, approximately 1,000 ml seawater was allowed to flow through each dish in one hour. Equivalent proportions of Tahitian *Isochrysis aff. galbana*, *Pavlova lutheri* and *Chaetoceros calcitrans*, based on dry weight at a final concentration of 15,000 cells ml^{-1}, was added to the flowthrough seawater to provide feed.

Statistical Analysis

GenStat (Payne et al. 2000) was used for all analyses, and differences between treatment means were evaluated for significance using least-significant difference testing (at a significance level of 0.05). The water jet detached 100% of the spat in the screen chamber within 2 min. Hence, this treatment had zero variation and was not included in the statistical analysis of detachment, and only control (normal seawater 33‰) and hypersaline (40‰) treatments were statistically compared. As the numbers of the attached spat varied among the individual screen chambers, the results of various detachment treatments were compared on the basis of percentage of total number of spat detached (i.e., at 120 min). Percentage detachment values were normalized by the angular transformation (arcsine of the square root of the proportions). The transformed data were analyzed using one-way ANOVA, with 3 replicate screen chambers per treatment. In addition to analysis of the total number of detached spat at 120 minutes, percentages of detachment in each cumulative interval were also subject to repeated measures ANOVA. For reattachment of young spat, the number of reattached animals was converted to the percentage and normalized by the angular transformation in each treatment, then also analyzed by one-way ANOVA. For movement of young spat, the distances of movement were analyzed by using two-way ANOVA, with the two treatments being "days" and "spat". The proportions of attached (vs. moved) animals were analyzed by using generalized linear regression analysis (McCullagh & Nelder 1989), using the binomial distribution and logit link, with the same treatments being fitted.

RESULTS

Qualitative Observations of Byssal Attachment and Movement

Newly settled postlarvae crawled actively using their foot. Non-active or immobile postlarvae were not observed to attach, and were easily moved by a gentle water current. Spat initially attached to the substratum with a fine and transparent byssus that was difficult to detect using a stereomicroscope, although its presence could be inferred by resistance to the water current. Upon reaching 1 to 2 mm, the byssus became thicker and could be observed microscopically (Fig. 1).

Shortly after byssal detachment by water jet, spat typically retracted into their shell for several seconds to minutes. Subsequently a spat would extend its foot from the byssal notch, using it to explore and crawl or attach to the substratum. Spat typically explored and crawled for several minutes before stopping, then continuing to explore the surrounding area with the foot. The cornet of the foot then pressed against a substrate, and seconds later the foot was retracted into the shell leaving a byssal filament that fixed the scallop to the substratum. *Amusium balloti* spat usually secreted two or three byssal threads. If disturbed, the spat would extend its foot again to explore the surrounding area and secrete another byssus, or release existing threads, and would crawl away using its foot and shell adduction. At about 4–5 mm, spat started to swim and ceased byssal attachment.

Inspection of PVC plates, nylon mesh and petri dishes indicated that 3 to 10 spat (out of 1,000 to 2,000) attached to the PVC plates or nylon mesh in contact with the bottom screen. No spat were found on PVC plates or nylon mesh that were off the bottom screen and no spat were caught in any of the petri dishes.

Spat Detachment

Based on the total number of spat detached after 120 min, one-way ANOVA indicated that hypersaline seawater (40‰) induced a significantly greater proportion (98%) of detachment compared with washing in normal seawater (33‰) (75%) ($P < 0.01$). The water jet (normal seawater) detached 100% of the spat in 1 or 2 min. Analysis of time intervals within the 120 min period (Fig. 2) indicated there was an interaction between time and treatments ($P < 0.01$), which occurred in 30 and 60 min intervals. Using salinity treatments, most detachment occurred during the first 60 min ($P < 0.01$). No mortality was observed within 24 h of detachment using any treatment.

Spat Reattachment

The rate of spat reattachment over time was significantly different (One-way ANOVA, $P < 0.01$; Fig. 3). Reattachment increased linearly, reaching a plateau of around 90% at 60 min, and remained stable up to 720 min. No mortality was recorded following this experiment.

Qualitative Measurement of Spat Movement

The average daily distance moved by spat over seven days was 17 mm, and tended to be greater in mid trial ($P < 0.01$, Fig. 4). However, there were large differences in distance moved between spat ($P < 0.01$). Figure 5 shows the average daily movement of individual spat over seven days with corresponding standard deviations. The longest mean daily distance travelled was 38 mm and the shortest mean distance was 1 mm. All animals moved at least once in seven days. Generalized linear regression analysis showed that for total numbers of attached animal and moved animals there was no statistical difference between different days ($P > 0.2$). However, individual spat showed differences in the proportion of times they were observed to be attached or to have moved over the seven days ($P < 0.05$).

DISCUSSION

Our investigation demonstrated that *A. balloti* consistently secreted a transient byssus, contrary to, and clarifying previous reports (Rose et al. 1988, Sumpton et al. 1990, Cropp 1992, Robins–Troeger & Dredge 1993). However, unlike most scallops (Sastry 1965, Bourne et al. 1989, Benigner & Le Pennec 1991), there is no evidence of the byssal attachment in *A. balloti* before and during early metamorphosis. The first appearance of byssal threads occurs after the spat begins to synthesize the dissoconch shell. Spat of shell height less than 1 mm do attach to the substratum, although it is difficult to detect byssus even under microscopic inspection because it is very thin and transparent (future electron microscopy may resolve this issue). The thickness and visibility of threads increases as the animal grows.

We do, however, find similarities between *A. balloti* and the reported behavior of other pectinids during byssal secretion (Benigner & Le Pennec 1991), specifically the crawling, and exploration phases prior to secretion, and the process of secretion itself.

Amusium balloti spat of 2–3 mm shell height secreted only 2 or 3 transient byssal filaments under our experimental conditions. Compared with other scallops *A. balloti* appears to have a much reduced byssus attachment. Gruffydd (1978) estimated the number

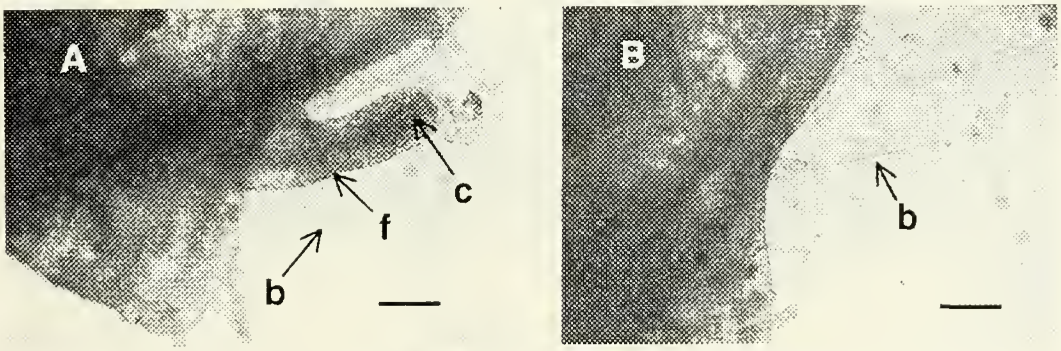

Figure 1. The foot, cornet, and byssus of *Amusium balloti* spat (shell height 2 mm) (A) Foot extended. (B) Foot retracted. b, byssal; f, foot; c, cornet. Scale bar = 10 μm.

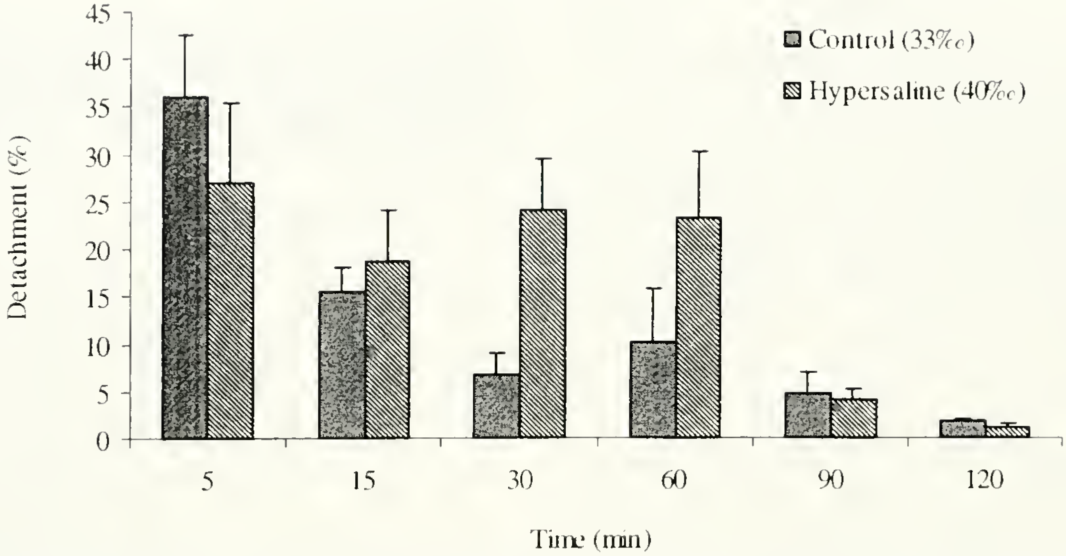

Figure 2. Sequential proportional detachment (mean and standard deviation of *Amusium balloti* spat (shell height 1.2 (±0.05 SE) mm) from settlement screens after exposure to normal (33‰) and hypersaline (40‰) seawater at cumulative time intervals of 5, 15, 30, 60, 90, and 120 min.

of byssal filaments secreted by *Chlamys islandica* to be 10–30, while *Placopecten magellanicus* juveniles secrete 3–7 filaments (Caddy 1972). *Amusium balloti* spat lose byssus when they reach a shell height of 4–5 mm, which is smaller than for most other scallops. For example, *Pecten maximus* ceases byssal attachment at 15 mm, *Argopecten gibbus* at 6–10 mm, (Brand 1991) and *Argopecten irradians* at 20–25 mm (Pohle et al. 1991). Other scallops such as *Chamys asperrimus* (Brand 1991) and *Chlamys farreri* (Wang et al. 1993) retain the ability to form byssus throughout life.

In culture, 1–2 mm *A. balloti* spat can crawl from the bottom to substrata (mesh and PVC plates) that are in contact with the bottom, although this appears to be uncommon. With our experiment design we found no evidence that spat can swim and attach to substrata raised above the bottom. However our experiment design did not categorically exclude the possibilities, either, that at this age spat were able to swim, but not able to attach to screen off bottom, or, that spat could swim but were not able to swim vertically more than 2 cm from the bottom. Future video surveillance techniques may overcome some of these technical limitations. Observations indicated that spat begin to swim at 4–5 mm, apparently losing the ability to synthesize new byssal filaments at the same time. With continued growth, spat demonstrate the active swimming noted in adults. According to Joll (1989), *A. balloti* is the fastest and longest distance swimmer amongst the scallops.

Results also indicated that most spat moved every day, although continuous movement patterns were not recorded. Since it is likely that spat movement is not in a single direction and perhaps more than once a day, our estimates of daily migration are likely to be underestimates of the actual distances moved. The change in

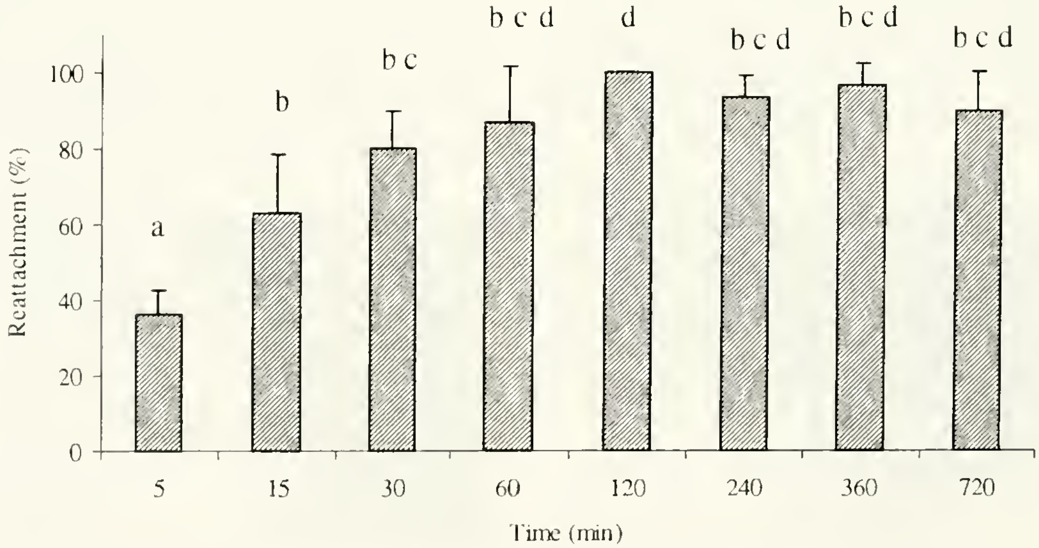

Figure 3. Reattachment rate (percentage with standard deviation) of *Amusium balloti* spat (shell height 1.5 (±0.05 SE) mm) Over 720 min following water jet detachment. Values sharing same superscript letter do not differ significantly ($P > 0.05$, ANOVA)

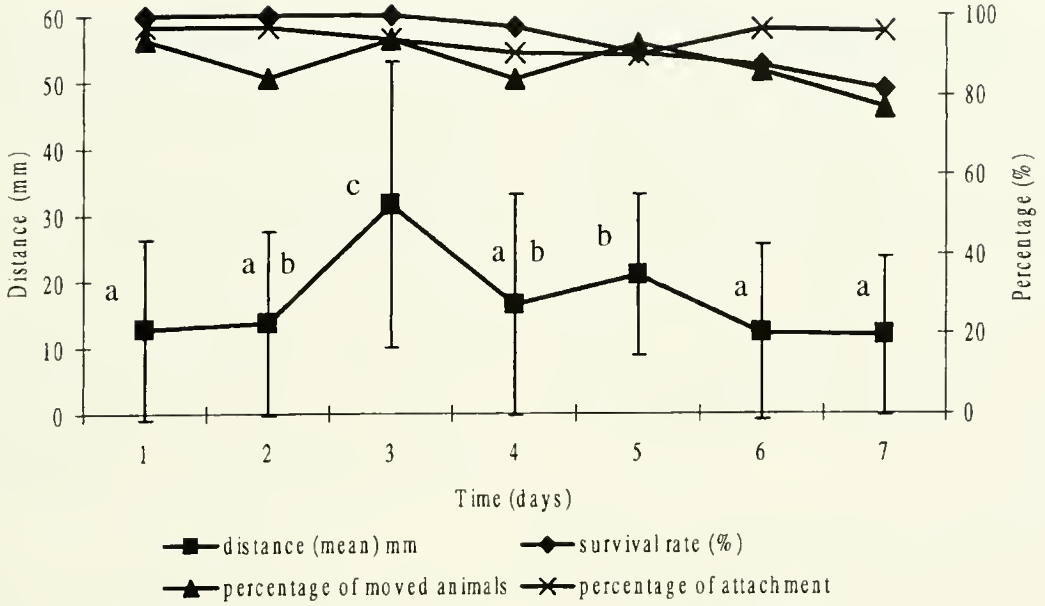

Figure 4. *Amusium balloti* spat (shell height 2 (±0.06 SE) mm), mean movement distances with standard deviation, daily proportions of moved and attached animals, and daily survival rates over seven days. Values sharing same superscript letter do not differ significantly ($P > 0.05$, ANOVA)

attachment rates over time for *A. balloti* young spat are similar to those reported for *Chlamys opercularirs* (L) (Paul 1980) and *Placopecten magellanicus* (Caddy 1972). The time required for most *A. balloti* spat to reattach is also similar to that reported for these species of scallop, with approximately 80% to 90% reattached after 30 and 50 min respectively (Caddy 1972, Paul 1980).

Scallops appear to be susceptible to the effects of siltation, particularly in relation to gills (Naidu & Scalpen 1976, Tettelbach et al. 1988). It has been suggested that byssal attachment to elevated substrates, by both juvenile and adult scallops, may help avoid burial by fine-grained sediments in areas of high near-bottom turbidity, as well as a means of avoiding predators (Bricelj & Shumway 1991, Pohle et al. 1991). *Amusium balloti* lives on medium to coarse sandy-mud seabed (Cropp 1994) where there is very fine sediment, but also no vertical substrate for juvenile attachment. Consequently *A. balloti* spat probably settle directly on the bottom with byssal attachment to coarse sand grains. As small spat, only a few filaments are required for them to attach onto the substrate and avoid being moved by currents, or perhaps concentrated and buried in areas of fine silt. The small number of filaments, in addition to minimizing the energy requirements of byssal production, would also enable more rapid detachment if the need arose, perhaps in response to predators. However, with the growth of young spat, sand grains are no longer effective anchors, even if the spat could secrete more and stronger byssal threads. Interestingly, after 4–5 mm, *A. balloti* spat rapidly develop a strong swimming capability, which is also likely to be an effective means of avoiding siltation and predation.

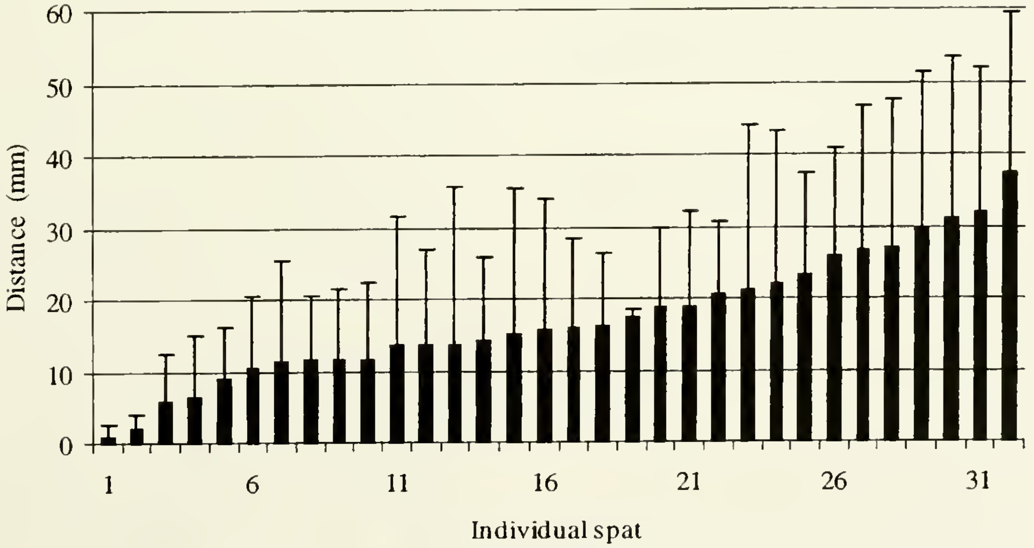

Figure 5. Mean daily movement of individual *Amusium balloti* spat ($n = 32$), (shell height 2 (±0.06 SE) mm), over 7 days, mean distance (mm) with standard deviation. Data arranged in ascending order.

Methods for artificial and controllable detachment of spat have important practical applications in hatchery production. Growing spat need to be frequently graded and transferred onto screens of successively larger surface area and mesh aperture sizes to reduce screen fouling and to offset increasing biomass (Heasman et al. 1994). In *Pecten fumatus*, mechanical methods, such as seawater jet and scrapers, can cause injury and subsequent high mortality, particularly in spat less than 5 mm shell height (Heasman et al. 1994) due to their more robust attachment. However, the delicate byssus of *A. balloti* is readily broken with a gentle stream of water does not cause mortality to the spat. A hypersaline (45‰) bath was found to be the most effective and safe means for inducing spat detachment in both *P. fumatus* (>95%) (Heasman et al. 1994) and *Pinctada maxima*, (>90%) (Taylor et al. 1997). This method was also effective for *A. balloti* with 40‰ salinity inducing 98% detachment within 2 h, compared with 75% in normal seawater (and after gentle rinsing).

A prerequisite for any aquaculture operation is the reliable, plentiful, and inexpensive supply of seed (juveniles). In most bivalve culture operations throughout the world, seed is obtained from the wild, although hatchery-produced spat is an alternative (Bourne 2000). Both approaches have been used successfully to produce scallop spat and in all documented cases, procurement of scallop relies on the attachment of spat via byssal threads to a solid substratum (Bourne et al. 1989, Ito 1991, Wang et al. 1993, Neima 1997, Bourne 2000). The results of this study demonstrate that *A. balloti* secrete byssus, consisting of a few fragile filaments. In addition, although spat are usually attached to a substratum by the byssus, they do move frequently. These characteristics explain why wild collection is unsuccessful, with water movement eventually removing most spat from collector bags, and supports the conclusion of Robins–Troeger and Dredge (1993), that fishery enhancement (and aquaculture) will be reliant on hatchery-reared spat.

The results of our present study also suggest that to culture *A. balloti* spat in the hatchery, we cannot use standard methodologies reliant on a robust byssal connection. However, this problem can be overcome by using screen chamber settlement systems. Downweller and upweller screen chamber settlement systems have been used successfully to rear many species of bivalve, such as oyster, clam, (Utting & Spencer 1991) and scallop (Heasman et al. 1998). Our work provides the first foundations for the development of a more reliable and commercial-scale hatchery production method for *A. balloti*, which has not previously been demonstrated. The potential for subsequent aquaculture or stock enhancement through large-scale hatchery production of spat may then be realized.

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INTRASPECIFIC VARIATION IN THE REPRODUCTIVE CYCLE OF THE TEHUELCHE SCALLOP *AEQUIPECTEN TEHUELCHUS* (PELECYPODA, PECTINIDAE), IN SAN MATÍAS GULF, PATAGONIA, ARGENTINA

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ABSTRACT The reproductive cycle of the tehuelche scallop, *Aequipecten tehuelchus*, from San Matías Gulf was studied for a two year period. Scallops were collected at monthly intervals from August 1997 to July 1999. Muscular and gonosomatic indexes were estimated and histologic sections of the gonad were prepared and used to determine gametogenic stages and to estimate the mean oocyte diameter. Gonosomatic index (GSI) data revealed a bimodal pattern, but the spring-summer spawn was larger and more synchronized than the autumn spawn. GSI and histologic observations had similar patterns. Massive spawning began in December. Gametogenesis lasted from June through late winter (September). The tehuelche scallop is a partial spawner. The spawning schedule showed between-year fluctuations, starting at any time during September through October, depending on the year. Partial spawnings and gonadal recovery alternated along the summer, until March. Spawning was followed by the reabsorption of unspawned gametes. A resting stage in the reproductive cycle occurred from late summer (March) to mid-autumn (May). In 1 of the two years studied, an early sexual maturation matched low temperatures. This suggests that in San Matías Gulf the reproductive cycles are not clearly synchronized with seasonal cycles of superficial seawater temperature and that other factors, presumably the amount of food supply, may be responsible for an anticipated gonadal maturation.

KEY WORDS: reproductive cycle, intraspecific variation, Aequipecten tehuelchus, san Matías Gulf

INTRODUCTION

The tehuelche scallop *Aequipecten tehuelchus* (D'Orb., 1846) is one of the most important commercially fished bivalves in eastern Patagonia (San Matías and San José gulfs). Landings have fluctuated from 4,700 to 100 tons during the last 30 years. Fishery measures include minimum commercial size, rotation of fishing areas, and selection of catch on board (Orensanz et al. 1991). The fishery operates during winter months to obtain maximum yield and to prevent capture during reproductive periods.

The tehuelche scallop is hermaphroditic (Christiansen & Olivier 1971). As in other pectinid species, the male gonad is white-yellow and the female gonad is orange ("coral"). Gamete development is easily viewed within the acini, and light varies with the gametogenic stage (Barber & Blake 1991). Christiansen et al. (1974) delineated the reproductive cycle of a San Matías Gulf population based on irregular samplings in the region. Fecundity ranges from 2 to 17 million eggs for scallop sizes of 35–90 mm (Orensanz et al. 1991). The reproductive cycle of the populations from San José Gulf (43°S) was studied by Lasta and Calvo (1978). Partial spawnings begin in late spring and pre- and post-spawning stages coexist as far as the end of the summer. Despite the high value of this fishery in San Matías Gulf (41°S), the reproductive cycle was not studied in detail until this work.

There is extensive literature on the gametogenic cycle and the timing of spawning in many bivalve species (Giesse & Pierce 1974, Sastry 1979, Newell et al. 1982, Barber & Blake 1991). For several pectinid species in the northern hemisphere, spawning occurs at higher temperatures and later in the year in southern populations (Barber & Blake, 1991) and is often more synchronized at higher latitudes (Bricelj et al. 1987).

The objective of this study is to describe the reproductive cycle of the tehuelche scallop of San Matías Gulf and to compare the reproductive patterns between years for the same population.

MATERIALS AND METHODS

Specimens of tehuelche scallop were collected from the northern bank of the San Matías Gulf (Orengo, 40°52′S; 64°32′W) (Fig. 1), at a depth of 18–24 m, between August 1997 and July 1999. Monthly samples of 20 specimens, with a mean shell height of 67.05 mm (SD: 10.1), were used to determine the gonosomatic index (GSI) and muscular index (MI) before fixation. Scallops were dissected; the shells removed; and the total weight (TW), soft parts wet weight (SPW), muscular weight, and gonad wet weight (GW) were recorded. The GSI was calculated using the equation (Giesse & Pierce 1974, Jaramillo et al. 1993):

$$GSI = \frac{GW \times 100}{SPW}$$

Mean GSI was estimated for each monthly sample. To analyze multiyear trends in reproduction, data collected in 1997 and 1999 were coupled to a GSI data set already available for the Orengo area from 1989 and 1991, and both data sets were related to sea water temperature. Minimal average values following high average

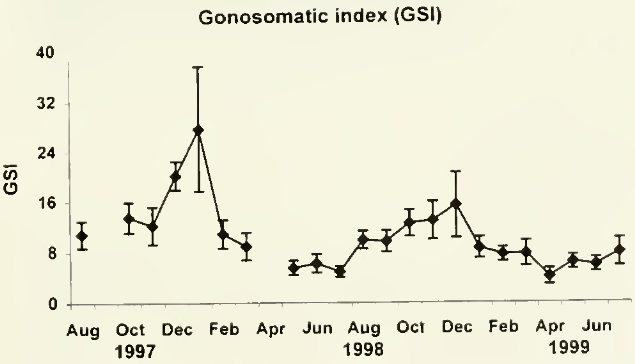

Figure 1. Temporal changes in the GSI of tehuelche scallop deployed in Playa Orengo, San Matías Gulf, from August 1997 to July 1999. Data points are mean values ± standard deviations; $n = 20$ scallops per sampling date.

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*Corresponding author. E-mail: maitenarvarte@canaldig.com.ar values were considered as indicators of spawning (Jaramillo et al. 1993, Avendaño & Le Pennec 1997).

The mean muscular index (MI = wt. of adductor muscle/TW × 100) was estimated monthly in a similar way than GSI and was compared with GSI data.

The gonads from those 20 specimens were then processed for histologic analysis. The whole gonad was fixed in Davidson's solution, stored in ethanol (70%), and then dehydrated using a series of increasing ethanol solutions (Howard & Smith 1983). Then, the samples were embedded in paraffin and sections 5- to 7-µm thick were cut and stained with Harris hematoxylin and eosin.

Gonad tissue was quantitatively examined using the schemes of Lasta and Calvo (1978), Dibacco et al. (1995), and Pazos et al. (1996) to assess developmental stages. The percentage of each gonadal stage was analyzed. The definition of each stage (early-mid mature, ripe, partial spawn, spawning and recovery, and spent) is provided in Table 1. Photomicrographs were taken using a standard microscope at magnifications of 100 and 400×. Oocytes were measured and analyzed according to Dibacco et al. (1995). Mean oocyte diameter was estimated by measuring the diameter of 250 oocytes of each individual. This process was repeated for five individuals from the total monthly sample. Only those oocytes with clear nucleolus were considered for the analysis.

The GSI and adductor MI indexes were compared by means of a simple ANOVA using data that had been subjected to an arcsine transformation $[\sqrt{(p)}]$ (Sokal & Rohlf 1979). The maximum values of the oocyte diameter were compared between years using a simple ANOVA.

RESULTS

Gonosomatic Indices

During this two year study, major peaks in GSI were observed in January and December 1998. Peaks were followed by decreases in GSI, representative of spawning events (Fig. 1). Maximum GSI was significantly different ($F_{1,39}$ = 88.88, $P < 0.001$) between these two reproductive periods. Average values of GSI ranged between 27.58 (SD: 9.93) and 4.83 (SD: 0.85) from January to July 1998, and from 15.30 (SD: 5.17) to 4.01 (SD: 1.11) from December 1998 to April 1999.

GSI increased between July and October, indicative of early stages of gametogenesis. GSI then reached maximal values from November to January and subsequently dropped over a short period, which indicated a massive spawn. Spawning continued to April, when the last spawning events were detected and after which the GSI was annually the lowest (May through June). A resting period of 1–2 mo was observed after the autumn spawn.

The magnitude of spring and autumn spawning periods, estimated by observing the extent of drops and peaks of the GSI, varied interannually, particularly for the spring spawn.

Histologic Analyses

Sperm and oocytes were observed together in the gonoduct (Fig. 2a). The male portion spawns over a longer period than the female gonad (Fig. 2b) and, consequently, the female gonad development is what determines the reproductive viability. For this reason gonadal cycle was described only for the female portion of the gonad.

Oocyte Characteristics

Absence of vitello was typical of the development oocytes, as well as a defined nuclei (Fig. 2c). Mature oocytes had a diameter around 50–60 µm, with a high quantity of vitello around a conspicuous nuclei (Fig. 2d and 2e). Reabsorbed oocytes (both mature and non-mature) remained attached to the follicle wall or free inside it. They were easily distinguished by the loss of the round-polyedric shape, typical of mature oocytes, and by the diffuse contour of the cell membrane (Fig. 2f). The central portion of the gonad, or the transitional zone between female and male portions, had a high frequency of atresic oocytes mainly in the ripe stage during August–October 1997 (Fig. 2h).

TABLE 1.

Main histological characteristics of gonadal maturity stages of the tehuelche scallop. Gonadal stages were determined by histological analysis and scored using a modification of Lasta and Calvo (1978).

Stages	Description
Early maturation	Follicles are well-defined. The lumen may occupy up to 50% of the follicle.The interfollicular space is highly visible. *Male:* few layers of cells (germ cells and spermatogonia) along the follicle wall. *Female:* presence of germ cells, oogonia, and primary oocytes with diameters varing between 10 and 25 µm.
Mid-maturation	The follicles occupy up to 75% of the gonad. Follicular lumen is much reduced. Interfollicular space is still present but limited. *Male:* follicles are full of spermatocytes I and II and spermatids. *Female:* stalked oocytes I, 25 to 40 µm, protrude into the follicular lumen but still attached to the wall (peduncled oocytes).
Ripe	The follicles are completely full of ripe gametes, and there is no interfollicular space. *Male:* spermatozoa occupy almost 100% of the follicles. They are in order and oriented with the flagella toward the centre of the follicle. *Female:* follicles are full of free oocytes up to 50 µm diameter. They have polygonal shapes, being pressed against each other.
Spawn and recovery	Ripe gametes are being released through the gonoducts. Presence of a second series of germ cells attached to the follicular wall. *Male:* presence of spermatocytes I and II, and spermatozoa are disordered in the follicular lumen. *Female:* presence of oocyte I attached to the wall and polygonal oocytes free and disordered in the follicular lumen.
Partial spawn	Follicles remain full of mature gametes, but some of them look lax because some gametes were released. Some phagocytes are present in the interfollicle space. *Male:* spermatozoa are disordered and separated in the follicular lumen. *Female:* remained oocytes still have polygonal shapes and they are free or attached at one side of the follicle.
Spent	Most follicles are empty. Interfollicular spaces become very noticeable. Phagocytic cells are very abundant. Residual gametes may or not be present, but with no signs of gametogenesis.

Gonadal Phases

Gonadal phases, considered as the percentage of individuals of each gonadal stage over the monthly sample, differed between 1997 and 1998 and 1998 and 1999 (Table 1, Fig. 3).

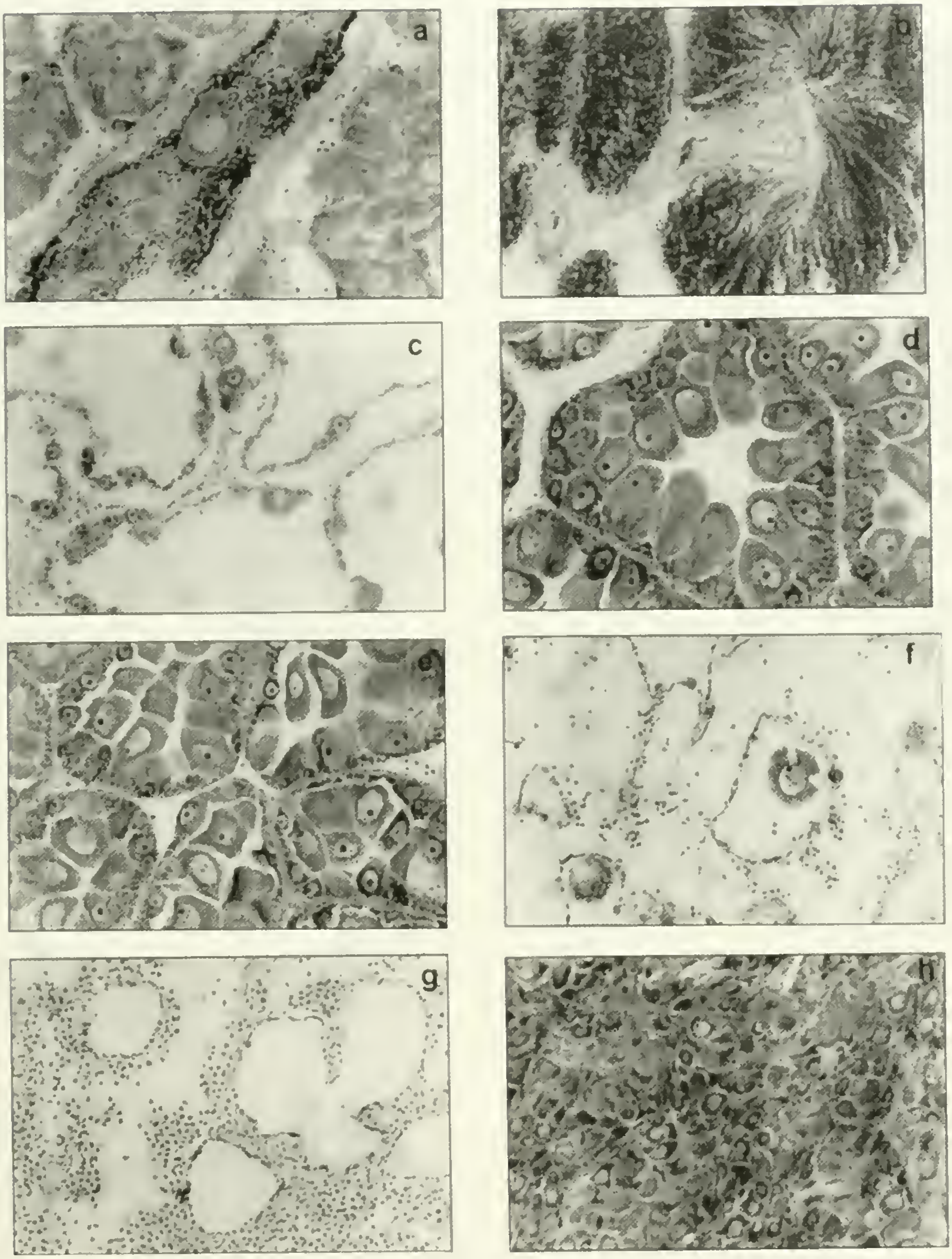

Figure 2. Photomicrographs of gonads of *Aequipecten tehuelchus* at different stages of a seasonal reproductive cycle. a, Sperm and oocytes in the same oviduct; b, partial male spawning; c–e, maturation; f, partial spawn; g, spent; h, atresic oocytes.

In 1997, 87% of the female gonads were "ripe" in August. Ripeness continued into the spring months, involving 90–100% of the total sample. The remaining individuals were spawning and recovering. A high percentage of individuals with atresic oocytes (80% in August–October) were considered ripe. A massive spawning (all individuals) occurred between December 1997 and January 1998, with individuals in partial spawning and recovery during this month. Partial spawns were observed in more than 90% of the population in February through March 1998 and most of the individuals were spent in May through July (Fig. 3).

In August 1998, the prevalent stage was mid maturation (90%). Proportions of total maturation during spring months (September through December 1998) were lower in magnitude than in the previous season, ranging from 55–85% of the sample. All individuals were in recovery in December 1998 and January 1999, indicating a spawning event had occurred. The same stage was observed in 70% of the individuals in February. Spent stages appeared gradually, increasing between March and June 1999 (Fig. 3).

Oocyte Diameter

Mean oocyte diameter reached its maximum value in December of both 1997 and 1998. Significant differences in the oocyte

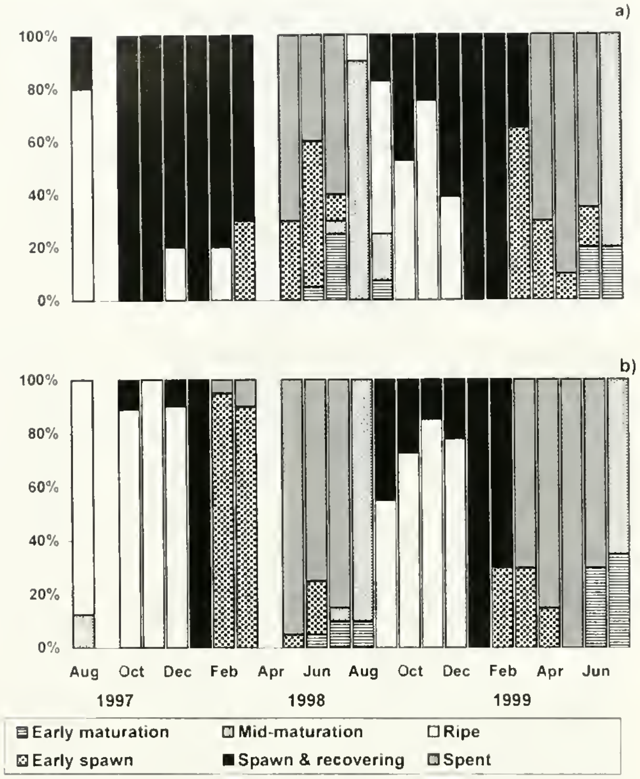

Figure 3. Diagram of the seasonal percentage distributions of the different gonadal phases in the tehuelche scallop gonads (a, male portion; b, female portion), during August 1997 to July 1999.

diameter were found between years ($F_{1,8} = 10.63$, $P < 0.05$) (Fig. 4). Maximum mean value in December 1997 was 41.98 µm (SD: 12.89), whereas a value of 36.04 µm (SD: 16.33) was found in December 1998.

Frequency distributions of oocyte diameters are presented in Figure 5. Mature oocytes were representatively observed in August 1997. Proportion of mature oocytes decreased in October and increased in November. The highest fraction of mature oocytes was observed in December of both years.

Mature oocytes were almost totally absent in January 1998 and the gonad was full of immature oocytes, indicating an intense proliferation. Diameter of these oocytes increased in February and they disappeared from the gonad in February and March. Two modes (10 and 35 µm) were present in March 1998.

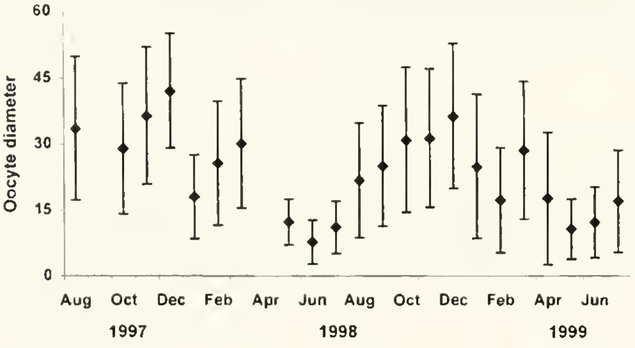

Figure 4. Mean oocyte diameter of tehuelche scallops, *Aequipecten tehuelchus*, in Playa Orengo, San Matías Gulf during August 1997–July 1999. Datapoints are mean values ± SD (*n* = 5 per sampling date).

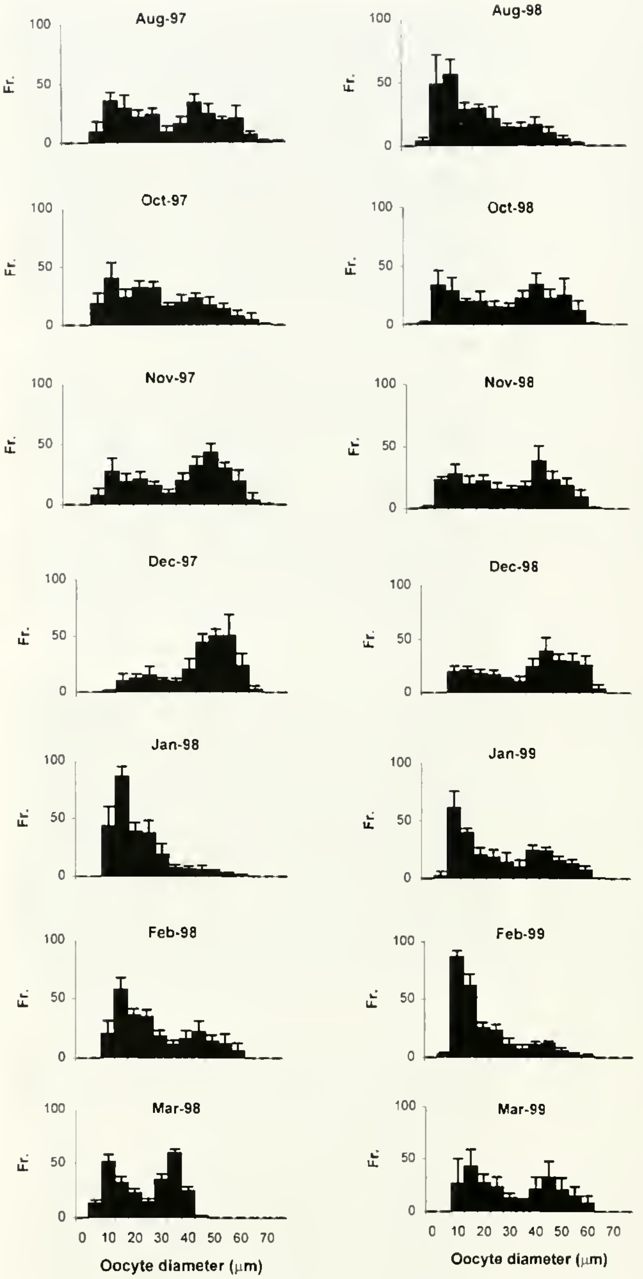

Figure 5. Frequency distribution of oocyte diameters of *A. tehuelchus* during the period from 1997 to 1999 (columns: mean values; bars: SD; *n* = 5 individuals per sampling date).

Prevalent stages found in August and September 1998 matched the initial cell series (early maturation). The co-existence of two generations of cells from September 1998 to March 1999 was characteristic of partial spawnings with recovery. A great fraction of mature oocytes disappeared from December 1998 to January 1999. In January and February, intense oocyte proliferation was observed. Numbers of mature oocytes increased again in March 1999 and decreased abruptly in April, indicating a fall spawning.

Inter-Year Variations of GSI and Its Relation With Water Temperature

Water temperature records of the 1989 and 1991 period showed slight variations between years. Differences between 1997 and

1998, however, were greater (Fig. 6a). Records of winter 1997–summer 1998 varied less ($\Delta \approx 1$°C) than records of 1998 and 1999. The highest monthly increases ($\Delta T > 1.3$°C) were recorded from September to December for the period 1997–1998 and from September to January in 1998–1999.

Figure 6b represents the variations of GSI during 1989–1991 and 1997–1999 seasons. Inter-year variations were similar for the first period. Maximum mean values oscillated between 16.15 (SD: 4.28) and 17.56 (SD: 6.02). During the 1997 and 1999 periods, differences between years were greater, ranging from 27.58 (SD: 9.93) to 15.30 (SD: 5.17).

Muscular Energy Reserves

MI reached its maximum mean value during winter-spring months, before the start of the reproductive season of 1997 to 1998 and 1998 to 1999 (Fig. 7). No differences in the MI were found between 1997 and 1998, for August, October, and November ($P > 0.05$). MI increased between January and July before gonadal development (Fig. 7).

DISCUSSION

The GSI of *A. tehuelchus* from the San Matías Gulf, studied over 2 consecutive years, indicated that this species undergoes its maximum reproductive activity during the spring–summer months. A small spawning occurred in autumn. Even when this pattern was repeated during the study period, seasonality and duration of spawnings were not identical between successive years. In both the 1997 to 1998 and 1998 to 1999 seasons, a massive spawning occurred between December and January. A second, lower-magnitude spawning occurred in March through April 1999. Even though a fall spawning was not supported by GSI in 1998, evidence of spawning was clearly observed in the oocyte frequency distribution and mean oocyte sizes.

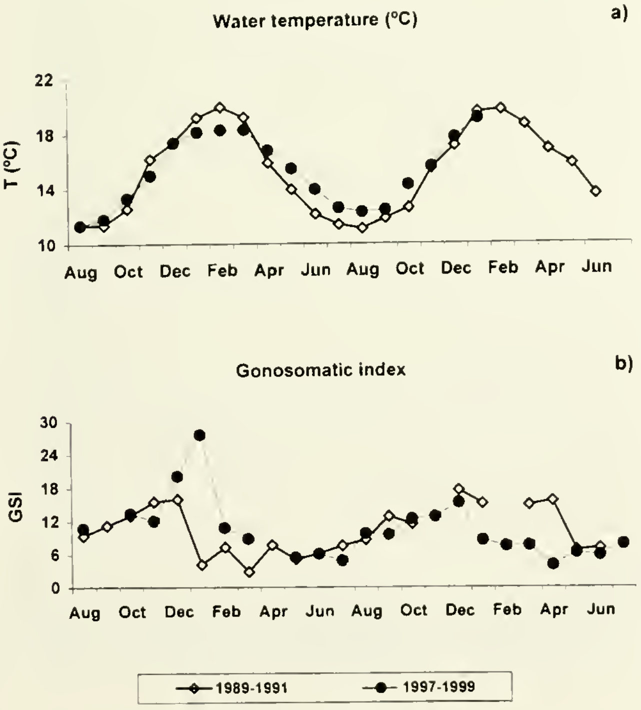

Figure 6. Inter-year variations of (a) the seawater temperature (T, °C) and (b) GSI during two study periods, 1989 to 1991 and 1997 to 1999.

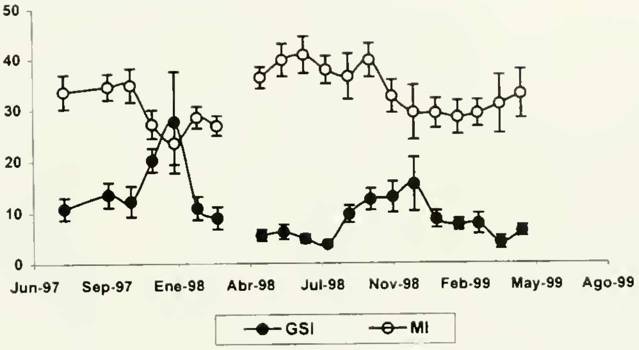

Figure 7. Variations of MI and GSI indexes from August 1997 to June 1999.

Temporal patterns of the reproductive condition of the tehuelche scallop in this study revealed some other differences between histological analyses and GSI monitoring. The main spawning in 1997 to 1998 season was detected from January to February by GSI and from December to January by histologic studies. In addition, the microscopy study on the female gonad of *A. tehuelchus* revealed the coexistence of different components of the oocyte series in those successive years. Therefore, the tehuelche scallop has asynchronous maturation and continuous spawnings within the same reproductive season.

The existence of an early mature stage in August was confirmed by the oocyte diameter frequency distribution. Nevertheless, the great percentage of atresic oocytes in August–October suggests that spawning did not occur. Greater than 50% atresia has also been observed in other pectinids (Lubet et al. 1987a, Lubet et al. 1987b, Avendaño & Le Pennec 1997, Román et al., unpublished data). Dibacco et al. (1995) suggested that during unfavorable environmental conditions, bivalves may have a mechanism to reabsorb and use the high energy content of mature oocytes. As in other pectinids (Motavkine & Varaksine 1983, Beninger 1987, Dorange & Le Pennec 1989, Avendaño & Le Pennec 1997), the highest levels of atresia in *A. tehuelchus* were observed just before spawning. These authors have suggested that atresia should be related with an energy deficiency, caused by low food availability. This could have occurred during the winter months in 1997 in San Matías Gulf, when lower water temperature was recorded.

Histologic observations of the female gonad during the fall spawning of both reproductive seasons showed a high relative proportion of oocytes in early stages, whereas larger oocytes were observed undergoing lysis. Metabolites produced by lysis of mature oocytes by phagocytic cells, present in large quantities at the end of the reproductive period, would implicate an important energy contribution for the recovery after the reproductive season. This phenomenon was previously observed for a variety of pectinids (Dibacco et al. 1995, Avendaño & Le Pennec 1997). Accordingly, we could infer that the second spawning of each season was smaller than the first, and constituted a minor contribution to that year class.

The utilization of the adductor muscle energy reserves for reproduction was cited for other pectinids (Barber & Blake 1991). Variations of MI observed along two annual cycles in this study showed a similar pattern, in which the energy stored in the adductor muscle is then diverted to the reproductive system, to satisfy energetic demands for the synthesis of gonad components (Gould et al. 1988). When the MI was compared between years, no clear variations were observed between 1997–1998 and 1998–1999. Therefore, energy demands from the muscle seemed to be similar. However, the extraordinary GSI values in 1997–1998 suggest a higher energy availability for the gonad development. Because muscular reserves are limited by muscular degradation, other exogenous mechanisms could help to sustain the energy budget for this development. A higher food availability during the spring 1997 was probably responsible for this extraordinary gonadal development. Thompson and MacDonald (1991) have indicated that temperate species such as *Placopecten magellanicus* are exposed to phytoplanktonic "blooms" in early spring and therefore have a short period of energy reserve accumulation. This early investment for reproduction would depend of the environmental conditions following the bloom (Dibacco et al. 1995).

de Vido de Mattio (1984) and Orensanz (1986) indicated that energy for gonadal development for *A. tehuelchus* in the San José Gulf seems to come more from food than from the energy stored in the muscle. In the San Matías Gulf, a decrease of MI before spawning indicates that this population uses both energy sources, food and muscle. As was observed in other pectinids (Langton et al. 1987, Barber & Blake 1991, Dibacco et al. 1995, Rheault & Rice 1996, Avendaño & Le Pennec 1997), exogenous factors, such as temperature and food availability, may produce great variations in the gonadal development and the spawning time of *A. tehuelchus*.

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REPRODUCTIVE AND RESERVE STORAGE CYCLES IN *AEQUIPECTEN OPERCULARIS* (L., 1758) IN GALICIA, NW SPAIN

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ABSTRACT A study was conducted on the reproductive and reserve storage cycles in *Aequipecten opercularis* (L., 1758) from deep waters (50–60 m) in the Ría de Arousa (Galicia, NW Spain). The reproductive cycle was studied by visual inspection of the gonad, the use of the gonad condition index, the gonad dry weight of a standard queen scallop 50-mm height, the average oocyte diameter and the percentage of the gonad occupied by oocytes. The same results were obtained with each of these methods. Following a resting period in autumn, which varied in length depending on the year, there was a period of recovery in winter, followed by a spawning period lasting until the end of summer. There were several partial spawnings, followed by recovery periods until the gonad was totally spent. Accumulated reserves in the adductor muscle (glycogen and proteins) and digestive gland (lipids) were used up during gametogenesis. The weight and condition indices of both organs and their reserve substrate contents showed parallel changes throughout the two years of the study.

KEY WORDS: *Aequipecten opercularis*, Ría de Arousa, breeding season, reproduction, energy reserves

INTRODUCTION

Aequipecten opercularis (L., 1758) is distributed along the North Atlantic coast, ranging between 30°N and 70°N, and also extends to the Mediterranean and the Adriatic Sea (Brand 1991). There is one commercial fishery of this species in Galicia, which in the 1960s caught up to 700 tonnes year^{-1}, although nowadays the catch rarely exceeds 100 tonnes year^{-1}.

Despite the commercial interest in *A. opercularis* in Galicia, investigation of the reproductive biology of natural populations in this region has not been performed. Only the reserve storage cycle and breeding season have been previously been described, by Román et al. (1996), for cultured queen scallop in the Ría de Arosa.

Mason (1983) and Barber and Blake (1991) reviewed the periods of gametogenesis in queen scallop populations in the North Atlantic. However, the information provided is scant and the reproductive behavior of geographically separated populations of the same species may vary (MacDonald & Thompson 1988, Bricelj et al. 1987, Devauchelle & Mingant 1991).

As gametogenesis, the storage and use of energy reserves in scallops is also cyclical and underlies the reproductive cycle. The importance of stored reserves in the reproductive cycle of pectinids in temperate waters is well documented (see Barber & Blake 1991 for a review). In general, protein and/or glycogen are stored in the adductor muscle and lipids in the digestive gland. Barber and Blake (1981) reported that in *Argopecten irradians*, the relative importance of different parts of the body as energy storage sites is highly adaptive, and results from genetic divergence or non genetic adaptation to different environments within the geographical range of the species.

This study is part of an investigation of the feasibility of cultivating *A.opercularis* in Galicia from spat obtained from natural environments using collectors. Studies of the reproductive behavior of this species are required to optimize spat collection for commercial purposes. The main objectives of this study are to describe gametogenesis, the breeding season, seasonal changes in weight of reserve storage organs and their biochemical composition in relation to the reproductive cycle, and the influence of environmental factors on gametogenesis and spawning.

MATERIALS AND METHODS

Study Area

The largest population of queen scallops in the Ría de Arosa are found in "The Channel" (Fig. 1), at a depth of between 50 and 60 m. The study was therefore centered on this area, although disperse, smaller banks are found in shallower waters.

Environment

From January 1996 until December 1997 weekly recording was made of temperature, salinity and chlorophyll ***a*** at a depth of 45 m using a CTD. From March to September 1996 fortnightly determinations were made of the particulate organic matter, by filtering a sample of 2 L of seawater, collected from a depth of 50 m, using pre weighed Whatman GFC/C filters (Whatman International Ltd., Maidstone, England). After drying at 60°C for 24 h, the filters were weighed then reweighed after combustion at 450°C for 12 h. The organic matter content was calculated as the difference between the two weights.

Animals

Adult specimens of *A. opercularis* were collected between January 26,1996 and November 18, 1997. Captures were made at fortnightly intervals in "The Channel" from a commercial fishing boat, by trawling. The animals measured between 35 and 65 mm in height, which corresponds to age year classes 1 and 2. The gonads of 10 scallops were placed in Bouin's fixative immediately following capture. The fixative was removed by frequent washing with 70% ethanol saturated with lithium carbonate and then stored in 70% ethanol until being processed. Classic histologic techniques were used; gonad was embedded in paraffin and serial sections of ovary (5 μm) stained using modified Wheatley's stain. Histologic study was only made of ovary tissue, using stereological methods (Paulet & Boucher 1991, Pazos et al. 1996) with a Visilog 5.1.1 image analyzer. The average diameter of the oocytes (Dm) and the

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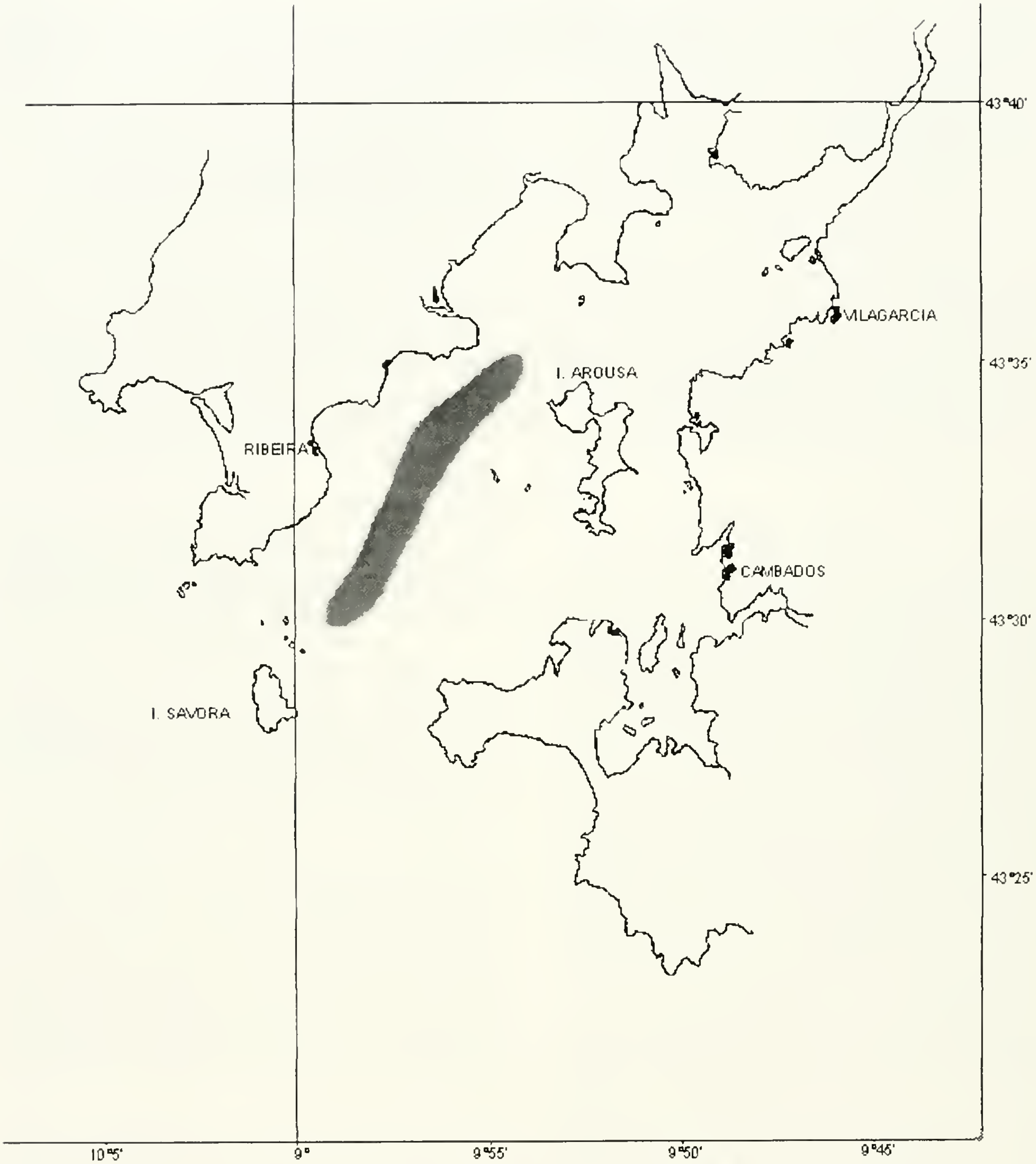

Figure 1. The Channel (shadowed area) in the Ría de Arosa.

percentage volume occupied by oocytes (OVF = % of ovary occupied by non atretic oocytes) were calculated.

Another 30 animals were transported to the laboratory in a coolbox and then maintained under running water for 18 h to allow sand to be expelled. The specimens were then opened and the soft tissue separated into 4 components: gonad, adductor muscle, digestive gland, and remaining tissues. After draining for 10 min on filter paper, the wet weight (WW) was determined; an aliquot of each organ was used to determine the dry weight (DW 100°C, 24 h). We recorded the appearance of the gonad, noting if transparent and colorless or if there were two clearly differentiated areas of color (white or cream in the testicle, orange or red in the ovary).

The height of the shell was measured to the nearest 1 mm, using calipers. The shells were dried (100°C, 24 h) and weighed. Condition indices were calculated for gonad (Gci), digestive gland (DGci), muscle (Mci) and remaining tissues (RTci) using the following equation:

CI organ = 100 × DWorgan/DWshell (Lucas & Beninger, 1985).

Regression equations were used to describe the relationships between shell height and the dry weight of each component, and the weight of each component for a standard animal having a shell height of 50 mm was calculated according to Taylor and Venn (1979).

Aliquots of muscle, digestive gland and gonad were lyophilized before biochemical analyses were carried out (glycogen and protein content of muscle and total lipid content of digestive gland and gonad) following previously described techniques for lipids and glycogen (Román et al. 1996). Protein contents were determined from the N content, measured using an elemental analyzer Carlo Erba 1108. The amount of N was multiplied by 6.25 to estimate the amount of proteins (Ansell 1974, Beninger 1982). Grams of lipids in gonads and digestive gland and glycogen and protein in muscle were estimated by multiplying the percentage of each reserve substrate by the previously calculated standard dry weight of each component.

Statistical Methods

One-way ANOVA was used to test for significant differences among the average values of the condition indices, Dm and OVF. The normality of the distribution of the variables was tested using the Kolmogorov–Smirnof test and the homogeneity of the variables using Bartlett's test. Where necessary, arcsine transformations ($x' = \arcsin\sqrt{x}$) were carried out. Correlation coefficients were calculated for the relationships among gonad indices, Dm and OVF, and among the condition indices of the reserve storage organs, the standard dry weight and biochemical composition.

RESULTS

Environment

The temperature varied little throughout 1996, ranging between 12.7°C and 14.4°C. In 1997 very high temperatures of 17 to 18°C were recorded in May and June. High values of 16.5 to 17°C were also recorded from the end of October until the middle of November. The chlorophyll ***a*** content was low in both years, ranging between 0.6 and 1.3 μg L^{-1}; the lowest values were registered between October 1996 and May 1997 and did not rise above 0.8 μg chlorophyll ***a*** L^{-1}. Particulate organic matter ranged between 0.3 and 2.7 mg L^{-1}. Salinity remained practically constant at 35.7–35.8‰ throughout most of the sampling period, with slight decreases occurring in winter to below 35.5‰. The minimum value, 34.2‰, was recorded in November 1997 (Fig. 2).

Gonad

During winter and spring of 1996 the gonads were well differentiated, with testicle and ovary clearly visible in all specimens. From July 23, 1996 onwards there was an increasing percentage (reaching a maximum value of 90% on September 10) of queen scallops with spent gonads, which were flat, colorless and watery; histologic analysis revealed empty follicles in the undeveloped gonads. In these queen scallops that were in a period of sexual repose, the average diameter of the oocytes and the percentage volume occupied by oocytes was considered to be zero (Fig. 3).

Following the period with the maximum percentage of sexually reposing animals, there was a period of gonad recovery, which involved only part of the population, peaking (at 60%) on October 26, then a new minimum (30%) was observed on November 19. From December 1996 onwards there was a new period of gonad recovery, which this time involved the whole population, reaching full development on January 28, 1997. Following a decrease observed on February 12, which affected 40% of the population, all animals had developed gonads. This situation persisted until the end of summer, then from August 12 until October 28, 1997 all of the population was in sexual repose and displayed spent gonads. In 1997, gonad recovery took place between October 28 and November 19.

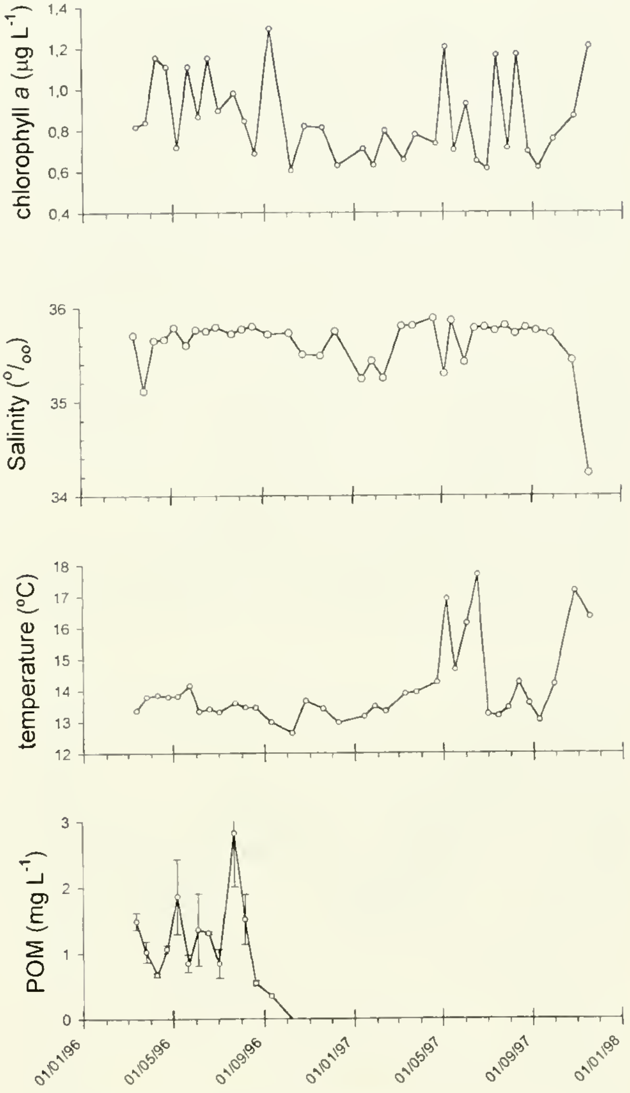

Figure 2. Environmental conditions in The Channel.

The value of the gonad condition index (Gci) ranged between 0.1 and 2.0 (Fig. 3). Maximum values corresponded to the period between March 13 and 26 1996 followed by a significantly lower value on April 10. There were then a series of recoveries followed by decreases to significantly lower values on June 4, July 2, and August 20; minimum values were recorded coinciding with gonads being totally spent. There was a new peak (significantly higher) on October 20, the gonads then became totally spent before undergoing recovery from November 19 onwards. The peak recorded on January 28, 1997 was followed by a significant decrease on February 12 and then again, as in 1996 there were a series of recoveries and decreases, with significant decreases observed on March 25, May 17, July 1, and August 12, which coincided with the

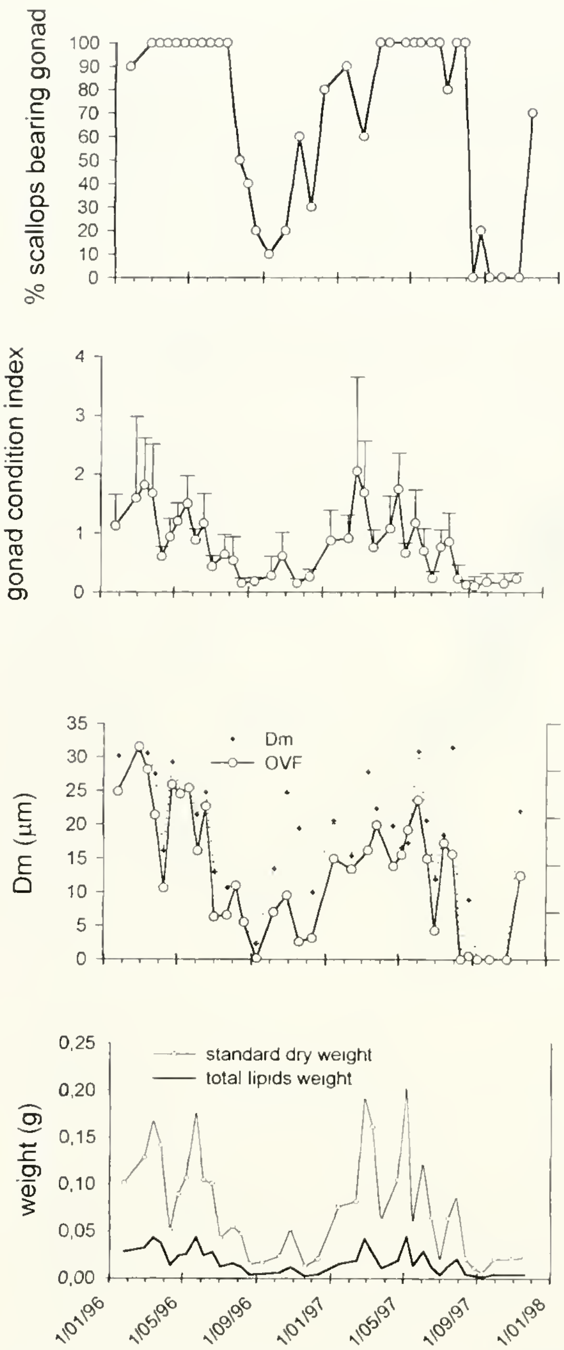

Figure 3. From top to bottom, a) percentage of scallops bearing gonad, b) gonad condition index, c) oocyte mean diameter and percentage volume of the ovary occupied by oocytes, and d) gonad dry weight of a standard 50 mm height scallop and lipid content of the gonad.

gonads being totally spent; recovery did not take place until November 19. The decreases were assumed to be associated with spawning.

Changes in the average diameter of the oocytes and of the percentage volume of the ovary occupied by oocytes (OVF) were similar (Fig. 3). Maximum values occurred in winter (February to March 1996) then a sharp decrease was observed on April 10, 1996. The values had significantly increased by April 24 and there was then a gradual decrease until reaching minimum values between September 10, and October 8, 1996, with two recoveries observed on June 19 and August 6. There were significant increases in both Dm and OVF between December 16 and January 14, coinciding with gonad recovery. In 1997 there were 4 peaks observed on January 28, March 10, June 3, and July 29 followed by decreases, which only on the latter date was not followed by gonad recovery.

The changes in all of the parameters studied followed the same pattern and each parameter was significantly correlated with the others: Gci vs. Dm (r_s = 0.588, $P < 0.0001$); OVF vs. Dm (r_s = 0.772, $P < 0.0001$), and Gci vs. OVF (r_s = 0.716, $P < 0.0001$).

The gonad dry weight of a standard queen scallop ranged between 0.006 and 0.204 g. The seasonal variations are shown in Figure 3. Although the mean percentage value of the total weight was low (9.0%) the range of values varied greatly, between 0.7% and 23.0% of the dry weight of the animal. There was a large variation in weight, which followed the pattern of variation of Gci.

Biochemical Composition of the Gonad

The percentage values of lipid contents are shown in Table 1. The total lipid content of the gonad of a standard queen scallop varied in a similar way to the Gci and to the standard DW, with maximum and minimum values coinciding with the maximum and minimum values of the condition index and the standard dry weight (Fig. 3).

Somatic Tissues

Seasonal changes in the standard dry weight of the muscle and the digestive gland, as well as in their respective condition indices and in their reserve substrate contents (lipids in digestive gland, glycogen and proteins in adductor muscle) are shown in Figure 4. Somatic growth began in early summer and was demonstrated by increases in dry weight, condition index and specific reserve substrate content, which reached maximum values between autumn and winter. There was then a decrease in the values of all of these parameters.

Adductor muscle accounted for most of the weight of the queen scallop, on average 54.5%; the mean dry weight of the muscle varied between 35.6% and 68.1%, representing the greatest variation in the weight of the animal. The dry weight of the muscle of a standard animal varied between 0.273 and 0.993 g, (i.e., the mean dry weight varied by up to 3 times). The condition index of the adductor muscle (Mci) varied in a similar way, ranging between 3.3 and 8.5.

Digestive gland accounted for a small proportion of the total weight of the animal (mean, 10.3%, range 6.9%–14.2%). Although the weight varied more than that of the muscle, ranging between 0.042 and 0.198 g, (i.e., varying by almost 5 times) the condition index (DGci) ranged between 0.5 and 1.7 (i.e., it varied by more than three times).

The dry weight of the remaining tissues accounted for, on average, 26.0% of the weight of the animal, ranging between 17.3% and 39.1% of the somatic components of the standard queen scallop. The dry weight of the remaining tissues of the standard specimen ranged between 0.144 and 0.310 g (i.e., it varied by approximately two times).

The percentage amounts of glycogen and proteins in the muscle and of lipids in the digestive gland in the gonad are shown in Table 1.

In January 1996, high values were recorded of standard dry weight of muscle, Mci, percentage, glycogen, and total protein and

TABLE 1.

Seasonal changes in the composition of *A. opercularis*.

Date	Digestive Gland % Lipids	Gonad % Lipids	Adductor Muscle % Glycogen	Adductor Muscle % Protein
Jan 25	57,52	28,07	14,74	71,68
Feb 28	42,70	25,27	7,28	80,19
Mar 13	32,04	25,88	5,19	80,22
Mar 26	34,96	26,63	5,32	77,34
Apl 10	27,40	27,04	4,27	71,95
Apr 24	28,72	26,89	4,41	77,61
May 07	22,08	24,20	3,34	80,17
May 23	35,00	24,67	3,47	72,19
Jun 05	33,75	23,30	4,49	69,38
Jun 19	44,26	27,89	4,39	76,00
Jul 02	42,77	29,14	6,11	72,51
Jul 23	50,55	29,41	6,84	70,64
Aug 06	54,86	25,87	7,85	69,77
Aug 20	61,02	27,05	9,21	73,42
Sep 10	48,04	29,57	16,68	67,23
Oct 08	54,81	26,71	18,02	64,44
Oct 26	49,50	22,64	18,92	69,33
Nov 19	44,34	18,86	14,03	72,30
Dec 10	41,61	21,80	12,56	70.01
Jun 14	47,20	20,61	13.03	74.83
Feb 12	62,45	23,54	8,55	74,89
Feb 25	41,81	22,12	5,63	75,66
Mar 10	34,63	17,32	8,20	74,52
Mar 25	28,66	18,21	6,25	73,07
Abr 22	25,76	18.54	2,65	77,87
May 06	31,80	22,03	6,61	77,02
May 17	25,38	23,73	2,93	76,26
Jun 03	36,65	23,73	2,76	74,26
Jun 17	39,01	18,95	5,76	78,27
Jul 01	39,31	22,31	6,36	73,45
Jul 15	44,40	22,11	8,44	—
Jul 29	49,11	23,70	8,48	—
Aug 12	52,66	23,87	11,98	—
Aug 26	57,81	30,00	13,91	72,10
Sep 09	59,36	25,67	14,74	71,58
Sep 29	57,64	23,31	14,79	72,76
Oct 28	60,75	21,28	18,68	66,25
Nov 18	62,99	19,91	14,90	68.80

glycogen content; these then decreased rapidly and minimum values were registered at the beginning of spring. During summer and autumn there was rapid somatic growth, with maximum weight values in November. Values of all parameters then began to decrease and the cycle was repeated, with minimum values occurring at the beginning of spring 1997, before beginning to increase once again. The percentage amount of proteins in the muscle showed a more irregular pattern of variation, with frequent oscillations between 70% and 80% between March and September. Following a minimum of 65% in November the values began to increase showing a similar pattern to that of the previous year.

The changes in standard dry weight of the digestive gland, DGci, percentage lipids, and total lipid content showed parallel variation, with the pattern being very similar to that observed for the studied muscle parameters. The greatest differences were that in both years growth of the digestive gland began later than that of the adductor muscle and also that in May of both years there was a sharp increase in growth, only found in the muscle in 1997.

There was no clear pattern of variation for the remaining tissues, or any correlation with the other somatic components.

There were highly significant positive correlations among condition index, standard dry weight and lipid content of the digestive gland and among condition index, standard dry weight and glycogen and protein contents of the muscle (simple regression, $P < 0.01$). Likewise, there were statistically significant correlations between the muscle and digestive gland condition indices ($P < 0.01$), between the "standard" dry weight of the muscle and of the digestive gland ($P < 0.01$) and between the lipid content of the digestive gland and glycogen and protein contents of the muscle ($P < 0.01$). Only the percentage amount of proteins in muscle correlated negatively with the other parameters.

Spearman's correlation analysis did not reveal any significant correlation between environmental variables and the parameters used to study reproduction and the cycle of reserve storage.

DISCUSSION

In this study we describe the spawning cycle and the cycles of storage and use of reserves in a population of *A.opercularis* from relatively deep waters (50–60 m) in the Ría de Arousa.

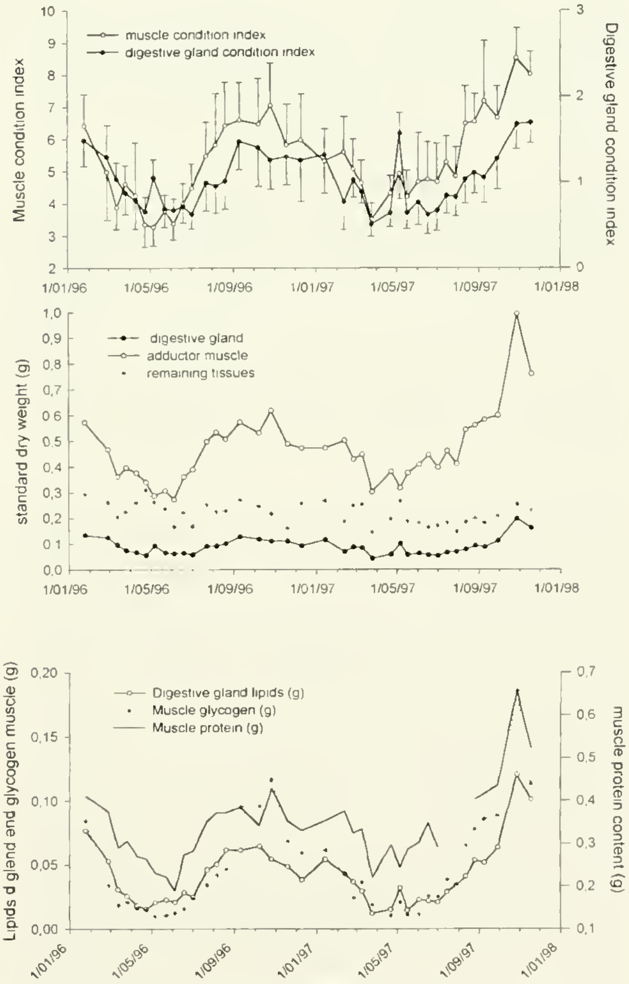

Figure 4. From top to bottom, a) muscle and digestive gland condition indices, b) dry weight of the somatic components of a standard scallop 50 mm height, and c) biochemical composition of the specific reserve substrates of the organs of energy storage.

Spawning Cycle

The spawning cycle of *A. opercularis* was studied by visual observation (presence or absence of developed gonad) as well as by quantitative (Gci) and qualitative methods (Dm and OVF) (Barber & Blake 1991).

Despite the simplicity of the first method, its use provided a general idea of when spawning took place—from winter until the middle of summer—there being a gradual decrease in the number of specimens with gonad as the summer progressed, culminating in the gonads being totally spent. There then followed a period of gonad recovery in autumn. Taylor and Venn (1979) also found the maximum number of spent scallops in summer in the Clyde Sea Area, although both the time of appearance of animals without gonad and the recovery period were earlier than found in the present study. It is possible that the rough estimate provided by this method would only be applicable in species or populations with very marked seasonal variations and in which gonads become totally spent.

The consistency of the results obtained by the different methods of establishing the different processes involved in the reproductive cycle (Gci, OVF, Dm) may be because Gci decreases due to release of mature, larger oocytes. As a result, there is an obvious decrease in the diameter of the remaining oocytes and of the percentage space occupied by them. Furthermore, as seen in the graphs outlining the changes in Dm and OVF (Fig. 2), increases in Gci coincided with cytoplasmic growth and vitellogenesis, (therefore larger oocyte diameter and greater percentage space occupied).

Spawning was indicated by the decrease in the Gci, the decrease in the mean diameter and the percentage space occupied as well as by the increase in the percentage number of spent gonads and the decrease in "standard" dry weight and lipid content.

Optimization of spat collection in natural environments depends on knowledge of the reproductive cycle, the changes in which must be monitored over time. The consistency of results independent of the method used allows monitoring of the reproductive cycle of this species without the need for use of sophisticated, expensive techniques by, simply recording the changes in Gci.

Following spawning, we recorded a period of gonad recovery, characterized by undifferentiated gonads, without gametes, as previously described by Allarakh (1979), who observed a very short recovery period of less than a month. In this study, this period lasted for 1 month in 1996 (September 10 to October 8) and for almost 2 months in 1997 (September 9 to October 28). In 1996 the period of sexual repose was shorter because there was partial maturation in autumn (from October 8), which was not observed in 1997. In both years gonad recovery, which involved the whole population, started at the end of autumn or beginning of winter and was rapid, as the first spawning took place in January; there were then a series of successive periods of maturation and spawning that lasted until the middle of summer. As the summer progressed, the number of individuals participating in reproduction gradually decreased (1996), or all suddenly displayed spent gonads (1997).

We conclude that the reproductive strategy of this species consists of a very protracted spawning period with frequent partial release of mature oocytes, followed by recovery due to the rapid maturation of oocytes present in the follicles, by vitellogenesis. These ovocytes are in turn spawned while a new cohort of oocytes matures. The successive recovery and spawning periods involve gradually less oocytes, until the gonads are totally spent; the final spawning is therefore not the most important. The whole population participates in reproduction during the period of greatest gametic activity, from February to July, but between August and January, only part of the population is involved.

Descriptions of the reproductive cycle of *A. opercularis* in northern areas, revised by Mason (1983) and Barber and Blake (1991), are mainly based on visual observation of gonads, although Taylor and Venn (1979) used the dry weight of gonad to describe the spawning cycle in Clyde Sea area; only Allarakh (1979) used histologic methods to study the reproductive cycle in scallops from Rade de Brest. In general these studies describe partial spawning taking place at different times, and the main spawning in autumn. Allarakh (1979) for example, described three natural spawnings in Rade de Brest, the first two in January to February and June to July and the third in September, ending with gonads being totally spent. Soemodihardjo (1974) and Paul (1978) reported various spawning periods in the Isle of Man, two partial spawnings in winter (January to February) and spring or the beginning of summer (May to July) and the main spawning in autumn (August to October). Amirthalingam (1928) observed spawning between January and July in Plymouth but did not record any spawning activity in autumn.

Our results are generally consistent with those of the authors cited, although the more frequent sampling and the inclusion of histologic analyses allowed confirmation that partial spawning takes place more often than previously described in other populations and that the final spawning that resulted in the gonads being totally spent, is not the most important, but merely the last before gonad recovery. In summary, partial spawning forms part of the sexual strategy consisting of staggered maturation of 4 to 5 cohorts of oocytes that are shed when mature, throughout a long breeding season that may span from January to July.

The generally small number of oocytes obtained by artificial stimulation of these animals is possibly due to this behavior (Le Pennec, 1982).

Conversion of the measurements obtained to those of a standard 50-mm animal allowed us to compare our results with those of Taylor and Venn (1979). In one of the two years of their study, these authors recorded a lower standard dry weight of gonad than in this study (range between 0.087–0.105 g), whereas in the other year the values were similar (0.170–0.198 g). In this study, the maximum values ranged between 0.168–0.204 g. This may be partly due to the lower frequency of sampling by Taylor and Venn (1979), who may have recorded dry weight values as they were rising or falling but not while at their maximum values, as we have observed considerable increases and decreases in weight within 15 days. A similar explanation may be given for the greater number of spawnings that were observed.

The lipid contents of scallops from the Ría de Arousa (maximum levels of 0.043 and 0.045 g in 1996 and 1997, respectively) were considerably higher than in those from the Clyde Sea Area (0.013–0.026 g).

Spawning took place at approximately the same times in both years, despite the large differences in temperature observed. Temperature therefore probably does not affect spawning, as previously thought by other authors; Broom (1976) investigated the possible relationship between spawning and changes in temperature, but with inconclusive results.

Cycles of Storage and Use of Reserves

By autumn somatic growth was completed, the muscle and digestive gland had reached their maximum weights and had maximum levels of reserves, the breeding season was over and gonads were spent. A new period of gonad growth began in November and from this time onwards in both years, the accumulated reserves began to diminish.

This decrease was associated with gametogenesis and the main spawning that affected the whole population during most of the breeding season. The final, partial, spawning that affects only part of the population, is apparently carried out using external energy enough to maintain gonad growth, without having to use the reserve energy stored in organs. In both years accumulation of reserves began (at the end of spring for muscle and at the beginning of July for digestive gland) even though spawning was still taking place.

The variation in the weights and indices of digestive gland and muscle indicate their status as reserve energy storage organs; the variation in the lipid content of the digestive gland and of glycogen and protein in the muscle indicate that these are the substrates used as energy reserves.

Although the percentage amount of protein in the muscle always remained high (64–80%), a large amount of protein was used (ranging between 0.189 and 0.657 g). The glycogen content varied greatly (3–18%), but the total amount used was relatively small (ranging between 0.010 and 0.185 g). As a consequence of fluctuations occurring during the cycle of storage and use of reserves, the weight of the muscle varied between 0.273 and 0.617 g and between 0.301 and 0.993 g in the first and second years, respectively. Observations were consistent with those of Taylor and Venn (1979) who describe a marked seasonal variation in the weight of the adductor muscle, with minimum values at the end of winter, gradual increases during spring and summer and maximum values in September to October, the weight sometimes doubling between March and October. According to these authors, and in agreement with our results, the seasonal variation in weight is due to changes in protein and carbohydrate contents.

Taylor and Venn (1979) attributed the variations in lipids of the remaining tissues to the digestive gland, although they did not carry out specific analyses of this organ. Observed in the Ría de Arousa were large variations in the lipid content of the digestive gland (minimum values of 0.012 and 0.016 g and maximum values of 0.133 and 0.198 g in 1996 and 1997, respectively), thereby confirming their speculations.

In contrast with the findings of Barber and Blake (1981), who observed that *Argopecten irradians concentricus* uses different substrates (glycogen, protein or lipids) of different origin (muscle or digestive gland) depending on the phase of gametogenesis, *A. opercularis* apparently uses all of its reserves simultaneously (muscle glycogen and proteins, as well as digestive gland lipids). However, these authors indicate that which are the important substrates, where they are stored and how their use is related to reproduction varies among species and among different populations of the same species. It should also be taken into account that *A. irradians concentricus* is semelparous, and has a straightforward reproductive cycle, with one spawning after which the animal dies, whereas *A. opercularis* is iteroparous, and spawns several times in one year for several years.

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KARYOTYPE ANALYSIS AND CHROMOSOME BANDING OF THE CHILEAN–PERUVIAN SCALLOP *ARGOPECTEN PURPURATUS* (LAMARCK, 1819)

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ABSTRACT The Chilean–Peruvian scallop, *Argopecten purpuratus*, is the southernmost representative of the genus and has become an aquaculture commodity in Chile as a result of 20 y of culture practice promotion after natural beds were almost exhausted by overexploitation. Chromosome preparations of *A. purpuratus* D-larvae from three samples, two from commercial hatcheries, and one from the few remaining wild populations were subjected to karyotype analysis by the usual measurements of chromosome pairs, Ag-NOR staining, as well as by the Hoechst 33258/Actinomycin D counterstain and restriction endonuclease banding techniques. The species has a modal diploid number of $2n = 32$, and the karyotype consists of 11 pairs of telocentric and five pairs of subtelocentric chromosomes, NF=32. The banding techniques, attempted for the first time in the species, served as a good complement for identifying a number of homologous chromosomes. The NOR was located terminally on the short arm and in the pericentromeric region in three chromosome pairs. The Hoechst 33258/Actinomycin D counterstain revealed tiny fluorescent heterochromatic areas in the centromeric region of chromosome pairs 1, 2, 4, 5, 6, 7, 8, 9, 11, 15, and 16, whereas a quenching zone was observed in chromosome pair 6. Endonuclease restriction banding revealed pericentromeric and telomeric bands in one chromosome pair (*Alu*I) and interstitial bands in other two chromosome pairs (*Hae*III). The karyotype and banding pattern provided in this work are likely to help an industry seeking for improved stocks. Such information will be also valuable for further investigating both intra- and interspecific genomic relationships in the Chilean Pectinids, where the number of species karyologically screened is still very limited.

KEY WORDS: *Argopecten*, karyotype, Chilean scallop, genetics, banding

INTRODUCTION

Classic techniques for chromosome analysis generally have permitted accurate assessment of chromosome numbers and morphology in a wide variety of aquatic invertebrate species (Thiriot-Quiévreux 1994). Yet the number of species investigated from the cytogenetic point of view is rather scarce, i.e., no more than 200 of approximately 15,000 species in the Bivalvia class (González-Tizón et al. 2000). Recent molecular and banding techniques have contributed to a better identification of whole or chromosome parts, and hence to a more detailed characterization of the genome organization in different organisms. Analysis of the distribution and composition of heterochromatin and identification of the nucleolar organizer regions (NORs) are among these advances (Insua et al. 1998; González-Tizón et al. 2000). In commercially exploited species, these techniques have enabled the obtaining of better karyotypes, although not fully standardized, allowing more reliable intra- or interspecific comparisons of genetic resources either for basic studies (evolutionary inferences) or for practical purposes (taxonomy, chromosomal manipulations).

The Chilean–Peruvian scallop, *Argopecten purpuratus*, is a functional hermaphrodite inhabiting sedimentary substrates in sheltered bays along the Pacific Ocean from Paracas Bay, Peru (13° South) to the Gulf of Arauco, Chile (37° South). The species used to be a commercially important member of the subtidal macro-invertebrate fauna along the northern part of the Chilean coast until overfishing almost exhausted natural beds. Since 1982, *A. purpuratus* has been farmed successfully and currently represents an important aquaculture product (Gajardo & Núñez 1992). Because of its economic importance, the species has been transplanted to southern Chile, where it does not occur naturally. Von Brand et al. (1990) ascribed it to the genus *Argopecten* instead of *Chlamys* as previously described based on the fact that both diploid number ($2n = 32$) and chromosome types (4 m+ 14 m-Sm+ 6St+ 8t) were shared with other Pectinids, particularly *Argopecten irradians* (Wada 1978). Instead, most *Chlamys* species studied so far exhibit a diploid number of $2n = 38$ and few bi-armed chromosomes (Komaru and Wada 1985).

A. purpuratus is replaced by other *Chlamys* species in the southern Chilean coast, such as *Chlamys patagonica*, *Chlamys amandi*, and *Chlamys vitrea*. As part of a broader effort aimed at the genetic characterization of *Argopecten purpuratus*, this study seeks to provide a more reliable karyotype description and chromosome pairing based on the chromosome index and banding analysis of this important aquaculture commodity. The information provided is likely to help in future cytogenetic studies of intra- and interspecific karyotype comparisons of Chilean scallops.

MATERIAL AND METHODS

Chromosome Preparations

Argopecten purpuratus D-larvae were obtained by artificial hatching of ripe animals from three different sources. Two samples were from commercial hatcheries, Cultivos Marinos Internacionales S.A (Caldera), northern Chile (24° South), and Germont S.A in southern Chile (Calbuco; 51° South), whereas the Quintay sample corresponds to animals collected in Laguna Verde (33° South). This latter location is one of the few natural beds remaining in the Central coast of Chile, close to Valparaiso.

TABLE 1.

Diploid chromosome number determination in three populations of *A. purpuratus*.

Locality	No. of Cells Observed	Chromosome Number 25	26	27	28	29	30	31	32	Modal 2n
Caldera	48	2	1	3	4	3	7	5	23	32
Calbuco	49			1	1	5	8	11	23	32
Quintay	28			1	2	4	4	5	12	32

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TABLE 2.

Chromosome measurements in three samples of *A. purpuratus*, mean relative length, 95% confidence interval ($\overline{X}$ ± ci), and standard deviation (SD) of the short arm (SA) and long arm (LA). Chromosome type according to the centromerie index (CI).

Locality	Chromosome No. of Pair	SA	LA	CI ± SD	Length ± SD (μm)	Type
		$\overline{X}$ ± ci (SD)	$\overline{X}$ ± ci (SD)			
Caldera (n = 9)	1	1.64 ± 0.17 (0.23)	7.75 ± 0.41 (0.56)	17.47 ± 1.93	4.28 ± 0.81	st
	2	1.24 ± 0.10 (0.13)^{a}	6.37 ± 0.36 (0.48)	16.26 ± 1.54	3.57 ± 0.83	st
	3	1.15 ± 0.08 (0.11)	5.60 ± 0.28 (0.37)	17.02 ± 1.37	3.10 ± 0.63	st
	4	1.10 ± 0.08 (0.11)^{a}	5.08 ± 0.39 (0.52)	17.87 ± 1.74	2.88 ± 0.53	st
	5	1.30 ± 0.19 (0.26)	4.66 ± 0.49 (0.65)	21.78 ± 3.20	2.78 ± 0.44	st
	6	0.61 ± 0.28 (0.37)^{a}	8.42 ± 0.30 (0.41)	6.61 ± 3.93	4.14 ± 0.83	t
	7	0.63 ± 0.20 (0.27)^{b}	7.24 ± 0.48 (0.65)	8.02 ± 3.50	3.65 ± 0.81	t
	8	0.55 ± 0.18 (0.24)^{b}	5.70 ± 0.35 (0.48)^{a}	8.86 ± 3.91	2.97 ± 0.78	t
	9	0.52 ± 0.22 (0.30)^{b}	5.14 ± 0.23 (0.31)^{a}	9.11 ± 5.20	2.64 ± 0.56	t
	10	0.48 ± 0.21 (0.28)^{b}	4.71 ± 0.37 (0.50)^{a}	9.45 ± 5.44	2.40 ± 0.46	t
	11	0.03 ± 0.06 (0.08)	5.89 ± 0.32 (0.43)	0.45 ± 1.34	2.80 ± 0.76	t
	12	0.05 ± 0.10 (0.14)	5.40 ± 0.38 (0.51)	0.87 ± 2.61	2.57 ± 0.70	t
	13	0.04 ± 0.08 (0.11)	4.98 ± 0.19 (0.26)	0.69 ± 2.06	2.33 ± 0.53	t
	14	0.00 ± 0.00 (0.00)	4.81 ± 0.23 (0.31)	0.00 ± 0.00	2.26 ± 0.57	t
	15	0.00 ± 0.00 (0.00)	4.55 ± 0.29 (0.40)	0.00 ± 0.00	2.15 ± 0.57	t
	16	0.04 ± 0.10 (0.13)	4.33 ± 0.29 (0.39)	1.02 ± 3.07	2.07 ± 0.55	t
Calbuco (n = 10)	1	1.47 ± 0.28 (0.39)	8.24 ± 0.74 (1.05)	15.08 ± 3.11	5.01 ± 1.84	st
	2	1.28 ± 0.27 (0.38)^{a}	6.57 ± 0.64 (0.91)	16.23 ± 4.09	4.02 ± 1.36	st
	3	1.26 ± 0.24 (0.34)	5.73 ± 0.39 (0.56)	17.96 ± 4.37	3.64 ± 1.36	st
	4	1.13 ± 0.22 (0.32)^{a}	5.18 ± 0.29 (0.42)	17.89 ± 4.88	3.30 ± 1.23	st
	5	1.00 ± 0.31 (0.44)	4.75 ± 0.83 (1.17)	17.10 ± 7.42	2.98 ± 1.06	st
	6	0.12 ± 0.15 (0.22)^{b}	8.07 ± 0.68 (0.97)	1.46 ± 2.67	4.34 ± 1.85	t
	7	0.10 ± 0.15 (0.22)^{a}	6.98 ± 0.62 (0.88)	1.47 ± 3.14	3.74 ± 1.62	t
	8	0.10 ± 0.15 (0.21)^{a}	6.30 ± 0.51 (0.72)^{a,b}	1.64 ± 3.50	3.40 ± 1.48	t
	9	0.06 ± 0.14 (0.19)^{a}	6.07 ± 0.46 (0.65)^{b}	1.08 ± 3.41	3.27 ± 1.41	t
	10	0.06 ± 0.12 (0.17)^{a}	5.80 ± 0.30 (0.43)^{b}	1.05 ± 2.88	3.15 ± 1.37	t
	11	0.07 ± 0.13 (0.19)	5.40 ± 0.30 (0.43)	1.33 ± 3.71	2.92 ± 1.23	t
	12	0.01 ± 0.02 (0.03)	5.31 ± 0.24 (0.34)	0.19 ± 0.59	2.86 ± 1.25	t
	13	0.00 ± 0.00 (0.00)	5.07 ± 0.26 (0.37)	0.00 ± 0.00	2.71 ± 1.17	t
	14	0.00 ± 0.00 (0.00)	4.85 ± 0.26 (0.37)	0.00 ± 0.00	2.58 ± 1.07	t
	15	0.00 ± 0.00 (0.00)	4.68 ± 0.27 (0.38)	0.00 ± 0.00	2.50 ± 1.06	t
	16	0.01 ± 0.02 (0.03)	4.32 ± 0.24 (0.34)	0.19 ± 0.60	2.31 ± 0.98	t
Quintay (n = 5)	1	1.23 ± 1.13 (1.13)	7.06 ± 1.59 (1.60)	14.69 ± 13.99	1.99 ± 0.42	st
	2	1.81 ± 0.20 (0.20)^{b}	6.30 ± 0.61 (0.61)	22.47 ± 3.22	1.94 ± 0.16	st
	3	1.46 ± 0.24 (0.24)	5.53 ± 0.35 (0.35)	20.84 ± 2.91	1.68 ± 0.15	st
	4	1.61 ± 0.18 (0.18)^{b}	4.85 ± 0.63 (0.64)	25.08 ± 3.97	1.55 ± 0.12	st
	5	1.28 ± 0.28 (0.28)	4.66 ± 0.72 (0.73)	21.63 ± 4.45	1.42 ± 0.14	st
	6	0.00 ± 0.00 (0.00)^{b}	7.78 ± 1.09 (1.09)	0.00 ± 0.00	1.87 ± 0.31	t
	7	0.00 ± 0.00 (0.00)^{a}	6.90 ± 0.23 (0.23)	0.00 ± 0.00	1.66 ± 0.16	t
	8	0.00 ± 0.00 (0.00)^{a}	6.57 ± 0.10 (0.10)^{b}	0.00 ± 0.00	1.58 ± 0.12	t
	9	0.00 ± 0.00 (0.00)^{a}	6.19 ± 0.18 (0.18)^{b}	0.00 ± 0.00	1.49 ± 0.13	t
	10	0.00 ± 0.00 (0.00)^{a}	5.95 ± 0.18 (0.18)^{b}	0.00 ± 0.00	1.43 ± 0.14	t
	11	0.00 ± 0.00 (0.00)	5.71 ± 0.11 (0.11)	0.00 ± 0.00	1.37 ± 0.10	t
	12	0.07 ± 0.16 (0.16)	5.51 ± 0.22 (0.22)	1.17 ± 2.61	1.34 ± 0.11	t
	13	0.18 ± 0.40 (0.40)	5.30 ± 0.22 (0.23)	2.82 ± 6.30	1.31 ± 0.15	t
	14	0.00 ± 0.00 (0.00)	5.18 ± 0.22 (0.22)	0.00 ± 0.00	1.24 ± 0.09	t
	15	0.00 ± 0.00 (0.00)	4.69 ± 0.15 (0.15)	0.00 ± 0.00	1.13 ± 0.10	t
	16	0.00 ± 0.00 (0.00)	4.16 ± 0.34 (0.35)	0.00 ± 0.00	1.00 ± 0.12	t

* Values with a different letter are significantly different (Tukey, $P < 0.05$).

The splash method was developed in our laboratory for D-larvae (24–48 h after hatch) according to the following protocol: initial colchicine (0.05%) treatment of a pool of D-larvae from each locality in sea water for 2 h, then mechanical disruption of cells, including centrifugation at 1000 rpm/10 min, hypotonic treatment in distilled/ seawater (1:1), washing to eliminate hypotonic solution (centrifuge at 800 rpm/10 min), fixation in methanol-acetic acid (3:1) for 20 min, and washing (centrifuging twice at 800 rpm/ 10 min). The pellet was re-suspended in 0.5 mL of cold fixative methanol/acetic acid (3:1) and a drop splashed onto clean slides and stained in 4% Giemsa. For diploid number determinations, between 28 and 49 of the best metaphase plates (without overlapping) were studied. These were then photographed on a Nikon Labophot microscope (with epifluorescence) for chromosome counting. In all populations, between 5 and 10 suitable cells were used for the estimation of the centromeric index (CI) following the protocol of Levan et al. (1964). Chromosome measurements were obtained and mean and standard deviation for CI were calculated for each chromosome. Total chromosome relative length (expressed as percentage) and the relative length of short (SA) and long arms (LA) over the total length of haploid complement were also obtained. Chromosome differences within and between karyotypes were evaluated from karyo-ideograms elaborated on SA and LA relative lengths (Spotorno 1985). Confidence intervals were established for SA and LA for each chromosome pair to assess the significance between comparisons. For chromosome arm number (NF) determinations, subtelocentric and acrocentric chromosomes were considered uni-armed according to Matthey (1945). This karyotype descriptor (NF), which only considers the main arm of a chromosome, is quite reliable as very often the size of the short arm of a subtelocentric chromosome is variable due to technical artefacts during spread preparations.

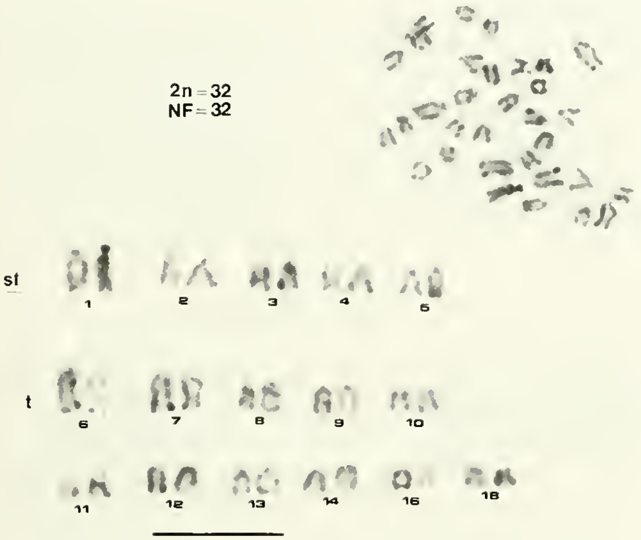

Figure 1. Karyotype of *Argopecten purpuratus*. $2n = 32$; NF = 32. Bar represents 10 microns.

Banding Methods

Howell and Black's (1980) silver-NOR staining method was used to identify NOR-bearing chromosomes. The counterstain-

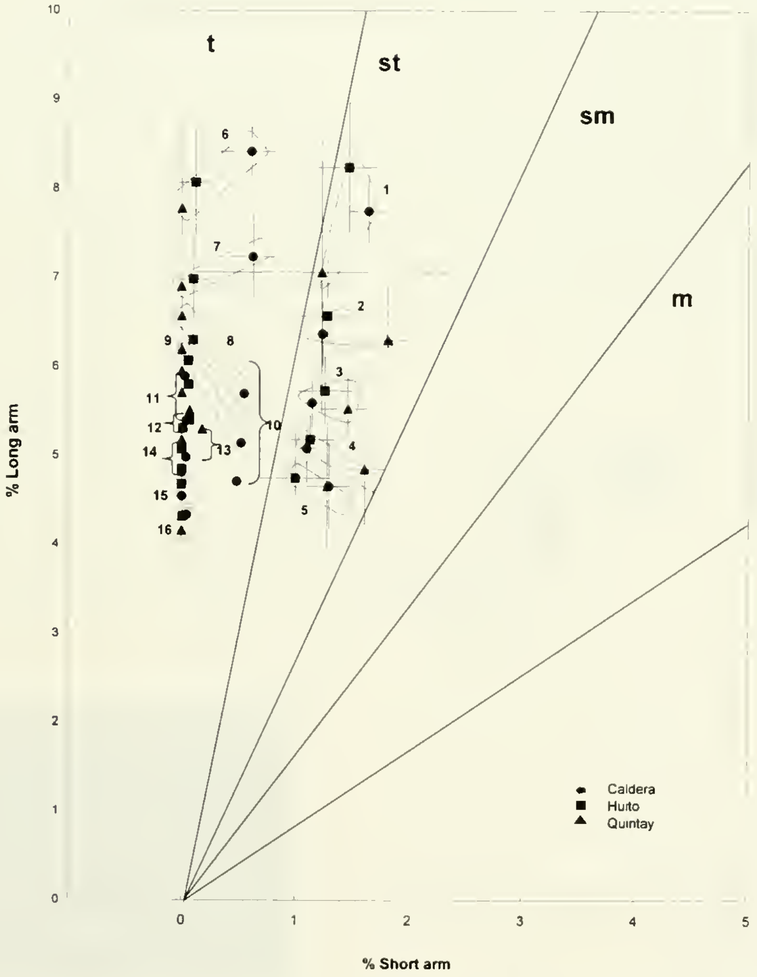

Figure 2. Karyo-idiogram for three populations of *A. purpuratus*. Diagonal lines separate chromosome morphologies in metacentrics (m), submetacentrics (sm), subtelocentrics (st), and telocentrics (t). Dotted lines show those chromosome pairs (identified by numbers) of the studied population that are easily distinguished from the rest. Each point in the karyo-idiogram represents a mean value (in percent) of the relative length of the short and the long arm of 16 homologous pairs. Bars represent 95% confidence intervals of the mean for short and long arms.

enhanced chromosome banding technique (Hoechst 33258/ Actinomycin D; Schweizer 1981) was used to identify heterochromatic regions. Metaphase plates were photographed using a Nikon epifluorescence microscope (filter block UV-2) with a Kodak T-Max 100 ASA film.

For restriction endonuclease (RE) banding, RE enzymes suspended in the appropriate buffer were added to one drop of an air-dried cell suspension and covered with a coverslip. Enzyme concentration and digestion time depended on each endonuclease type, as follows: for *Eco*RI, 0.5 U/μL for 5 h; for *Bam*HI, 0.5 U/μL for 8 h; for *Alu*I, 0.3 U/μL for 7 h; and for *Hae*III, 1.7 U/μL for 5 h. Slides were incubated in a moisture chamber at 37°C, washed in distilled water, and stained in 4% Giemsa solution for 10 min.

RESULTS

The modal diploid number of 32 was established for all three *A. purpuratus* populations screened (Table 1). The CI revealed that all karyotypes had similar chromosomal constitutions (5 pairs of subtelocentrics and 11 pairs of telocentrics (Table 2 and Fig. 1). The karyo-ideogram showed that various chromosome pairs could be easily identified because of size differences (pairs 1 to 7). Interpopulational chromosome differences were evident, particularly for the short arm (ANOVA, $P < 0.05$) for chromosome pairs 2, 4, 6, 7, 8, 9, and 10 (Table 2 and Fig. 2).

The Ag-NOR staining revealed three pairs of NOR-bearing chromosomes (Fig. 3) with NOR location varying, i.e., it was pericentric in the long arm of one pair and telomeric in the short arm of two other pairs (see Fig. 3). The number of NOR-bearing chromosomes was in agreement with the number of nucleoli observed in the interphase nucleus (3 to 6, Fig. 3b).

The Hoechst/Actinomicin D counterstain (Fig. 4) revealed a tiny positive fluorescent heterochromatic block in the centromeric area of chromosome pairs 1, 2, 4 to 9, 11, 15, and 16 (Fig. 4). This stain produced a quenching zone in the middle of the long arm of pair 6, which corresponded to an A-T low-content chromatin sequence, according to the pair specificity of the Hoechst 33258. Figure 5 shows the pattern after restriction endonuclease digestion. Only two REs, *Alu*I and *Hae*III, produced a banding pattern in such a way that pericentric and telomeric bands were evident in one chromosome pair (*Alu*I) and interstitial bands in two pairs (*Hae*III).

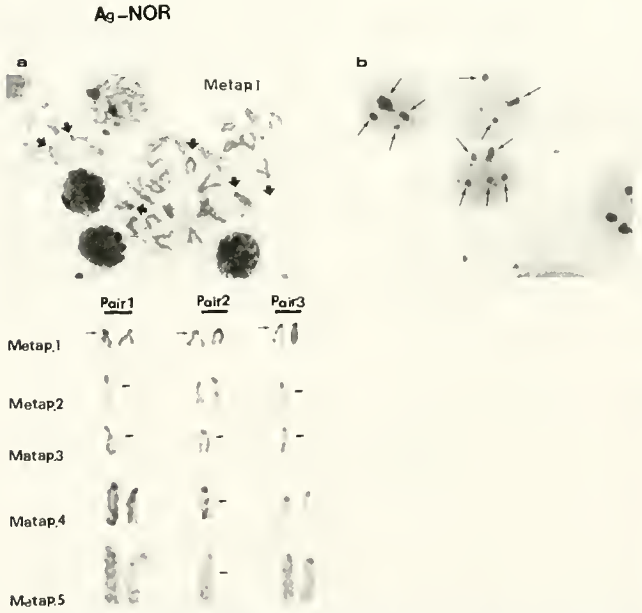

Figure 3. Ag-NOR staining. (a) Metaphase plate showing six NOR-bearing chromosomes. Below, NOR-bearing chromosomes obtained from different metaphase plates. (b) Resting nuclei showing 3 to 5 nucleolus.

DISCUSSION

This work confirms the chromosome number of $2n = 32$ reported for *A. purpuratus* (Von Brand et al. 1990; Canello et al. 1992; Alvarez-Sarret & Lozada 1992; Winkler et al. 1993). However, the chromosome formulae obtained (5 st + 11 t chromosome pairs) differs from that reported by Von Brand et al. (1990), who indicated the presence of 2 m pairs, 7 m-sm pairs, 3 st pairs, and 4 t pairs in the karyotype. Such a difference is likely to be produced by the fact that in the latter study the karyotype was tentatively arranged using the size criteria without the chromosome measurements required to determine the centromeric position. Our results consistently show, in all three samples that were analyzed, a chromosome complement of sub-telocentric chromosome types (see Fig. 2). *A. purpuratus*, along with other related species, such as *A. irradians irradians* (Wada 1978) and *C. nobilis* (Komaru and Wada 1985), are among the few Pectinids sharing a diploid number of 32. Instead, the majority of species studied (i.e., genus *Chlamys*, *Pecten*, *Patinopecten*, and *Placopecten*) exhibit $2n = 38$ (Thiriot-Quiévreux 1994; Insua et al. 1998), with *Aequipecten opercularis* ($2n = 26$) being the exception (Beaumont & Gruffydd 1974).

Molluscs and crustaceans are among the most primitive groups considering those karyologically compared by Thiriot-Quiévreux (1994). Nevertheless, a typical diploid number does not emerge in most groups, Ostreidae being a special case in which a diploid number of $2n = 20$ is common and very likely the ancestral one. Some marine species frequently exhibit greater numbers of chromosome than those of freshwater species, although in some cases, for example, within the bivalvia, both an increase and decrease are evident. The diploid number in most animal species ranges widely, between 12 and 40 (White 1978). Whereas evolution to higher chromosome numbers in certain aquatic organisms is often paralleled by an increase of subtelocentric and telocentric chromosomes, probably by fission of chromosomes (see review by

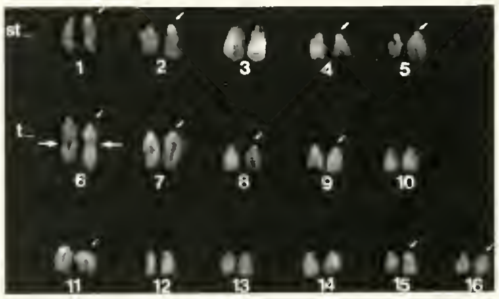

Figure 4. Hoescht 33258/ Actinomycin D counterstain. Intense fluorescent bands are indicated by small arrows. Large arrows depict a less fluorescent area in chromosome pair 6.

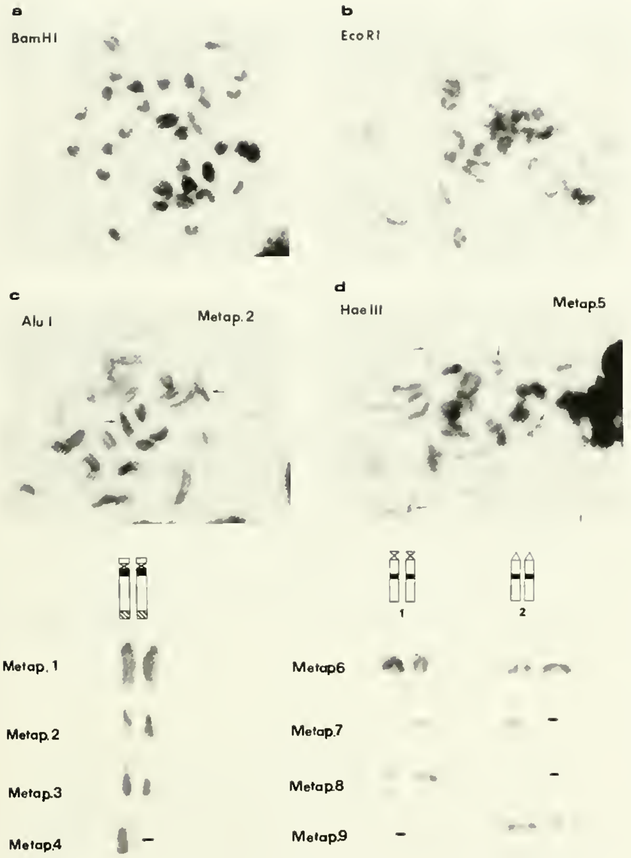

Figure 4. RE digestion: (a) *Bam*HI (no digestion); (b) *Eco*RI (no digestion); (c) *Alu*I (+ digestion), photo depicts a pericentromeric band in one pair of subtelocentric chromosome (arrows); (d) *Hae*III (+ digestion) depicts an interstitial band in two chromosomes. Below, banded chromosomes obtained from different metaphase plates.

Thiriot-Quiévreux, 1994: 379). Such phenomenon is likely to be related with specialization or adaptation to a certain type of marine environment. As mentioned previously, the diploid number in Pectinids varies between 26 and 38; hence, the chromosome formulae observed for *A. purpuratus* could be considered a derivative state within the group. Although it is debatable whether such a trend could yet be inferred in a group with few species exhibiting standardized, or comparable, karyotypes, it is worth mentioning that *A. purpuratus* is one of the southernmost *Argopecten* representatives. Indeed, this group originated in the tropical/subtropical region of the Caribbean and Atlantic (Von Brand et al. 1990). *A. purpuratus* along with *A. opercularis* (Beaumont & Gruffydd 1974) are among the few Pectinids of 14 species karyotyped so far with $2n = 32$ and $2n = 26$, respectively (see Insua et al. 1998).

Our results (2n and NF) and similar information from other species within the group (reviewed by Méndez et al. 2000), suggest that NF (Matthey 1945) is a valuable descriptor for species comparisons and to evaluating factors affecting chromosome shape variations such as chromosome rearrangements (White 1978). Because of technical procedures, the size of the short arm can vary widely and this can be deduced from the deviation observed between the average values of the centromeric index (CI) in Table 2 as well as from the presence or absence of short arms in the telocentric chromosomes.

The combination of classic and banding techniques used in this work allowed a better description of the karyotype and a more reliable pairing of certain homologous chromosomes. Reproducible banding patterns are still uncommon in aquatic invertebrates; hence, chromosome markers described in this work, for the first time in *A. purpuratus*, are considered valuable for an improved and reliable characterization of the species karyotype and its variations. Three NOR-bearing chromosome pairs were preliminary identified in the karyotype with the NOR regions, varying its location in the chromosomes (Fig. 3). Further analysis of these chromosome regions (using chromomycin A3 and a rDNA probe) will be required to clarify a probable polymorphism for both number, position, or NOR size in *A. purpuratus*. The three NOR-bearing chromosome pairs observed in this work are within the range reported for Pteriomorphia subclass (1–4 pairs; Martínez-Lage et al. 1997; Insua et al. 2000). The Ag-NOR number reported for *A. purpuratus* is higher in comparison with *A. opercularis*, which shows only one pair (Insua et al. 1998).

The combined use of fluorescent dyes and RE digestion allowed the detection of different kinds of chromatin in *A. purpuratus*, suggesting specific DNA composition of certain chromosome sectors. The Hoechst 32258/Actinomycin D counterstain highlights heterochromatic areas in humans (Schweizer 1981) and lower vertebrates (Colihueque et al. 2001). Although we observed highly fluorescent areas in the *A. purpuratus* karyotype, these were restricted to the centromeric regions of few chromosomes, indicating that heterochromatin is not common in this species (see Fig. 4). However, the differential response to digestion with restriction enzymes is also evidence of chromatin differences in *A. purpuratus* chromosomes. For example, the positive detection (no bands), at least preliminarily, of sites for *Alu* I (AGÅCT) and *Hae*III (GC↓CC; see Fig. 5) indicates that these target sequences should be highly repeated for the digestion to be evident (Sánchez et al. 1991). It should be stressed, however, that heterochromatic areas are few and mainly restricted to the centromeric areas as revealed by the fluorescent banding. Digestion with RE of fixed metaphase plates is not so common in invertebrates, with the exception of *Drosophila* (Mezzanotte 1986). Therefore, the results of this work confirm the finding of Martínez-Lage et al. (1994), who indicated that the digestion of bivalve chromatin produces specific chromosome bands (Fig. 5). However, the pattern of digestion is simple and restricted to few chromosomes similar to what is observed in fishes (Sánchez et al. 1991) and mammals (Burkholder 1989).

The farming of the Chilean scallop was greatly promoted by the fact that natural beds were almost depleted. Nowadays, a blooming, fast-growing industry is still far from realizing its potential because of the limited, or unreliable, production of quality seeds (Gajardo & Núñez 1992). This is in part related with proper broodstock management. By providing a better karyological description (quantitative measurements and banding pattern of chromosomes), this work offers a reliable and comparable way to evaluate the chromosome formulae of *A. purpuratus* and hence, a proper way for comparing probable differences among the few wild populations remaining, or hatchery stocks, if any can be identified yet. This is expected to assist to the management, improvement and conservation of this economically valuable resource. At the same time, it will help in the understanding of the processes affecting chromosome evolution in this species as well as in the bivalvia class.

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MOLECULAR CLONING AND CHARACTERIZATION OF A FRUCTOSE-1,6-BIPHOSPHATE ALDOLASE cDNA FROM THE DEEP-SEA SCALLOP *PLACOPECTEN MAGELLANICUS*

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ABSTRACT The deep-sea scallop *Placopecten magellanicus* is an important member of commercial fisheries along the coast of Northeastern United States and Atlantic Canada. A cDNA encoding a glycolytic pathway enzyme fructose-1,6-biphosphate aldolase was isolated from a sea scallop adductor muscle-specific cDNA library and sequenced from both directions. The full-length cDNA is a 1627 base-pair (bp) long sequence that has a 62 bp 5′ untranslated region, a 1092 bp open-reading frame, and a 473 bp 3′ untranslated region including a 24 bp polyA tail. The open-reading frame encodes a 39.3 kDa protein with 363 amino acids. The protein has 183 nonpolar, 94 polar uncharged, and 86 polar-charged amino acids. Several amino acids show bias for codons with G/C at their third position. The cDNA has 28 unique restriction sites, including common restriction enzymes such as *Sac*I, *Bam*HI, *Taq*I, and *Bst*EII. The aldolase is a highly conserved protein with 66% sequence identity with that of *Schistosoma mansoni*, 65% with that of *Drosophila melanogaster*, 62% with that of *Homo sapiens*, and 57% with that of *Oryza sativa*. Southern blot analysis indicates that aldolase in sea scallops belongs to a family with 4 to 10 putative genes. Northern blot analysis shows that this gene is expressed only in adductor muscles. Hybridization of an aldolase cDNA probe to genomic DNA from several individuals revealed restriction fragment length polymorphisms at several loci, indicating potential use of this cDNA as a marker in genetic studies of sea scallops.

KEY WORDS: aldolase, cDNA, sea scallops, restriction fragment length polymorphisms, codon bias

INTRODUCTION

Fructose-1,6-biphosphate (FBP) aldolase, technically known as D-glyceraldehyde-3-phosphate lyase (EC 4.1.2.13), is a long-lived, ubiquitous, glycolytic pathway enzyme. In glucose metabolism, it catalyzes cleavage of FBP into two 3-carbon molecules, glyceraldehyde 3-phosphate and dihydroxyacetone phosphate (DHAP), and in gluconeogenesis, it catalyzes the reaction in reverse order. This enzyme is also involved in gluconeogenesis and in the Calvin Cycle in plants. In the Calvin Cycle of higher plants, aldolases were reported to be bifunctional (Flechner et al. 1999) because they catalyze both the reversible condensation of DHAP to glyceraldehyde 3-phosphate and DHAP and erythrose-4-phosphate to sedoheptulose-1,7-biphosphate. Cytosolic aldolase in strawberry was found to be related to fruit ripening (Schwab et al. 2001).

The aldolases are distinguished as two classes based on their two distinct mechanisms of catalyzing glycolytic reactions, requirement of divalent cations in catalysis, subunit structures, pH optima, and substrate affinity (Rutter 1964). The two classes of aldolases share very little in their sequence homology (Alefounder et al. 1989, Plaumann et al. 1997) and are considered to be independent evolutionary lineages (Flechner et al. 1999). The class I enzymes are tetramers of 40 kDa identical subunits, each with an active site (Lys 229) located in the core of the enzyme (Lai et al. 1974, Samson et al. 1997). These enzymes have the same molecular weight and subunit structure and catalyze the overall reaction through Schiff base formation with ketoses of sugar substrates and can be inhibited by borohydride reagents (Sawyer et al. 1988). Although they are prevalent in animals and higher plants, they also occur in green algae and a few prokaryotes grown under autotrophic conditions (Sauve & Sygusch 2001). The class II enzymes are usually dimers with certain exceptions where they are tetramers, and are found in bacteria, yeast as well as other fungi and some green algae grown under heterotrophic conditions (Rutter 1964, Plaumann et al. 1997). These aldolases are EDTA sensitive, stabilize the enol intermediate of the reaction by using a divalent cation, usually Zn^{2+} or Fe^{2+}, and their activities are enhanced by monovalent cations.

In vertebrates, there are three classes of class I enzymes: aldolase A (isolated from muscle), aldolase B (isolated from liver), and aldolase C (isolated from brain). This work deals with the class IA group. The classes have immunologic differences, different kinetics, different chromosomal locations, and different gene sequences. Some class I enzymes are expressed only in specific tissues. For example, aldolase A is present in muscle and red blood cells; aldolase B in the liver, kidneys, and small intestine; and aldolase C in neuronal tissues (Penhoet et al. 1966, Penhoet et al. 1969, Tolan et al. 1987). These enzymes were reported to be three distinct proteins, yet are structurally closely related. Their functions are different, but homologous. In invertebrates, so far no such tissue specificity of an aldolase gene has been reported.

The sea scallops are an important fishery in the Northeastern coastal regions of the US and in Atlantic Canada (Black et al. 1993). This species has been the subject of several genetic studies in recent years (Volckaert & Zouros 1989, Patwary et al. 1994a, Patwary et al. 1994b, Patwary et al. 1996, Patwary et al. 1999, Pogson 1994, Pogson & Zouros 1994, Gjetvaj et al. 1997). As a part of our continuing effort to characterize cDNAs in this species, we report here a first bivalve full-length FBP aldolase cDNA and show that it is potentially useful as a probe in genetic studies of sea scallop and other related species. The cDNA information would also contribute to enhancing the understanding of structure-function relationships of aldolase genes in bivalves.

MATERIALS AND METHODS

Sea scallops were obtained from commercial beds in Nova Scotia and from the Marine Biology Supply Center, Woods Hole, MA. The methods of DNA extraction and cDNA library construction are as described by Patwary et al. (1996).

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Isolation of FBP Aldolase cDNA Clones

As described earlier (Patwary et al. 1999), a small portion of adductor muscle-specific Uni-ZAP XR cDNA library was plated and 130 plaques were randomly cored and stored at 4°C in SM buffer. Bluescript clones containing cDNA inserts were rescued according to the supplier's protocol (Stratagene, La Jolla, CA). inserts were partially sequenced from the 5′ end, and one of these clones was identified as a truncated aldolase cDNA. An additional 150 plaques were randomly cored from a set of new plates and stored in SM buffer. The cDNA inserts from each of these recombinant Uni-ZAP XR lambda clones were separately amplified (Patwary et al. 1996) by polymerase chain reaction (PCR) using SK and KS primers (Stratagene), the PCR products were fractionated in agarose gels, and the they were transferred onto positively charged nylon membranes using standard protocols (Sambrook et al. 1989). The blots were hybridized with aldolase cDNA labeled with ^{32}P-dCTP as previously described (Patwary et al. 1999), exposed to X-ray films, and two positive clones were identified. These two cDNA clones, PmC 82 and PmC 83, were rescued into pBluescript from their recombinant uni-ZAP XR lambda vectors and sequenced completely from both directions. The 5′ truncated cDNA sequence end is obtained using a 5′RACE system version 2.0 (Invitrogen Corporation, Chicago, IL).

Preparation of RNA and Northern Blotting

Total RNA for northern blotting was extracted separately from several tissues pooled from at least six animals of both sex. The collected tissues were immediately fixed in RNA*later*^{TM} (Ambion Inc. Austin, TX) and stored at −20°C. Each pooled sample was ground in a separate baked mortar using a baked pestle. RNAs were extracted according to the protocol that came with Ambion's ToTALLY^{TM} RNA Kit. After quantification, RNA samples were precipitated by adding salt and 95% ethyl alcohol and stored at −180°C. The quality of RNA samples was checked in a formaldehyde gel containing ethidium bromide at the time of Northern analysis. Northern blotting, preparation of ^{32}P probe, prehybridization, and hybridization were performed as described in Patwary et al. (1999).

Preparation of Probe for Restriction Fragment Length Polymorphism (RFLP) Detection

To detect polymorphisms among sea scallops, the entire coding region of FBP aldolase cDNA was used as a probe. The cDNA was labeled with alkali-labile Dig-11-dUTP by PCR. The 25 μ PCR reaction was prepared on ice. The final concentration of PCR components were as follows: *Taq* polymerase buffer 1x; $MgCl_2$ 1.5 mM; dATP, dCTP, dGTP 100 μM each; dTTP 84 μM; dig-dUTP 14 μM; nested primers flanking coding regions 20 ng each; plasmids with cDNA insert 1 ng; and *Taq* DNA polymerase 1U. A Gene-Amp 9600 thermocycler (Perkin Elmer, Norwalk, CT) was used to perform PCR reactions. The thermocycler was programmed for 31 cycles. Cycle 1 was at 94°C for 4 min, followed by 29 cycles each of 94°C for 30 sec, 55°C for 30 sec, and 72°C for 2 min and the final cycle of 94°C for 30 sec, 55°C for 30 sec, and 72°C for 5 min. A small portion of the labeled PCR and unlabeled PCR (control) products were subjected to an agarose gel electrophoresis and the slower mobility of labeled PCR products in comparison to unlabeled control confirmed successful labeling. The probe was cleaned using nick columns (Pharmacia Biotech, Upsala, Sweden) and quantified by comparing hybridization with known dig-labeled DNA on a dot blot.

Preparation of Genomic Blots

Ten micrograms of each DNA sample was digested to completion with 40 units of appropriate restriction enzymes at 37°C. Spermidine to a final concentration of 5 mM was included in each reaction to improve digestion. A small quantity of each sample was fractionated in a mini agarose gel to verify the extent of digestion. The digested DNA samples were fractionated in 0.8% regular molecular biology grade agarose gels in TAE (0.04 M Tris-acetate, 0.0001 M EDTA) buffer together with dig-labeled molecular weight marker III (Boehringer Mannheim). The digested DNA samples were vacuum transferred to positively charged nylon membranes. DNA blots were rinsed in TAE buffer, air dried, and baked at 80°C for two hours before hybridization with the probe.

The blots were prehybridized in an oven at 40°C for three to four hours in hybridization buffer containing deionized formamide (50%), sodium citrate (SSC) and sodium chloride (5x each) *N*-lauroylsarcosine (0.1%), SDS (0.02%), blocking reagent (2%), and denatured yeast RNA (100 μg/mL). Hybridization with the probe was performed for 18 to 24 h in fresh buffer containing 10 ng/mL denatured probe. Blots were washed twice in 2x SSC, 0.1% SDS for 10 min, once in 0.5× SSC, 0.1% SDS for 15 min, and once or twice in 0.2× SSC, 0.1% SDS for 20 min. Detection of labeling was done according to the Boehringer Mannheim protocol.

RESULTS AND DISCUSSION

As a part of our ongoing effort to identify cDNAs that have potential as probes in genetic studies of sea scallops, over one hundred randomly selected phagemid clones with cDNA inserts were partially sequenced from the 5′ end. Through an amino acid sequence homology search (BLAST P) one of these clones was identified as the cDNA encoding FBP-aldolase. This cDNA sequence was highly truncated at the 5′ end and it was used as a probe to screen additional Uni-ZAP XR clones from the adductor muscle-specific cDNA library. Two clones designated PmC 82 and PmC 83 were isolated through this screening process. PmC 82 was determined to have the entire open reading frame (ORF). Clone PmC 83, although it is bigger than the first clone, is still shorter by several bases in the 5′ coding region. The clone PmC 82 is 1546 bp long excluding the polyA tail. It has only five base pairs in its 5′ untranslated region, a 1092 bp ORF and a 449 bp 3′ untranslated region. Figure 1 shows a full length cDNA that includes additional 57 bp at 5′ end obtained by using the RACE system. The ORF encodes 363 amino acid residues with a predicted molecular mass of 39, 343 Da. These amino acid residues are 183 nonpolar, 94 polar uncharged, and 86 polar charged. There are 45 alanine and 3 tryptophan residues in the sequence, and these two are the most heavily represented and least represented amino acids, respectively (Table 1).

The G/C content for the coding region of the cDNA is 57.7%, and the distribution of these two nucleotides in the gene at first, second, and third positions are 81%, 43%, and 71.4%, respectively. A similar distribution was also observed in the aldolase gene of *Thermus aquaticus* (Sauve & Sygusch 2001). The high G/C content at the third position of the codon tend to be higher than the overall G/C content in exons of many genes of other organisms including humans. There is a considerable bias in codon-usage for a number of amino acids in FBP aldolase gene. This bias is particularly prominent in the usage of codons for leucine, isoleucine, valine, glutamine, asparagine, lysine, and aspartic acid (Table 1). For example, 25 of the 31 leucine residues are encoded by two codons (CUC, CUG), although there are four

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   1 cgtgtaggcagtccttccccagaagcatccagacgcatttgtgatccagcgaaagaaccaag ATG CCA ACC TTT  74
   1                                                                M   P   T   F    4

  75 CCA CAG TAC CTG AGT CCA GAG AAG GAG GAG GAG CTC CGA AAC ATT GCC AAC GCC ATA GTA 134
   5 P   Q   Y   L   S   P   E   K   E   E   E   L   R   N   I   A   N   A   I   V    24

 135 GCT CCT GGC AAG GGG ATC CTA GCC GCT GAC GAG TCA ACA GGT TCA GTT GGG AAG AGG TTC 194
  21 A   P   G   K   G   I   L   A   A   D   E   S   T   G   S   V   G   K   R   F    44

 195 GCC CCC ATC AAA GTA GAG AAC ACG GAG GAG AAT CGT CGC CGG TAC AGA GAA CTG CTG TTC 254
  31 A   P   I   K   V   E   N   T   E   E   N   R   R   R   Y   R   E   L   L   F    64

 255 ACC ACC GAC AAC GCT ATC TCC GAA AAC ATC AGC GGC GTC ATC ATG TTC CAC GAA ACG TTT 314
     T   T   D   N   A   I   S   E   N   I   S   G   V   I   M   F   H   E   T   F    84

 315 TAC CAG AAG ACG GCC GAC GGC GTG CCC TTC ACG AAG GTT CTC CAG GAC AAG AAC ATC ATT 374
     Y   Q   K   T   A   D   G   V   P   F   T   K   V   L   Q   D   K   N   I   I    104

 375 CCA GGC ATC AAG GTG GAC AAG GGT GTC GTA CCA CTG ATG GGC ACG GAC AAC GAA TGT ACC 434
     P   G   I   K   V   D   K   G   V   V   P   L   M   G   T   D   N   E   C   T    124

 435 ACA CAG GGT CTG GAT GGC CTG AGT GAG AGG TGT GCC CAG TAC AAG AAA GAT GGC GCC CAG 494
     T   Q   G   L   D   G   L   S   E   R   C   A   Q   Y   K   K   D   G   A   Q    144

 495 TTT GCC AAA TGG AGG TGC GTG CTA AAG ATC CAG AAG GAG ACG CCG TCC TAC CAG GCC ATG 554
     F   A   K   W   R   C   V   L   K   I   Q   K   E   T   P   S   Y   Q   A   M    164

 555 CTG GAG AAC GCT AAC GTC CTC GCC CGC TAC GCC AGT ATC TGT CAA CAG AAT GGC CTG GTG 614
     L   E   N   A   N   V   L   A   R   Y   A   S   I   C   Q   Q   N   G   L   V    184

 615 CCC ATC GTC GAG CCA GAG GTC CTC CCC GAC GGT GAA CAT GAT CTG GAC ACT GCA GAA AAA 674
     P   I   V   E   P   E   V   L   P   D   G   E   H   D   L   D   T   A   E   K    204

 675 GCT ACA GAA CAG GTG CTT GCG TTT ACC TAC AAG GCT TTG GCC GAC CAC CAC GTG TTC CTA 734
     A   T   E   Q   V   L   A   F   T   Y   K   A   L   A   D   H   H   V   F   L    224

 735 GAA GGG ACC CTC CTC AAA CCC AAC ATG ATC ACG GCC GGT ATG TCG TGT AGT AAG AGG GGC 794
     E   G   T   L   L   K   P   N   M   I   T   A   G   M   S   C   S   K   R   G    244

 795 ACG CCT GCC GAG AAT GCC CGC GCG ACA GTG CTC TGC CTC AGT AGG ACC GTC CCT CCC GCA 854
     T   P   A   E   N   A   R   A   T   V   L   C   L   S   R   T   V   P   P   A    264

 855 GTC GCC GGT GTG ACG TTC CTG TCC GGT GGT CAG TCA GAG GAA GAC GCG TCC ATC AAC CTC 914
     V   A   G   V   T   F   L   S   G   G   Q   S   E   E   D   A   S   I   N   L    284

 915 AAC GCC ATC AAC ACA GAC TCC GGC CGC AAA CCC TGG CCG CTG ACC TTC TCC TTC GGC CGA 974
     N   A   I   N   T   D   S   G   R   K   P   W   P   L   T   F   S   F   G   R    304

 975 GCG CTC CAG GCC AGC GTA CTC AAA ATT TGG CAG GGC AAG GAC GAA AAT GTG GCC GCA GCA 1034
     A   L   Q   A   S   V   L   K   I   W   Q   G   K   D   E   N   V   A   A   A    324

1035 CAA AAG CAG CTC ACC ATG AGG GCA AAG GCC AAC GGT TTG GCA GCG CTG GGC AAA TAC CAG 1094
     Q   K   Q   L   T   M   R   A   K   A   N   G   L   A   A   L   G   K   Y   Q    344

1095 GGA GAC GCG GCG AGT GCG GCT GCA GCG GAT TCT CTG TTT GTG GCG CAG CAC GCC TAC TAG 1154
     G   D   A   A   S   A   A   A   A   D   S   L   F   V   A   Q   H   A   Y   *    363

1155 acaccagccacgcttacctccccttatctacaccatagtgtaaccctatactgaacaatgtcagagacaaattttcaca 1233
1234 cgtttatattttaacgaacaatattgttgttagatgcattttgatgcggtgtatgttgatgaagagattaagtaataat 1312
1313 gtatgtttttacattcggggtaacctacaatgtgatggatattaaaacatattttagatattttttttctaggtagaaa 1391
1392 tctcagctctaggcataatatgtttataatgttaatgtgtgaatttgtcacctactgttttgttgacgtcagcattttg 1470
1471 tttactgttatttatttgctttaatatcagaacaggttatgcctgtgaagaattaaataatgaatctgctataatttct 1549
1550 gtgatccctcaattgattatactttgtcagtattaaaaaccattatatgcatgtaaaaaaaaaaaaaaaaaaaaaa  1627
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Figure 1. Nucleotide sequence of the sea scallop fructose-1,6-biphosphate aldolase cDNA and the deduced amino acid sequence of the enzyme. The nucleotide residues are numbered from the 5′ end. The amino acid residues are numbered from first in-frame methionine (M). The potential polyadenylation signal is bolded. Underlines indicate primers for amplification of 3′UTR. The star indicates the stop codon. Note that the 57 bp obtained by RACE system is added to the 5′ end of the clone PmC 82.

TABLE 1.

Codon usage in fructose-1,6-biphosphate aldolase gene.

UUU	phe	F	5	UCU	ser	S	1	UAU	tyr	Y	—	UGU	cys	C	4
UUC	phe	F	8	UCC	ser	S	6	UAC	tyr	Y	9	UGC	cys	C	2
UUA	leu	L	—	UCA	ser	S	3	UAA	OCH	Z	—	UGA	OPA	Z	—
UUG	leu	L	2	UCG	ser	S	1	UAG	AMB	Z	1	UGG	trp	W	3
CUU	leu	L	1	CCU	pro	P	3	CAU	his	H	1	CGU	arg	R	1
CUC	leu	L	12	CCC	pro	P	7	CAC	his	H	4	CGC	arg	R	4
CUA	leu	L	3	CCA	pro	P	6	CAA	gln	Q	2	CGA	arg	R	2
CUG	leu	L	13	CCG	pro	P	2	CAG	gln	Q	16	CGG	arg	R	1
AUU	ile	I	3	ACU	thr	T	1	AAU	asn	N	4	AGU	ser	S	6
AUC	ile	I	13	ACC	thr	T	9	AAC	asn	N	14	AGC	ser	S	2
AUA	ile	I	1	ACA	thr	T	5	AAA	lys	K	8	AGA	arg	R	1
AUG	met	M	7	ACG	thr	T	9	AAG	lys	K	16	AGG	arg	R	6
GUU	val	V	2	GCU	ala	A	7	GAU	asp	D	4	GGT	gly	G	9
GUC	val	V	7	GCC	ala	A	21	GAC	asp	D	13	GGC	gly	G	13
GUA	val	V	4	GCA	ala	A	7	GAA	glu	E	10	GGA	gly	G	1
GUG	val	V	10	GCG	ala	A	10	GAG	glu	E	15	GGG	gly	G	3

In each block, the first, second, third, and fourth columns are codons, three-letter abbreviations for amino acids, one-letter abbreviations for amino acids, and number of amino acid residues, respectively.

other codons for this amino acid. Likewise, one (CAG) of the two codons for glutamine code for 16 of the 18 residues of this amino acid. This conspicuous nonrandom usage of synonymous codons in the FBP aldolase gene in sea scallops is consistent with the bias found in highly expressed genes of several other organisms (Sharp & Li 1986, Sharp et al. 1988). This strong codon-usage bias may be the result of selection for translation efficiency and accuracy in highly expressed genes like FBP aldolase.

The number of FBP-aldolase amino acid residues in different organisms varies from 358 in *Oriza sativa* to 366 in *Caenorhabditis elegans*. However, a wide range of organisms such as *Drosophila*, rat, and *Xenopus* have 363 residues as in sea scallops. Based on the high amino acid sequence homology, we conclude that this is a subunit of class I aldolase. Because of its essential role in glucose metabolism, the primary structure of this enzyme has remained highly conserved across kingdoms. The rate of evolution of aldolase was estimated to be about 4% amino acid residue changes every 100 million years (Sawyer et al. 1988). The muscle-specific aldolase evolved at an even slower rate, with only about 2% amino acid residue changes per 100×10^6 years (Freemont et al. 1988). The BlastP search (Altschul et al. 1997) results show that the sea scallop aldolase is approximately 66% identical with that of *Schistosoma mansoni*, 65% with that of *Drosophila melanogaster*, 64% *Salmo salar*, 63% with *Gallus gallus* and *Xenopus leavis*, 62% with human muscle aldolase A, 62% with *caenorhabditis elegans*, 57% with *Oryza sativa*, and 55% *Plasmodium falciparum*.

The approximate number of genes encoding FBP-aldolase in sea scallop was established by Southern blot analysis. Hybridization of cDNA coding region probe with a blot containing DNA samples from a single sea scallop but digested with several restriction enzymes produced signals of varying intensity in all lanes (Fig. 2). From the number of signals we estimate that sea scallops have a family of 4 to 10 FBP-aldolase genes. Aldolase constitutes a medium-sized gene family in comparison to the estimated family size of 12–15 for the actin gene (Patwary et al. 1996) and 1 to 3 for the tropomyosin gene (Patwary et al. 1999) in sea scallops. The organization of FBP aldolase as a small multigene family was also observed in *Euglena* (Plaumann et al. 1997).

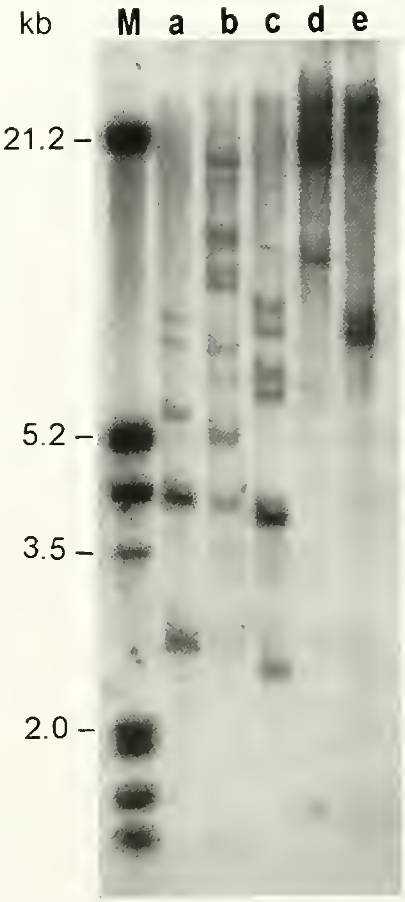

Figure 2. Detection of fructose-1,6-biphosphte aldolase gene in the sea scallops. In this Southern hybridization, digoxigenin-labeled aldolase coding region probe was hybridized to genomic DNA from a single animal. Each lane has 10 μg of DNA digested with *Eco*RI (lane a), *Eco*RV (lane b), *Hind*III (lane c), *Sal*I (lane d), and *Xba* (lane e). M is digoxigenin-labeled DNA molecular weight marker (Boehringer Mannheim).

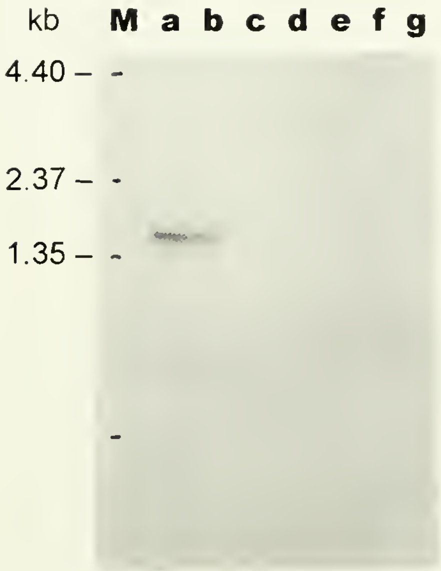

Figure 3. Northern blot analysis. A 3′ untranslated cDNA region was labeled with ^{32}P and hybridized to blots each lane with 12 μg of pooled total RNA obtained from adductor muscles (lanes a and b), gonads (lane c), hearts (lane d), livers (lane e), mantles (lane f), and gills (lane g). M is RNA molecular marker lane.

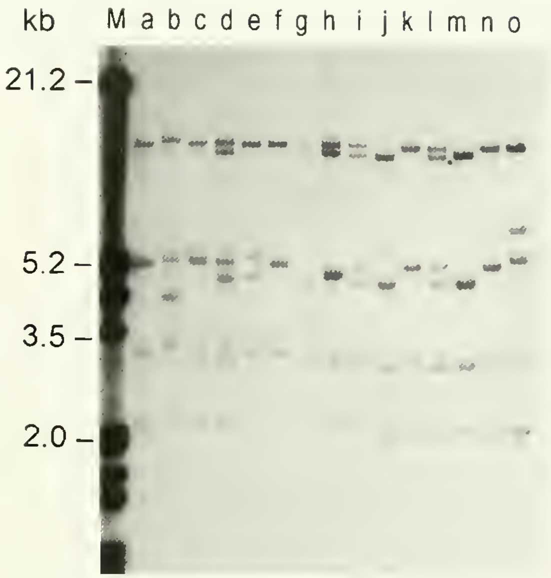

Figure 4. RFLPs in the region surrounding the aldolase genes in sea scallops. Each lane has 10 μg of genomic DNA from a different animal obtained from five different sea scallop beds (a–c from Yarmouth, d–f from Sable Island, g–i from Georges Bank, j–l from Browns Bank, and m–o from Newfoundland) digested with restriction enzyme *Acc*I and hybridized with the complete aldolase cDNA coding region. M is digoxigenin-labeled DNA molecular weight marker (Boehringer Mannheim).

Figure 3 shows that the size of sea scallop FBP aldolase messenger RNAs in the Northern blot corresponds closely to the length of the sea scallop FBP aldolase cDNA (Fig. 1). This result suggests that the clone PmC-82 represents a FBP aldolase cDNA with a very small truncation at its 5′ untranslated region. We have used the 3′ non-translated region of cDNA as a probe in Northern hybridization to determine the tissue specificity of aldolase expression, and obtained signals in the adductor muscle lanes only (Fig. 3). In absence of any indication of RNA degradation in the gel or in the blot, we conclude that the aldolase gene in question is expressed tissue-specifically in the adductor muscle of sea scallop. This finding suggests that, like vertebrates (Penhoet et al. 1966, Penhoet et al. 1969), sea scallops may have different class I aldolase genes expressed in different tissues. The extent of structural differentiation of these genes and the pattern of their distribution among different sea scallops tissues remain to be studied.

We examined the utility of FBP-aldolase cDNA as a probe to reveal polymorphisms. The probe revealed RFLPs in three loci in an *Acc*I-digested blot (Fig. 4) and in two loci in a *Dde*I-digested blot (data not shown). Pogson and Zouros (1994) and Pogson (1994) obtained three types of RFLPs in sea scallops when they used unidentified sea scallop cDNAs as probes. These were restriction site polymorphisms, polymorphisms caused by variable numbers of tandem repeats (VNTR) and complex fingerprinting patterns (Pogson 1994). In this study the FBP aldolase cDNA probe revealed restriction site polymorphisms at the approximately 15 kb and 3 kb loci and a VNTR type polymorphism at the 5 kb locus in the *Acc*I digested genomic blot (Fig. 4). Each of these loci has one or two bands, suggesting that the genomic DNA was completely digested and that the gene lacks restriction sites within it for the enzyme concerned. This finding of polymorphisms at multiple loci supports the presence of multiple copies of the aldolase gene probably distantly placed in the genome. Some of these genes possibly carry one or more variable sized introns and or VNTRs within their introns or in their flanking regions, producing multiple alleles at a particular locus. These results demonstrate that FBP cDNA is a useful addition to our collection of cDNAs that can be used as a probe to reveal RFLP markers for varieties of genetic studies in deep-sea scallops and possibly in other related bivalves. The isolation of the first bivalve aldolase cDNA may also assist in understanding the mechanism of aldolase function in this unique group of organisms.

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COMPARATIVE ANALYSIS OF OOCYTE TYPE FREQUENCIES IN DIPLOID AND TRIPLOID CATARINA SCALLOP (*ARGOPECTEN VENTRICOSUS*) AS INDICATORS OF MEIOTIC FAILURE

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ABSTRACT Differences between diploid and triploid female mollusks in their gametogenic process had previously centered on comparing the general gametogenic cycle, not the oocyte types in each ploidy class, which by their presence or absence might be used as indicators of completion of specific meiotic events. In this study we compared oocyte type frequencies at three different stages of the gametogenic cycle of triploid and diploid catarina scallops. Regardless of age of sampling (81 d, 118 d, 205 d of grow out), the most abundant oocyte type in diploids was always the vitellogenic, and in triploids the previtellogenic. The frequency of occurrence of vitellogenic oocytes in triploids was only 4.7–19% of that in diploids, and that for postvitellogenic oocytes was only from 0–8% of that in diploids. This indicates that the oocyte stage in triploids at which gametogenesis was largely arrested was the previtellogenic oocyte, at meiosis I. The principal cause for the meiotic arrest might be associated with problems during zygotene at the time of synapsis between the three homologous chromosomes in triploids as previously suggested by other authors.

KEY WORDS: triploid, oocyte-frequency, scallop, *Argopecten ventricosus*

INTRODUCTION

Previous research comparing diploid and triploid female mollusk gonad development have centered on comparisons of the gametogenesis cycle as described for diploids. Those studies have led to the general conclusion that triploid gametogenesis is retarded when compared with diploids (Allen & Downing 1986, Allen & Downing 1990, Allen 1987, Komaru & Wada 1989, Cox et al. 1996). Whereas some studies have based the comparative analysis of diploid and triploid gametogenic cycle in the qualitative occurrence of some particular type of oocyte at an age (Cox et al. 1996), a different approach to study the abnormal gonad development in triploid females is to estimate oocyte type frequencies in each ploidy class at different stages of the gametogenic cycle as it has been done for some fish species (Carrasco et al. 1998, Felip et al. 2001). The most common method of comparing diploids and triploids (by gametogenesis cycle) results in that it is not clear if the delayed gametogenesis in triploids is caused by a lower developmental rate of most oogenic stages, or by an inhibitory effect resulting in delay or an arrest of a particular stage so further development is impaired, or by both. What is clear is that a final consequence of the triploid condition at the end of the gametogenic cycle is the occurrence of partial or total sterility observed for most mollusk species, measured as the qualitative observation of low numbers of either spermatozoa or mature oocytes (Komaru & Wada 1989, Allen & Downing 1990, Guo & Allen 1994a, Guo & Allen 1994b, Cox et al. 1996, Eversole et al. 1996).

As with other mollusk species, in the functional hermaphrodite catarina scallop, *Argopecten ventricosus*, gametogenesis of triploid scallops is apparently delayed when compared with diploids, resulting in partial sterility of triploids. The partial sterility results in fecundity being largely reduced in triploid females, with the male gonad generally not developing further than the spermatocyte stage, and becoming replaced by female germinal cells later in the life cycle of triploids (Ruiz-Verdugo et al. 2000, Ruiz-Verdugo et al. 2001, Maldonado-Amparo & Ibarra 2002). However, as is the case with most other mollusk species, an oocyte stage at which gametogenesis is arrested has not been clearly identified. The objective of this study is to compare oocyte type frequencies between diploid and triploid catarina scallops at three different ages to define if such a stage exists.

MATERIALS AND METHODS

Definition of Oocyte Types and Their Size

Because oocyte types in catarina scallop have not been previously described, we first defined the oocyte stages based on those described by Dorange and LePennec (1989) and Saout (2000) for another scallop, *Pecten maximus*. The oocyte stages described by those authors included oogonia, previtellogenic oocyte, vitellogenic oocyte, and postvitellogenic oocyte. An additional oocyte stage was described for catarina scallop, mature oocytes, following the description from Longo (1983). The definition of meiotic stages occurring in previtellogenic oocytes of catarina scallop were based on those described for *Pecten maximus* by Saout (2000).

To obtain high definition photomicrographs of each oocyte stage, samples of diploid and triploid catarina scallop gonads were taken from five scallops of each ploidy group at 7 months old. These scallops were produced following the same methodology as Ruiz-Verdugo et al. (2000), inhibiting formation of the second polar body with cytochalasin-B (0.5 mg L^{-1}), and grown under the same conditions. All gonad samples were fixed similarly as for electronic microscopy studies (Komaru et al. 1994) to be able to obtain semi-thin (2 μm) sections, with the following modifications. The gonads were fixed in 2% gluteraldehyde in phosphate buffer (0.2 M) with an adjusted pH to 7.4 for 24 h. After this time, all samples were washed twice, each during 30 min, in a washing solution (9 g NaCl, 0.14 g KCl, 0.12 g $CaCl_2$, 0.2 g $NaHCO_3$, 2 g glucose, in 1000 ml distilled water). The samples were then progressively dehydrated passing them sequentially from 30% alcohol to absolute alcohol. The rest of the histologic process was the same as with H&E. Photomicrographs taken with an Olympus BX-41 microscope with an integrated camera (CoolSNAP-Pro Color) were digitalized and measurements of oocyte types diameters of each ploidy class were taken with the image analysis program SigmaScan Pro5, obtaining the area by digitalizing contours and estimating diameters. A total of 30 cells of each type were measured per ploidy.

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Quantification of Oocyte Types

After defining the type of oocytes in catarina scallop, we proceeded to obtain oocyte types frequencies. The diploid and triploid catarina scallops used in the evaluation of numbers of oocyte types in this study were those used by Ruiz-Verdugo et al. (2000) for the study of gametogenesis between triploids and diploids. In that study oocyte types present in each ploidy class at different ages were not comparatively quantified. Those triploids were produced by inhibition of polar body 2 with 0.5-mg cytochalasin-B L^{-1}, and the scallops were reared for one year at Bahía Magdalena, B.C.S. Mexico.

Gonad samples from three ages were evaluated for both ploidy groups. Samples were taken at 81 d of grow out when vitellogenesis was beginning in diploids, at 118 d as an intermediate gametogenesis stage in which advanced vitellogenesis was evidenced by

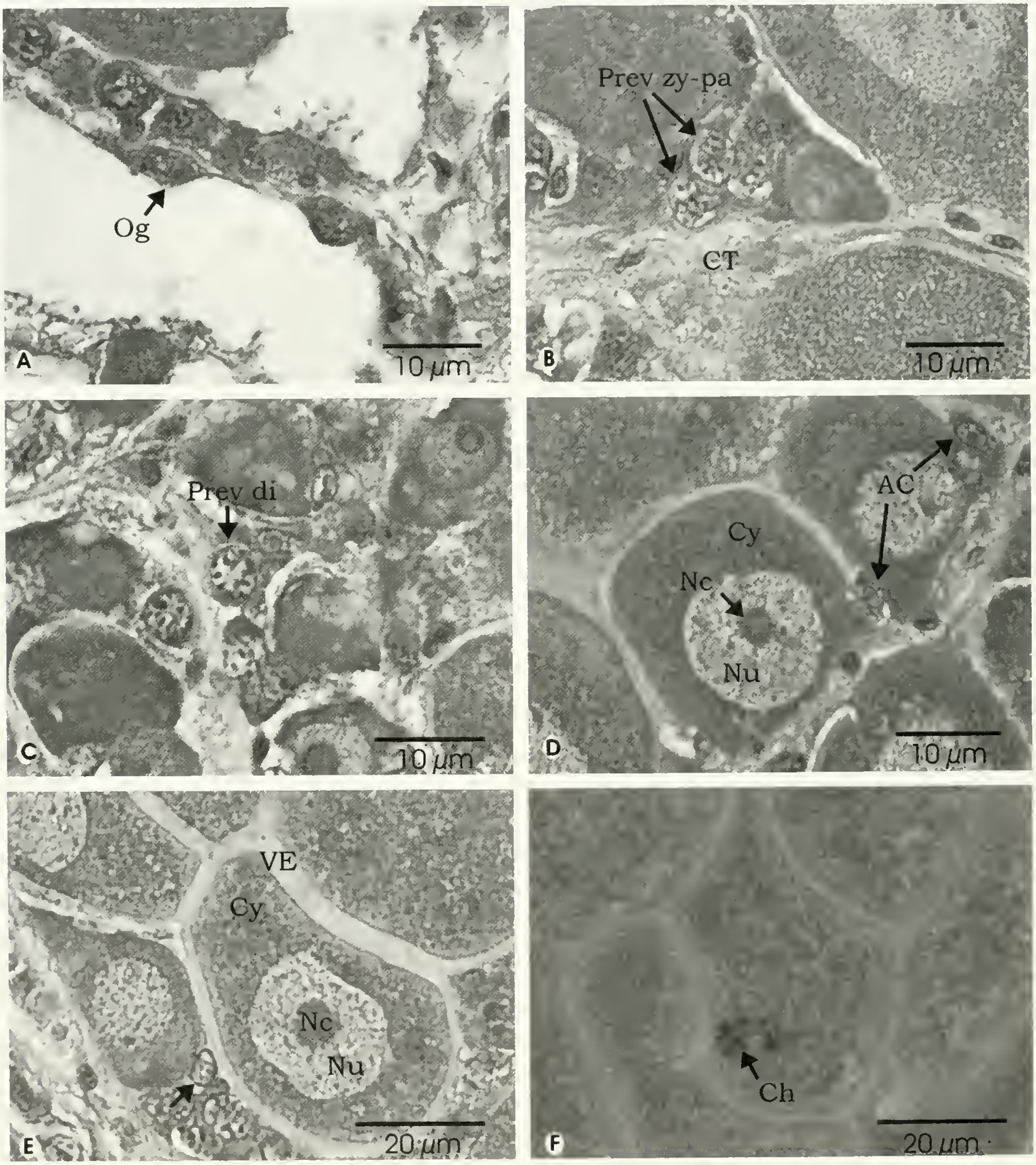

Figure 1. Diploid oocytes in catarina scallop. Light microscopy (×100). (A) Oogonia (Og); (B) Previtellogenic oocytes in zygotene-pachytene (Prev zy-pa); (C) Previtellogenic oocyte in diplotene (Prev di); (D) Vitellogenic oocyte; (E) Postvitellogenic oocyte; (F) Mature oocyte. AC = auxiliary cells; Ch = chromosomes in metaphase; CT = connective tissue; Cy = cytoplasm; Nc = nucleolus; Nu = nucleus; VE = vitelline envelope.

TABLE 1.

Mean cytoplasm diameters (minimum—maximum) of each oocyte type ($n = 20$) for each group, diploid (2N) and triploid (3N), in catarina scallop (*Argopecten ventricosus*).

	Cytoplasm Diameter, µm (range)	
Oocyte Types	**Diploid**	**Triploid**
Oogonia	4.57 (3.98–5.12) a	4.85 (4.08–5.46) b
Previtellogenic	6.95 (5.75–8.34) a	7.81 (7.24–8.76) b
Vitellogenic	37.34 (11.99–49.31) a	32.77 (10.29–55.54) a
Postvitellogenic	43.84 (35.25–50.54) a	50.35 (38.77–58.93) b

Different letters between columns indicate significant differences between ploidy classes.

the presence of large numbers of vitellogenic and postvitellogenic oocytes, and at 205 d of grow out when mature oocyte stages occurred in diploids and spawning had taken place in some scallops. For the quantification of each oocyte type the same digitalization process that was used for definition of oocyte types was followed. For each ploidy class 9 to 10 gonads (organisms) were randomly sampled, and for each gonad all oocyte types in 20 randomly selected acini were counted.

Statistical Analyses

Differences in size of oocyte types between ploidy groups were analyzed with a single factor (ploidy) analysis of variance for each oocyte type. Means were compared with a Duncan test. Differences in numbers of oocyte types between ploidy groups were analyzed with a multivariate mixed ANOVA model, in which scallops were a random effect, and age and ploidy were fixed effects. For the only fixed interaction (ploidy by age), *post hoc* comparisons were done between treatment means using a Tukey test (Sokal & Rohlf 1981). Significance for all analyzes was established at $P < 0.05$.

RESULTS

Oocyte Types in Catarina Scallop

The oocyte types occurring in diploid catarina scallop are depicted in Figure 1, and their mean sizes in Table 1. Those stages included oogonia (diameter 4.57 µm) attached to the acinus wall, previtellogenic oocytes with an average diameter of 6.95 µm in which the prophase I stages of zygotene-pachytene and diplotene could be distinguished (based on Saout 2000). The first association of oocytes to auxiliary cells characterized vitellogenic oocytes, with an average diameter of 37.34 µm, but ranging from 11.99 µm to 49.31 µm. Postvitellogenic oocytes, in which a clearly defined vitelline envelope was present, had an average diameter of 43.84 µm. Few mature oocytes, those in which the germinal vesicle was broken, were observed in these scallops, and therefore not measured.

In triploid catarina scallop (Fig. 2, Table 1), oogonia also attached to the acinus wall were significantly larger (4.85 µm) than those in diploids. Previtellogenic oocytes (diameter 7.81 µm) were also larger and differed from diploids in that they were the most abundant oocyte type, and in that they were surrounded by empty spaces, probably a consequence of cell lyses. Vitellogenic oocytes diameter (32.77 µm) in triploids was not significantly different from diploids, although in triploids most of the few observed vitellogenic oocytes were still pedunculated. Postvitellogenic oocytes (diameter 50.35 µm) were larger than in diploids and free in the lumen of the acinus. No mature oocytes were observed in triploid scallops.

Analyses of Numbers of Oocyte Types

Both, ploidy class and age were significant effects ($P < 0.0001$) in the analysis for frequencies of all oocyte types. There was a significant interaction between ploidy class and age, and also the triple (random) interaction effect was significant. At 81 d and 118 d of grow out, diploids had significantly less oogonia and previtellogenic oocytes than triploids, and more vitellogenic, postvitellogenic, and mature oocytes than triploids (Table 2). At 205 d diploid and triploids practically showed no oogonia, but diploids still had significantly less previtellogenic oocytes and more vitellogenic, post-vitellogenic, and mature oocytes than triploids. In triploids evaluated at this late age the most common oocyte type was still the previtellogenic oocyte.

Beside the differences within age for numbers of oocyte types between ploidy groups, and the difference in the most common oocyte type between diploid and triploid scallops at any age (Fig. 3), diploids had an increasing number of vitellogenic oocytes and a decreasing number of previtellogenic oocytes from 81 d (15.26 and 6.89, respectively) to 118 d (29.47 and 0.7, respectively). Triploid scallops had an increasing number of previtellogenic oocytes and a decreasing number of vitellogenic oocytes during the same period (27.82 and 2.19 at 81 d, and 85.53 and 1.4 at 118 d). There was a decrease in numbers of vitellogenic oocytes found in diploids from 118 d (29.47) to 205 d (24.64), which was paralleled

TABLE 2.

Mean numbers of oocyte types at each age and for each ploidy group, diploid (2N) and triploid (3N) of catarina scallop, *Argopecten ventricosus*.

Oocyte Types						
Age	**Ploidy**	**Oogonia**	**Previtellogenic**	**Vitellogenic**	**Postvitellogenic**	**Mature**
81 d	2N	1.43 b	6.89 b	15.26 c	1.07 b	0.09 a
	3N	6.62 b	27.82 c	2.16 a	0.00 a	0.00 a
118 d	2N	0.00 a	0.66 a	29.47 e	6.62 cd	0.65 b
	3N	0.35 a	85.53 e	1.40 a	0.03 a	0.00 a
205 d	2N	0.03 a	0.01 a	24.64 d	5.76 c	2.74 c
	3N	0.00 a	77.06 d	4.76 b	0.47 ab	0.00 a

Differeot letters within columns (oocyte types) indicate significant differences between ploidy groups and ages.

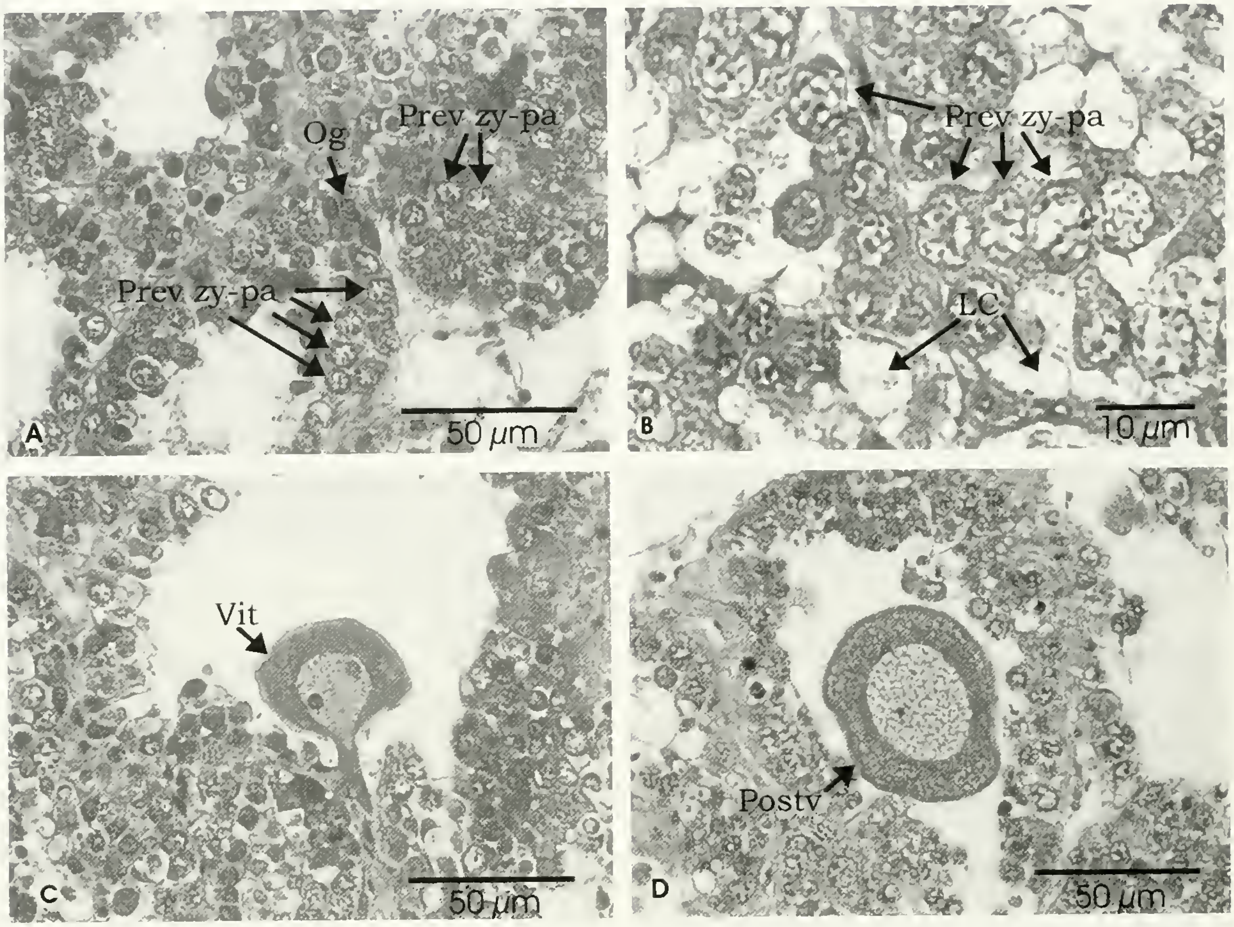

Figure 2. Triploid oocytes in catarina scallop. Light microscopy. (A) Oogonia (Og), and large numbers of previtellogenic oocytes in zygotene-pachytene (Prev zy-pa) (×40). (B) Closer view of Prev zy-pa (×100). (C) Isolated vitellogenic oocyte (Vit) (×40). (D) Isolated postvitellogenic oocyte (Postv) (×40). LC = Cell lysis.

by an increase in mature oocytes (0.65 at 118 d, and 2.74 at 205 d). Among triploids there was a decrease in number of previtellogenic oocytes from 118 d (85.53) to 205 d (77.01), which paralleled an increase in numbers of vitellogenic oocytes (1.40 at 118 d, and 4.76 at 205 d).

The significance of the triple random interaction (ploidy, age, and organism) was caused by differences among diploid and triploid individuals in the number of the different types of oocytes each showed at the different ages evaluated, with the largest differences occurring among triploid individuals for the number of previtellogenic oocytes present per individual, followed by the number of oogonia and vitellogenic oocytes. In diploids, variation between individuals was seen especially for numbers of vitellogenic oocytes, but the variation was not as marked as that seen for previtellogenic oocytes among triploids (Fig. 4).

DISCUSSION

We have demonstrated in this study that in catarina scallop the number of late developmental oocyte stages (vitellogenic, postvitellogenic, and mature) is largely reduced in triploids when compared with those developing in diploids, and that the number of early oocyte stages in triploids, especially the previtellogenic oocyte, is in a large frequency from the beginning to the end of the reproductive cycle of diploids. A large reduction in fecundity, measured as number of mature or spawned eggs, as a consequence of triploidy has been observed for oysters and clams (Guo & Allen 1994c, Utting et al. 1996), as well as for the catarina scallop (Ruiz-Verdugo et al. 2001). The sporadic occurrence of vitellogenic oocytes in triploid scallops appears to be a random event, as significant variation between individuals was seen. The low number of vitellogenic oocytes translates into a reduction in fecundity of 86% at 81 d, 95% at 118 d, and 81% at 205 d of growth. However, when post-vitellogenic oocytes are considered, the reduction in fecundity was larger, 100% at 81 d, 99% at 118 d, and 92% at 205 d.

Because the first sampling analyzed already contained diploid scallops with advanced stages of development, not just the first oocyte stages, we were unable to determine whether there was an actual delay in triploid scallops in the initial occurrence of the first oocyte stages (oogonia and previtellogenic oocytes) when compared with diploids by oocyte frequency counts. In this study the scallops shell height at the earliest sampling (81d) was close to 3 cm (Ruiz-Verdugo et al. 2000), and it is known that the first age of sexual maturation for catarina scallop when grown at the same environment is a shell height of 2 cm (Cruz et al. 2000). Further studies at ages or sizes smaller than the ones sampled in this study are necessary to clarify whether there is an actual delay in early oogenesis of triploid scallops when compared with diploids.

In fish, studies comparing numbers of oocyte types between diploids and triploids (Carrasco et al. 1998, Felip et al. 2001), have

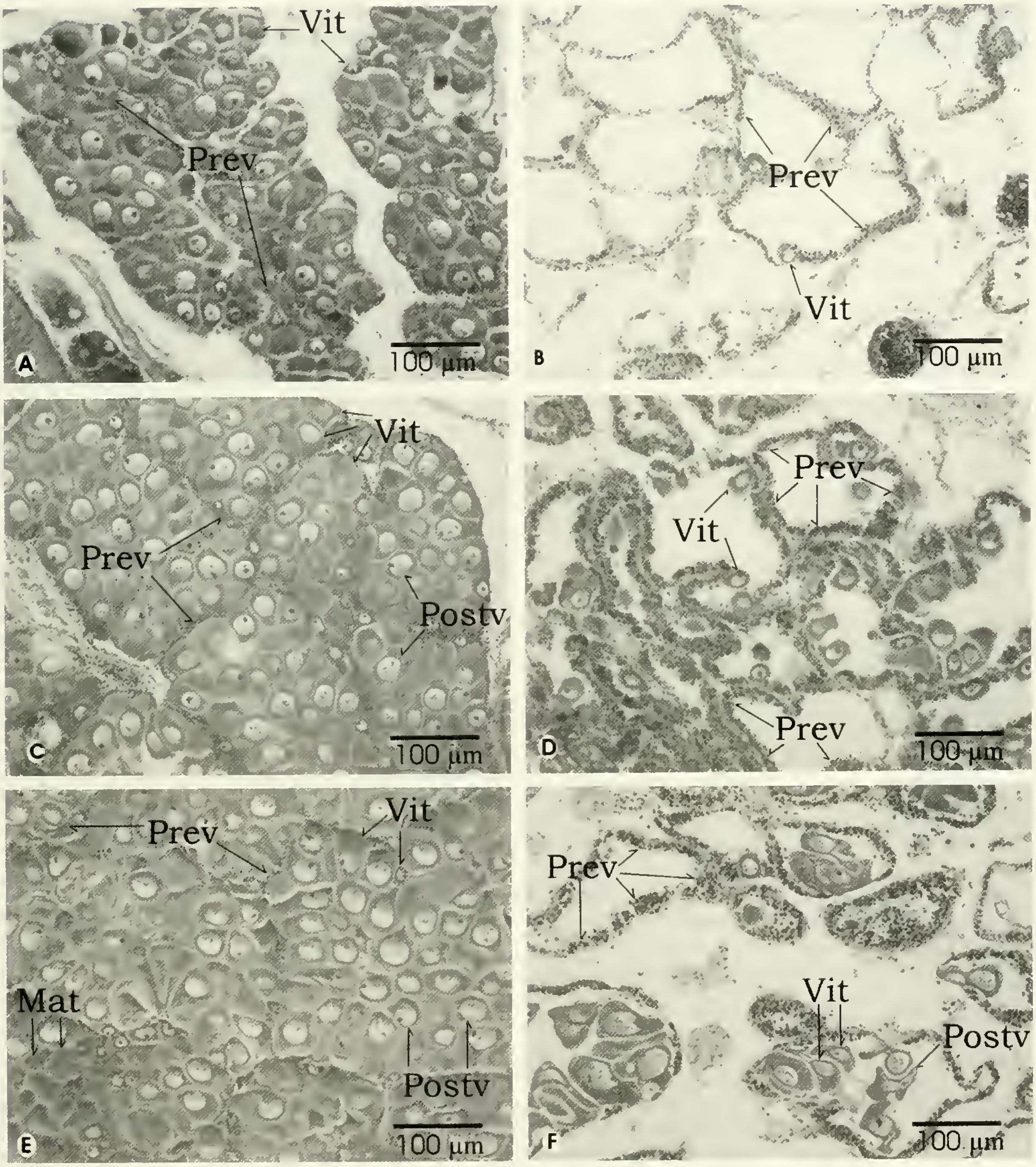

Figure 3. Diploid (left) and triploid (right) catarina scallop female gonad structure at 81 d (A & B), 118 d (C & D), and 205 d (E & F) of grow out. Light microscopy (×20). In diploids at 81 d (A) there were abundant vitellogenic oocytes (Vit), and few previtellogenic (Prev); by 118 d (C) postvitellogenic oocytes (Postv) were abundant; by 205 d (E) some mature oocytes (Mat) were also present. In triploids previtellogenic oocytes were the most common type at all ages although a few vitellogenic (B) were already present at 81 d, increasing in numbers by 118 d (D); some postvitellogenic oocytes were evident by 205 d (F).

also indicated that there is a difference between ploidy groups in the type of oocytes most frequently occurring, with the most common one in triploids being the previtellogenic oocyte. This points toward the first oocyte stage at which the triploid condition results in an inhibitory effect of further development being the previtellogenic oocyte, such that more advanced oocyte stages occur at much lower frequencies. In scallops it is known that only those previtellogenic oocytes that complete the pachytene stage and enter the diplotene stage of meiosis I will begin the process of vitellogenesis and become mature (Beninger & Le Pennec 1991, Dorange & Le Pennec 1989). Our results clearly point to the fact that oogenesis in triploid catarina scallop (*Argopecten ventricosus*) was arrested at the previtellogenic oocyte, and the reduced numbers of ooyete stages other than the previtellogenic type in triploids point to the halt of oocyte development at prophase of meiosis I, before the diplotene stage. In the male gonad part of this hermaph-

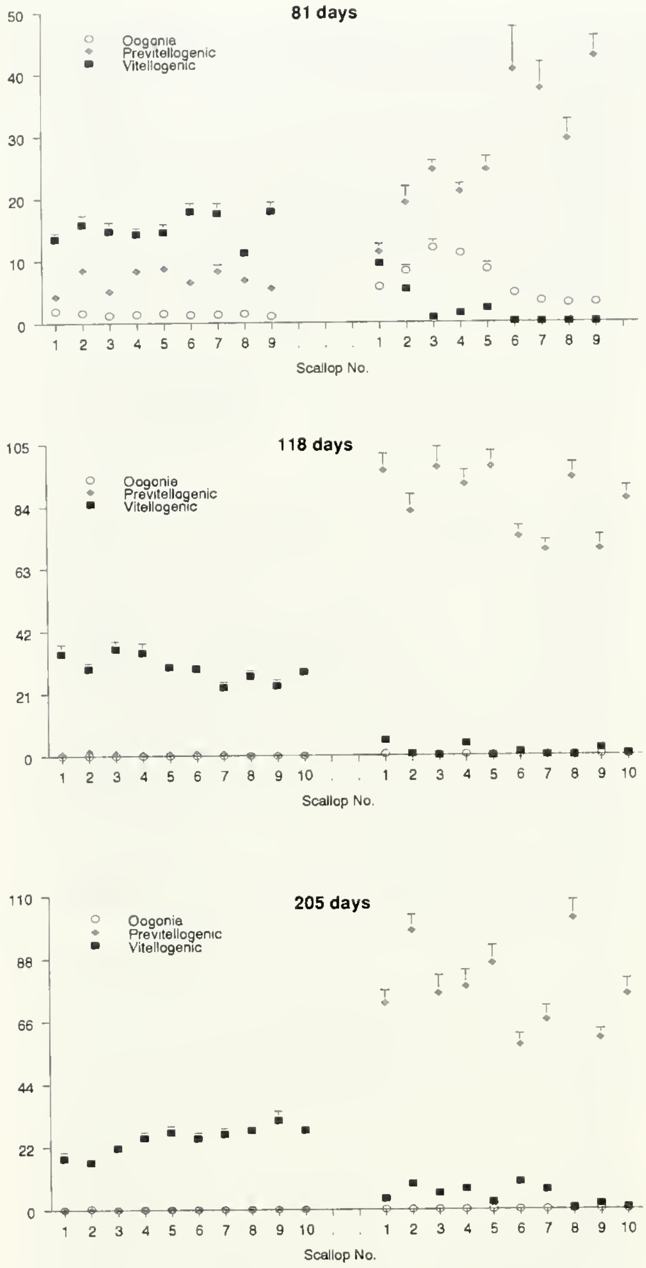

Figure 4. Frequencies of oocyte types (oogonia, previtellogenic, vitellogenic) per scallop in both, diploids and triploids, at each of the evaluated ages.

rodite species it is known that the halt in spermatogenesis occurs also at prophase I (Maldonado-Amparro & Ibarra 2002).

Until now, the cause of the sterility in triploid mollusks had been mostly ascribed to the inability of chromosomes to synapse, align, and segregate. However, at least for one species, the Pacific oyster, it has been demonstrated that synapses and segregation of chromosomes can occur in triploid oocytes even if in a lower number of oocytes than in diploids and in an abnormal fashion (Guo & Allen 1994c). In other mollusk species, including the catarina scallop, female triploids are known to be able to produce mature oocytes, even if in largely reduced numbers when compared with diploids (Guo & Allen 1994a, Utting et al. 1996, Ruiz-Verdugo et al. 2001). Among some species of male triploid mollusks, meiosis I and II are able to proceed through sperm formation although in reduced numbers when compared with diploids (Komaru & Wada 1990, Guo & Allen 1994a, Cox et al. 1996, Maldonado-Amparo & Ibarra 2002), and the produced sperm is generally aneuploid.

Because mollusks do not have to go through the completion of meiosis I to form mature oocytes, Allen (1987) proposed that the fact that oogenesis is arrested in triploid mollusks must be a consequence of meiotic difficulties arising at pre-synaptic or synaptic stages, after chromosome replication, rather than difficulties in pairing of homologous for segregation in metaphase I of meiosis. Our results support this same conclusion. The finding that few oocytes proceeded to the vitellogenic oocyte in triploids points toward the halt of meiosis being just before the diplotene stage at prophase I. The regulatory mechanisms to explain that arrest of meiosis remain to be investigated. One possible regulatory mechanism might be associated with the known "meiotic checkpoints" described for a number of organisms (Murakami & Nurse 1999, Murakami & Nurse 2000, Roeder & Bailis 2000, Tarsounas & Moens 2001), with the "recombination or pachytene checkpoint" being one of the most interesting ones to explain the arrest of meiosis in oocytes of triploid organisms, as it provides with a genetic, rather than just a mechanical mechanism for the observed arrest of further development. Meiotic checkpoints, also called "housekeeping mechanisms", are known to act by means of protein complexes signaling abnormal chromosome behavior, with effector proteins acting to delay or arrest the meiotic process (Roeder & Bailis 2000).

In conclusion, partial sterility in the female gonad of the catarina scallop is related to a halt in previtellogenic oocytes. As it is known that only those previtellogenic oocytes that complete the diplotene stage of meiosis I become vitellogenic, we conclude that the sterility is caused by problems during chromosome synapsis and recombination, that is, during the zygotene and pachytene stages of meiosis I. Why some oocytes are able to complete the vitellogenic process in spite of the demonstrated general arrest at previtellogenic oocyte is not known, but recent studies in triploids of other species point toward the existence of a correction mechanism of trivalent and tetravalent formation when homologous pair for recombination, which appears to be sex specific (Gui et al. 1991, Gui et al. 1992, Gui et al. 1995; Oliveira et al. 1995, Zickler & Kleckner 1999), and explains triploids differences between sexes in sterility.

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LOW TEMPERATURE, BUT NOT AIR EXPOSURE SLOWS THE RECUPERATION OF JUVENILE SCALLOPS, *PLACOPECTEN MAGELLANICUS*, FROM EXHAUSTING ESCAPE RESPONSES

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ABSTRACT Marked changes in temperature as well as periods of air exposure are common during scallop seeding operations. We examined whether the escape responses of cultured juvenile sea scallops *Placopecten magellanicus* (35–45 mm shell height) of the size used for seeding were hindered by such stresses and how the performance of stressed scallops changed within the week following thermal change. Tagged cultured scallops were either transferred to 8°C or maintained at 18°C; escape responses from starfish were measured 8 times during the following 156 h. The second study combined the stress of air exposure (4 h) with that of transfer to colder temperatures. Transfer of scallops from 18 to 8°C significantly reduced the clapping rates and maximum number of claps in a series at every sampling time (12 to 156 h) following thermal transfer. After 15 min of recuperation from exhaustive escapes, the percent initial claps was lower than that of control scallops (~40–50% vs. 60–70%). The time spent closed after exhaustive exercise was similar between the cold-stressed and control scallops, except at the first sampling time (6.5 and 2.7 min, respectively). Escape performance was not modified by 4 h air exposure. While decreased performance under cold stress could be ascribed to direct, kinetic effects of temperature, certain aspects of the swimming response improved during the 156 h at 8°C. However, the scallops did not completely acclimate their clapping rate to the thermal change within 6 d, suggesting that if a cold stress accompanied seeding, cultured scallops may remain vulnerable to starfish predation during a prolonged period. Seeding operations should therefore try to minimize thermal shock.

KEY WORDS: *Placopecten magellanicus*, escape responses, aquaculture, temperature, emersion, acclimation

INTRODUCTION

The sea scallop, *Placopecten magellanicus*, is a cold-water species that does well at temperatures ranging from 5°C–15°C, with optimum growth occurring at about 10°C (Naidu et al. 1989). Depending on previous thermal acclimation history, temperatures above 21–23.5°C can cause scallop mortality (Dickie 1958). Specific thermal regimes are known to influence growth, to provide stimuli for synchronous spawning (Bonardelli et al. 1996) and to condition predator-prey interactions (Hatcher et al. 1996). Low temperatures can delay larval growth and increase larval mortality as a consequence of longer exposure to predators (Dickie 1955, Young-Lai & Aiken 1986). High temperatures have frequently been held responsible for mass mortalities of scallops since the 1920s in the southwestern Gulf of St. Lawrence (Dickie & Medcof 1963). Since most of these mortalities occurred in the summer at depths of less than 12–20 m, oscillations in the thermocline were likely to have led to exposure of scallops to lethally high temperatures. Moreover, warm water debilitates sea scallops and increases their susceptibility to predation (Dickie & Medcof 1963). Thus, the sea scallop, which performs best at 10°C ± 5°C, can be exposed to thermal fluctuations that modify its performance in its natural habitat and during aquaculture operations.

Aerial exposure and handling prior to seeding may weaken scallops, rendering them more vulnerable to predation. In summer and autumn, scallops can take more than 3 days to recover from such stress (Fleury et al. 1996). While some bivalves are well adapted for air exposure, scallops such as *Pecten maximus* are unable to withstand a progressive respiratory acidosis, and some may die after approximately 72 h of emersion (Duncan et al. 1994). Further, adult saucer scallops *Amusium japonicum balloti* suffer appreciable mortality when exposed to air more than 2 h (Dredge 1997), while mortality of juvenile *P. maximus* increased significantly after 15 h of dry transport (Maguire et al. 1999a). Desiccation stress and air exposure reduce both the righting and recessing activities of juvenile scallops (Maguire et al. 1999b, Minchin et al. 2000). During air exposure of scallops, the adenylate energy charge (AEC) in the striated muscle decreases markedly, but recovers after a few hours in aerated seawater (Maguire et al. 1999a, Maguire et al. 1999b). When scallops, *Chlamys opercularis* (4–5 cm in diameter), were exhausted and then allowed to recover in the air, the adductor muscle AEC of the scallop increased to 92% of its original value within 30 min. However, final recovery of muscle energetic status only occurred in aerated seawater (Grieshaber 1978).

Since metabolic pathways and the contractile machinery are temperature-sensitive (Olson & Marsh 1993), escape responses may decrease during short-term exposure to cold. Subsequent acclimation to such cold temperatures is likely to compensate for this reduction in performance. For example, scallops living at greater depths compensate for the prevailing lower temperatures through an increase of membrane fluidity by adjustments of membrane fatty acid composition (Napolitano et al. 1992). Thus the escape responses of scallops may be temporarily reduced by changes in temperature.

During bottom seeding operations, juvenile scallops can be exposed to marked changes in temperature as well as periods of air exposure. For example, the scallops may be transferred from a warmer rearing temperature (i.e., during suspension in pearl nets) to a cooler temperature on the bottom. Both air exposure and thermal change during seeding operations may hinder the development of escape responses. In this study, we examined whether such stresses contribute to the loss of juvenile scallops after seeding, by reducing their escape response capacity or their recuperation from exhausting escape responses.

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MATERIALS AND METHODS

Scallop Collection, Storage, and Tagging

Spat were collected east of the Îles-de-la-Madeleine in September 1997 (Fig. 1). Thirteen months later, juveniles (17–23 mm shell height) were transferred for intermediate culture in pearl nets (35-cm square base, with a 6 mm mesh netting) at a density of $100 \cdot \text{net}^{-1}$. From June to September 1999, the scallops were maintained at a density of 20 individuals per pearl net (35-cm square base, with a mesh size of 9 mm). For our experiments, we selected individuals between 35–45 mm of shell height (maximum dorsal-ventral distance). Hallprint™ labels (4 × 8 mm) were glued on the upper valve using cyanoacrylate adhesive to identify the scallops during sequential tests.

Cold Shock Experiment

On September 19, 1999, 64 scallops were brought in an icebox from their suspended culture site in the Havre-aux-Maisons lagoon to the laboratory (emersion time <10 min; see Fig. 1). Temperature in the lagoon was measured hourly to the nearest 0.1°C by a Sealog-T V 1.04 thermograph during this period. Water temperature in the lagoon was approximately 18°C the week before retrieving experimental scallops (Fig. 2A). Hence, we used 18°C as our control treatment and 8°C to simulate the cold stress experienced during transfer from suspension culture in the lagoon to the seeding ground (Fig. 1), since autumn seeding may expose scallops to such bottom temperatures. The scallops were equally divided between 2 well-aerated 200-L tanks, one at 18°C (control) and the other at 8°C (thermal stress). The photoperiod was maintained at natural day lengths (12 h light and 12 h dark). Salinity ranged from 29.0–30.5‰. No food was supplied and seawater was filtered (1 μm) and UV-sterilized.

Cold Shock with Air Exposure Experiment

A second group of scallops was brought to the laboratory on September 26th to compare the impact of air exposure coupled with cold stress (thermal stress + air (TSA) with that of air exposure alone [control + air (CA)]. The transfer from warm (15.5°C) (see Fig. 2) to low (8°C) temperature was preceded by a 4 h emersion (18°C) during which the scallops were sprayed with seawater every 30 min to keep them damp. As in the former experiment, 32 scallops were placed at 8°C and 32 others were kept at 18°C. Scallops were maintained in the same conditions as indicated earlier.

Evaluation of Escape Responses

Scallops were put in 33 × 28 × 12 cm-basins and tested separately after a minimum of 2 min without disturbance. An escape

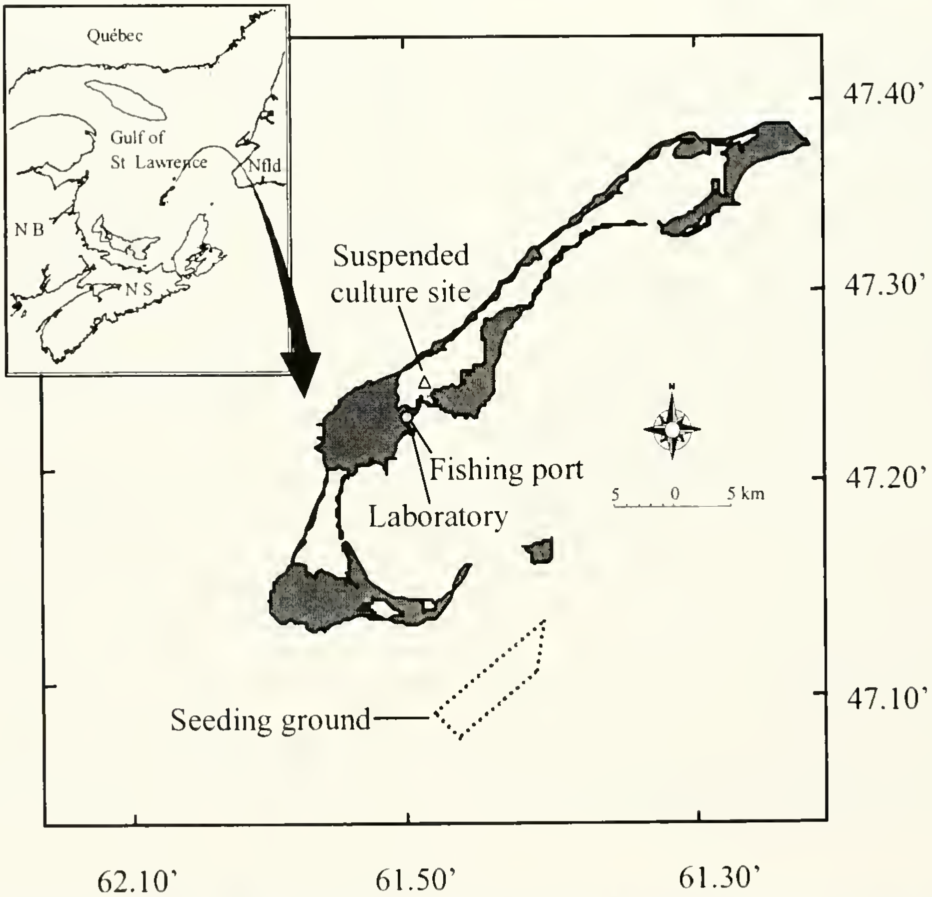

Figure 1. Location of sites for sea scallop suspension culture in pearl nets (Havre-aux-Maisons lagoon) and seeding on the sea bottom (Chaîne-de-la-Passe fishing ground) in the Îles-de-la-Madeleine. Inset shows location of the study site in the Gulf of St. Lawrence, eastern Canada.

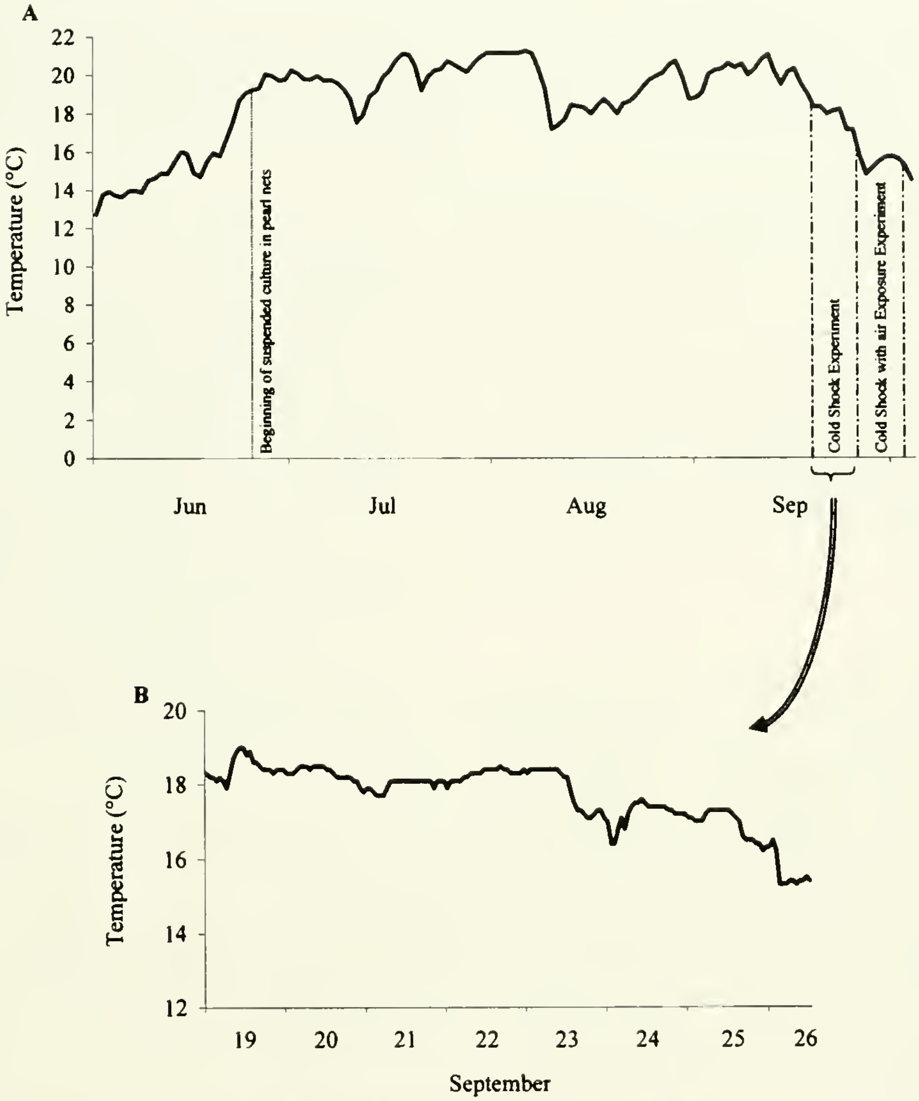

Figure 2. Temperature measured in the Havre-aux-Maisons lagoon (A) between early June and early October and (B) for the week (September 19–26) that scallops of the Cold Shock with Air Exposure Experiment spent in the lagoon, while scallops of the Cold Shock Experiment were being tested. Data were collected hourly with a precision of 0.1°C using a Sealog-T thermograph.

reaction consisted of a jumping or a swimming response following contact with the arm of a starfish. Time, number of valve claps (adductions) and the maximum number of claps in a series were counted until repeated stimulation did not elicit a clap within 1 min of the previous clap (exhaustion). Once the scallop was exhausted, it was left in its aerated basin for 15 min. Then the escape response was quantified a second time. Each escape parameter evaluated during this second test was called "response after 15 min of recuperation". Response time, defined as the time from initial contact of a starfish with the scallop mantle to first valve adduction, was measured in seconds. At the end of the escape response, the scallop was classified either as a "swimmer" or a "jumper". A "swimmer" performed several series of claps (3 claps or more) in response to predator stimulation, whereas a "jumper" did not clap more than twice in a row. The escape responses of twenty-four scallops from each treatment were measured 12, 24, 36, 48, 60, 84, 120, and 156 h after transfer. The remaining eight scallops from each treatment were left undisturbed in their respective tanks all week. These reference scallops (from the Cold Shock with Air Exposure Experiment) allowed us to examine the effect of repeated swimming behavior on the levels of macromolecular reserves in the muscle. The experiments on the impact of thermal transfer were carried out a week before those examining the combined impact of air exposure and thermal change. Whenever a percentage of recuperation is given for an escape parameter, it represents the ratio of the performance during the second stimulation (after 15 min of recuperation) relative to the initial response. These values are useful in that they show the extent to which a particular response returns to initial values after a short recuperation.

The *Asterias vulgaris* (radius of 5.5–7.5 ± 0.25 cm) used to provoke the escape responses were collected in the lagoon Le Bassin (southern end of the Îles-de-la-Madeleine) and kept in tanks containing filtered, continuously aerated seawater either at 18°C or 8°C (holding capacity of ~80 and 180 L, respectively) 30 h before the first stimulation. The starfish used in a particular escape response test were haphazardly chosen among 12 individuals at the experimental temperature. The same starfish was used for the two tests carried out on a given scallop at a given sampling time.

Biometric Measurements and Macromolecular Reserves in Adductor Muscle

Dissections of the scallops used for the Cold Shock with Air Exposure Experiment were carried out the day after the last escape response tests. The adductor muscles were immediately frozen on dry ice and maintained on dry ice for approximately 1 mo before transfer to a −80°C freezer at Université Laval. Other soft tissues were dried to constant mass at 60°C to determine their water content. We calculated a "muscle index" (muscle mass/mass of total soft tissues minus muscle mass) to examine the relative contribution of the adductor muscle. Muscle protein concentrations were measured using the bicinchoninic acid method with bovine serum albumin (BSA) as a standard (Smith et al. 1985). Muscle carbohydrate levels were determined using the phenol-sulfuric-acid method of Dubois et al. (1956), as modified by Martinez (1991).

Statistical Analyses

Clapping behaviors were compared using repeated measures ANCOVAs following the MIXED procedure (SAS 1999). The two main factors, Treatment (control vs. stress) and Time (12, 24, 36,..., 156 h after start of experiment) were considered as fixed effects. Individuals ($n = 24$) nested within Treatment were considered as a random effect. Time was better defined with a spatial power covariance structure (SP(POW) command) since it followed an exponential distribution. The Interaction term, Treatment × Time, was always included in models. Comparisons were only made between 2 treatments at a given sampling time using the difference of least squares means (LSMEANS TIME × TREATMENT / DIFF command). *P*-values associated with comparisons between treatment means at a given time came from these least squares means contrasts. The stability of escape performance over time for specific treatments (ex: Time × Treatment CA) was assessed by tests of effect slices (see Table 2). To meet requirements of normality and homoscedasticity of residuals, parameters may have been transformed using logarithms, square root or inversion although untransformed values are shown in the figures. If a behavioral response after 15 min recuperation exceeded 200% of the initial response, it was removed from the analysis (see notes in Tables 1, 2, and 3).

Multiple pairwise comparisons (Scheffe) were used to test for specific differences when ANOVAs showed significant effects (Table 4). Normality was assessed using the Shapiro–Wilk's test and homogeneity of variances by a Brown–Forsythe's test (SAS 1999). A probability level (α) of 0.05 was used.

RESULTS

Our first experiment compared the escape behaviors of scallops transferred from warm (18°C) to colder water (8°C) with those of scallops maintained in 18°C. The second examined the impact of such a thermal transfer when it followed 4 h of air exposure. Most of the escape response parameters (except clapping time and time spent closed) for control (control and control + air) scallops showed higher values than for cold stressed (cold and cold + air) scallops (Figs. 3, 4, 5, 6). The difference between control (control + air) and cold (cold + air) treatments was also apparent when considering the scallops' capacity for recuperation 15 min after the initial response (Figs. 4B, 4C, 4D; 6B, 6C, 6D; Tables 1, 2, 3).

Cold Shock Experiment (Control vs. Cold-Stressed)

Scallops responded to the first contact with the starfish by clapping their valves for a mean of 1.6 and 3.5 min before exhaustion (no clap within 1 min of the previous clap), during which an average of 51 and 45 claps was observed for control and cold-stressed scallops, respectively (Fig. 3A, 3C). Although the number of claps only differed between treatments at a few sampling times, control scallops had a significantly higher clapping rate and a greater number of maximum claps in a series at every test (Fig. 3E, 3G; Table 1). Control scallops kept a relatively constant clapping time throughout their successive encounters with starfish, whereas cold-stressed scallops showed a gradual reduction of their initial clapping time (Fig. 3C; Table 2). Initial clapping time differed between the two treatments throughout the 168 h. Clapping time after 15 min of recuperation did not vary throughout time for either group (Table 2). Cold-stressed scallops only showed longer clapping times than control scallops at 12, 60, and 156 h (Fig. 3D). In contrast, the gap between control and cold-stressed scallops for total number of claps, clapping rate and maximum number of claps in a series was similar or increased after 15 min recuperation (Fig. 3B, 3D, 3H).

The changes in escape behaviors during the experimental period provided indications of thermal acclimation (cold-stressed) and habituation (control and cold-stressed). Cold-stressed scallops improved some aspects of their responses while control scallops remained constant or worsened. Control scallops decreased the total number of claps and maximum claps in a series during successive sampling events while cold-stressed scallops maintained their values (Fig. 3B, 3G; Table 2). Control scallops showed steady clapping time and rate and time spent closed while cold-stressed scallops shortened their clapping time, increased their clapping rate and decreased the time closed (Fig. 3C, 3E; 4A; Table 2). The other parameters shared downward (Fig. 3A) or steady trends (Fig. 3D, 3F, 3H) in both groups (Table 2).

The time during which scallops kept their valves closed after exhaustion was similar in the treatments ($P \geq 0.058$; Fig. 4A; Table 1), except at the first observation period when cold-stressed scallops stayed shut more than twice as long as the control scallops ($P < 0.0001$). At virtually all observation periods, control scallops recuperated more of their initial response (i.e., in term of percentage) than cold-stressed scallops (Fig. 4B, 4C, 4D). This was particularly clear for the total number of claps (Fig. 4B).

Cold Shock with Air Exposure Experiment (Control + Air vs. Cold-Stressed + Air)

In this experiment, all scallops were initially exposed to air (18°C) for 4 h to simulate conditions during transfer from cultivation sites to seeding grounds. Control scallops were then returned to warm water (18°C) whereas cold-stressed scallops were transferred to 8°C. Scallops from these treatments made an average of 50 claps in the first escape test (Fig. 5A). At this time, the 1.5 min clapping time of control scallops was significantly shorter than the 2.5 min time for cold-stressed scallops ($P = 0.0002$) (Fig.

TABLE 1.

Statistical analysis of the effects of treatment and time on escape performance of juvenile sea scallops from Cold Shock Experiment. The scallops were transferred from pearl nets in the lagoon (18°C) to seawater at either 18°C (control) or 8°C (cold-stressed) in the laboratory to simulate the cold stress accompanying seeding operations. Each scallop (n = 24 per treatment) was measured during each sampling time (12, 24, 36, 48, 60, 84, 120 and 156 h).

	Initial Response				**Response After 15 min of Recuperation**		
	df	*F*	*P*		*df*	*F*	*P*
				Number of claps			
Treatment	1	2.63	0.11		1	45.44	<0.0001
Time	7	4.73	<0.0001		7	2.31	0.026
Treatment × Time	7	1.08	0.37		7	1.22	0.29
				Clapping time			
Treatment	1	33.67	<0.0001		1	13.33	0.0007
Time	7	7.33	<0.0001		7	1.54	0.15
Treatment × Time	7	5.78	<0.0001		7	1.83	0.081
				Clapping rate			
Treatment	1	23.25	<0.0001		1	63.12	<0.0001
Time	7	2.44	0.019		7	0.80	0.59
Treatment × Time	7	1.71	0.11		7	1.30	0.25
				Maximum number of claps in a series			
Treatment	1	48.79	<0.0001		1	104.38	<0.0001
Time	7	3.26	0.0023		7	0.44	0.88
Treatment × Time	7	0.67	0.70		7	1.16	0.32
				Time spent closed			
Treatment	1	3.68	0.061		n.a.	n.a.	n.a.
Time	7	4.39	<0.0001		n.a.	n.a.	n.a.
Treatment × Time	7	3.09	0.0036		n.a.	n.a.	n.a.
				% initial number of claps†			
Treatment	n.a.	n.a.	n.a.		1	49.86	<0.0001
Time	n.a.	n.a.	n.a.		7	2.47	0.018
Treatment × Time	n.a.	n.a.	n.a.		7	0.49	0.84
				% initial clapping rate‡			
Treatment	n.a.	n.a.	n.a.		1	15.54	0.0003
Time	n.a.	n.a.	n.a.		7	1.11	0.35
Treatment × Time	n.a.	n.a.	n.a.		7	0.12	0.99
				% initial maximum number of claps in a series§			
Treatment	n.a.	n.a.	n.a.		1	26.39	<0.0001
Time	n.a.	n.a.	n.a.		7	1.33	0.24
Treatment × Time	n.a.	n.a.	n.a.		7	1.53	0.16

† 1, ‡5, or §7 observations were removed from the analysis as recuperation exceeded 200%.
n.a. = not applicable.

5C). Thus, initial clapping rate was higher for control scallops (38.7 vs. 23.6 claps · min^{-1}). Control and cold-stressed scallops had, respectively, average maxima of 10 and 7 claps in a row (Fig. 5E, 5G).

One day (24 h) after re-immersion, the escape performance of cold-stressed scallops resembled that of control scallops. The initial number of claps of cold-stressed scallops remained constant throughout the experimental period while it gradually decreased for control scallops (Fig. 5A; Table 2).). The initial clapping time was only greater for cold-stressed scallops at the 84th (P = 0.032) and the 120th h (P = 0.0002) (Fig. 5C). Initial clapping rates remained similar between 24 and 156 h (Fig. 5E; Table 3). In spite of the slightly greater mean values for control scallops, the 2 treatments did not differ in the initial maximum number of claps in a series, except at the 48th h (P = 0.038) (Fig. 5G; Table 3).

After the15 min recuperation, control and cold-stressed scallops differed in terms of total number of claps for the first six sampling periods ($P \leq 0.046$) (Fig. 5B). This difference was alleviated at 120 and 156 h after transfer, when both groups made 22–24 claps. After recuperation, the clapping time of the two groups never differed (Fig. 5D; Table 3), even though the clapping time of cold-stressed scallops remained steady, whereas that of control scallops decreased slightly (Table 2). After recuperation, clapping rate and maximum claps remained stable for control and cold-stressed scallops (Table 2). Clapping rate tended to be higher for control scallops (Fig. 5F). Control scallops consistently made a maximum of 7–8 claps in a series in contrast to 4–5 claps for cold-stressed scallops (Fig. 5H; Table 3). Overall, cold-stressed scallops improved their performance relative to that of control scallops with time after transfer, either sustaining their performance as control scallops decreased (Fig. 5A, 5B; Table 2) or improving their performance while control scallops remained stable (Fig. 5E, 5G; Table 2).

During the first test 12 h after re-immersion, the time spent closed after exhaustion differed between control and cold-stressed scallops (3.8 vs. 6.7 min, respectively P = 0.0019). Subsequently,

TABLE 2.

Temporal stability of escape performance by juvenile sea scallops either maintained at 18°C (control: C), transferred from 18°C to 8°C (thermally-stressed: TS), air-exposed for 4 h (control + air: CA) or air-exposed for 4 h during a transfer from 15.5°C to 8°C (thermally-stressed + air: TSA). Tests of effect slices analogous to the ANCOVAs shown in Tables 1 and 3. Each scallop (n = 24 per treatment) was measured at each sampling time (12, 24, 36, 48, 60, 84, 120, and 156 h).

	Initial Response			Response After 15 min of Recuperation		
	df	*F*	*P*	*df*	*F*	*P*
Number of claps						
Time × Treatment C	7	3.30	0.0021	7	2.94	0.0053
Time × Treatment TS	7	2.51	0.016	7	0.59	0.77
Time × Treatment CA	7	3.03	0.0042	7	2.93	0.0055
Time × Treatment TSA	7	1.10	0.36	7	1.04	0.40
Clapping time						
Time × Treatment C	7	1.73	0.10	7	1.92	0.065
Time × Treatment TS	7	11.38	<0.0001	7	1.44	0.19
Time × Treatment CA	7	4.24	0.0002	7	2.28	0.028
Time × Treatment TSA	7	6.50	<0.0001	7	0.45	0.87
Clapping rate						
Time × Treatment C	7	1.04	0.41	7	0.62	0.74
Time × Treatment TS	7	3.12	0.0034	7	1.48	0.17
Time × Treatment CA	7	1.93	0.065	7	0.84	0.56
Time × Treatment TSA	7	6.72	<0.0001	7	0.97	0.45
Maximum number of claps in a series						
Time × Treatment C	7	3.13	0.0033	7	0.54	0.81
Time × Treatment TS	7	0.80	0.59	7	1.06	0.39
Time × Treatment CA	7	1.04	0.40	7	0.74	0.64
Time × Treatment TSA	7	3.06	0.0040	7	0.76	0.62
Time spent closed						
Time × Treatment C	7	1.32	0.24	n.a.	n.a.	n.a.
Time × Treatment TS	7	6.16	<0.0001	n.a.	n.a.	n.a.
Time × Treatment CA	7	1.72	0.10	n.a.	n.a.	n.a.
Time × Treatment TSA	7	4.54	<0.0001	n.a.	n.a.	n.a.
% initial number of claps						
Time × Treatment C	n.a.	n.a.	n.a.	7	1.63	0.13
Time × Treatment TS	n.a.	n.a.	n.a.	7	1.33	0.24
Time × Treatment CA	n.a.	n.a.	n.a.	7	0.84	0.56
Time × Treatment TSA	n.a.	n.a.	n.a.	7	0.79	0.60
% initial clapping rate						
Time × Treatment C	n.a.	n.a.	n.a.	7	0.77	0.61
Time × Treatment TS	n.a.	n.a.	n.a.	7	0.46	0.86
Time × Treatment CA	n.a.	n.a.	n.a.	7	1.80	0.087
Time × Treatment TSA	n.a.	n.a.	n.a.	7	2.41	0.021
% initial maximum number of claps in a series						
Time × Treatment C	n.a.	n.a.	n.a.	7	1.65	0.12
Time × Treatment TS	n.a.	n.a.	n.a.	7	1.19	0.31
Time × Treatment CA	n.a.	n.a.	n.a.	7	0.63	0.73
Time × Treatment TSA	n.a.	n.a.	n.a.	7	1.22	0.29

n.a. = not applicable.

both control and cold-stressed scallops maintained their valves closed 4–7 min (Fig. 6A; Table 3). Towards the end of the observations, control scallops stayed closed longer than cold-stressed scallops with this trend becoming significant at 156 h (P = 0.044).

As noted during the first experiment, control scallops recovered more of their initial responses than cold-stressed scallops (Fig. 6B, 6C, 6D; Table 3). This difference was again particularly marked for the percent initial number of claps (Fig. 6B). The overall recovery of control scallops was between 65–75% compared with 42–52% for cold-stressed scallops.

Evaluation of the Impact of Air Exposure

Statistical comparison of the escape performance (number of claps, clapping time, clapping rate, etc.) of the control groups from the two experiments did not reveal any differences. As the values for each escape parameter were similar, with P-values ranging from 0.12 to 0.94, 4 h of air exposure did not seem to affect the escape response capacity of the scallops.

Sixty to ninety percent of the air exposed scallops responded to the starfish within 5 sec of the initial contact whether they were

TABLE 3.

Statistical analysis of the effects of treatment and time on escape performance of juvenile sea scallops from Cold Shock with Air Exposure Experiment. A 4 h air exposure during the transfer of scallops from pearl nets in the lagoon (15.5°C) to seawater at either 18°C (control) or 8°C (cold-stressed) in the laboratory was used to simulate the cold stress with air exposure accompanying seeding operations. Each scallop (*n* = 24 per treatment) was measured during each sampling time (12, 24, 36, 48, 60, 84, 120, and 156 h).

	Initial Response			Response After 15 min of Recuperation		
	df	*F*	*P*	*df*	*F*	*P*
Number of claps						
Treatment	1	2.85	0.010	1	20.40	<0.0001
Time	7	2.93	0.0055	7	1.93	0.064
Treatment × Time	7	1.20	0.30	7	2.04	0.050
Clapping time						
Treatment	1	7.78	0.0077	1	1.94	0.17
Time	7	6.60	<0.0001	7	1.96	0.060
Treatment × Time	7	4.13	0.0002	7	0.77	0.61
Clapping rate						
Treatment	1	1.89	0.18	1	7.35	0.0094
Time	7	3.86	0.0005	7	1.03	0.41
Treatment × Time	7	4.80	<0.0001	7	0.78	0.61
Maximum number of claps in a series						
Treatment	1	3.20	0.080	1	15.94	0.0002
Time	7	2.03	0.051	7	0.76	0.62
Treatment × Time	7	2.06	0.047	7	0.73	0.64
Time spent closed						
Treatment	1	0.11	0.74	n.a.	n.a.	n.a.
Time	7	2.98	0.0049	n.a.	n.a.	n.a.
Treatment × Time	7	3.28	0.0022	n.a.	n.a.	n.a.
% initial number of claps						
Treatment	n.a.	n.a.	n.a.	1	57.24	<0.0001
Time	n.a.	n.a.	n.a.	7	0.77	0.61
Treatment × Time	n.a.	n.a.	n.a.	7	0.85	0.54
% initial clapping rate†						
Treatment	n.a.	n.a.	n.a.	1	9.32	0.0038
Time	n.a.	n.a.	n.a.	7	3.24	0.0025
Treatment × Time	n.a.	n.a.	n.a.	7	0.98	0.45
% initial maximum number of claps in a series†						
Treatment	n.a.	n.a.	n.a.	1	15.65	0.0003
Time	n.a.	n.a.	n.a.	7	0.75	0.63
Treatment × Time	n.a.	n.a	n.a.	7	1.10	0.36

n.a. = not applicable.

† observations were removed from the analysis as recuperation exceeded 200%.

cold-stressed or not (Fig. 7C, 7D). Interestingly, scallops from the Cold Shock Experiment (without air exposure) took more time (5–15 sec) than those given 4 h air exposure before initiating an escape response (Fig. 7A, 7B). Very few individuals (1 to 3 out of 24) required more than 15 sec following the initial contact with the starfish.

In both experiments, control scallops swam in virtually all tests (Fig. 8), while a greater proportion of cold-stressed scallops re-

TABLE 4.

Carbohydrate and protein contents in the adductor muscle, and muscle index of juvenile sea scallops that were used in repeated escape tests or that remained inactive during the Cold Shock with Air Exposure Experiment. Mean (SE, *n*). In a given line, values for treatments that do not share the same letter differed ($P < 0.05$) according to Scheffe's multiple comparisons.

	Control (18°C) + Air		Thermally-stressed (8°C) + Air	
Variahle	Repeated Escapes	Rest	Repeated Escapes	Rest
Muscle index†	65.7 (1.2, 24)^a	68.7 (2.5, 8)^a	66.4 (1.6, 24)^a	59.7 (2.9, 8)^a
Carbohydrate concentration (mg · g^{-1} wet tissue)	10.6 (0.8, 24)^a	17.5 (1.9, 8)^b	11.1 (1.1, 24)^a	17.5 (1.6, 8)^b
Protein concentration (mg · g^{-1} wet tissue)	145.4 (2.0, 24)^a	138.7 (3.8, 8)^a	138.9 (2.5, 24)^a	139.6 (5.3, 8)^a

† $(M_{muscle}/(M_{digestive\ gland} + M_{other\ soft\ tissues})) \times 100$.

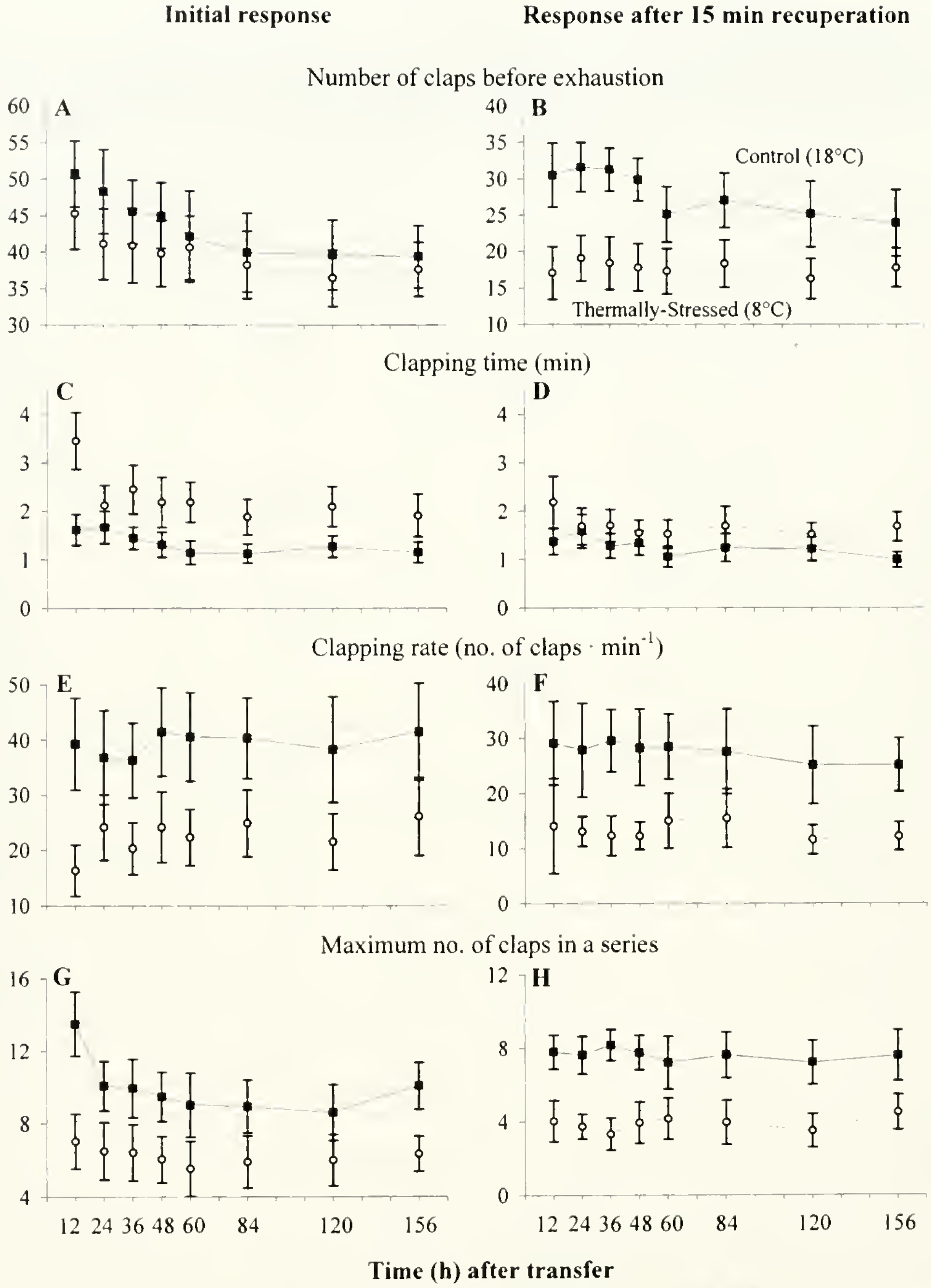

Figure 3. Effect of a transfer from 18°C to 8°C (thermal stress) vs. maintenance at 18°C (control) in seawater on the escape behaviors of juvenile sea scallops, *Placopecten magellanicus*, measured before and after 15 min of recuperation from exhaustive exercise. Means are shown for all parameters. Vertical bars indicate 95% confidence limits and $n = 24$ for each treatment.

sponded with jumps. In the experiment with air exposure, jumps were again more frequent for cold-stressed individuals, particularly within the first 60 h.

Biometrics and Muscle Energetics

At the end of the Cold Shock with Air Exposure Experiment, we compared shell characteristics and soft tissues of cold-stressed and control scallops. Furthermore, we compared resting scallops with those that had taken part in escape tests to assess the impact of the repeated escape measurements on tissue masses and muscle reserve levels. Shell mass was marginally smaller in resting scallops ($P = 0.040$). However, we found no differences between the adductor muscle mass ($P = 0.67$), digestive gland ($P = 0.13$) and the mass of other soft tissues (wet and dry; $P \geq 0.26$) of scallops that were induced to swim and those left undisturbed. Nonetheless, the muscle index of resting scallops exposed to air prior to the cold stress was slightly lower than that of control scallops ($P = 0.051$) (Table 4).

Muscle carbohydrates were decreased in scallops used for escape tests (Table 4). The phasic muscle of resting scallops contained about 18 mg of carbohydrates · g^{-1} wet tissue in comparison

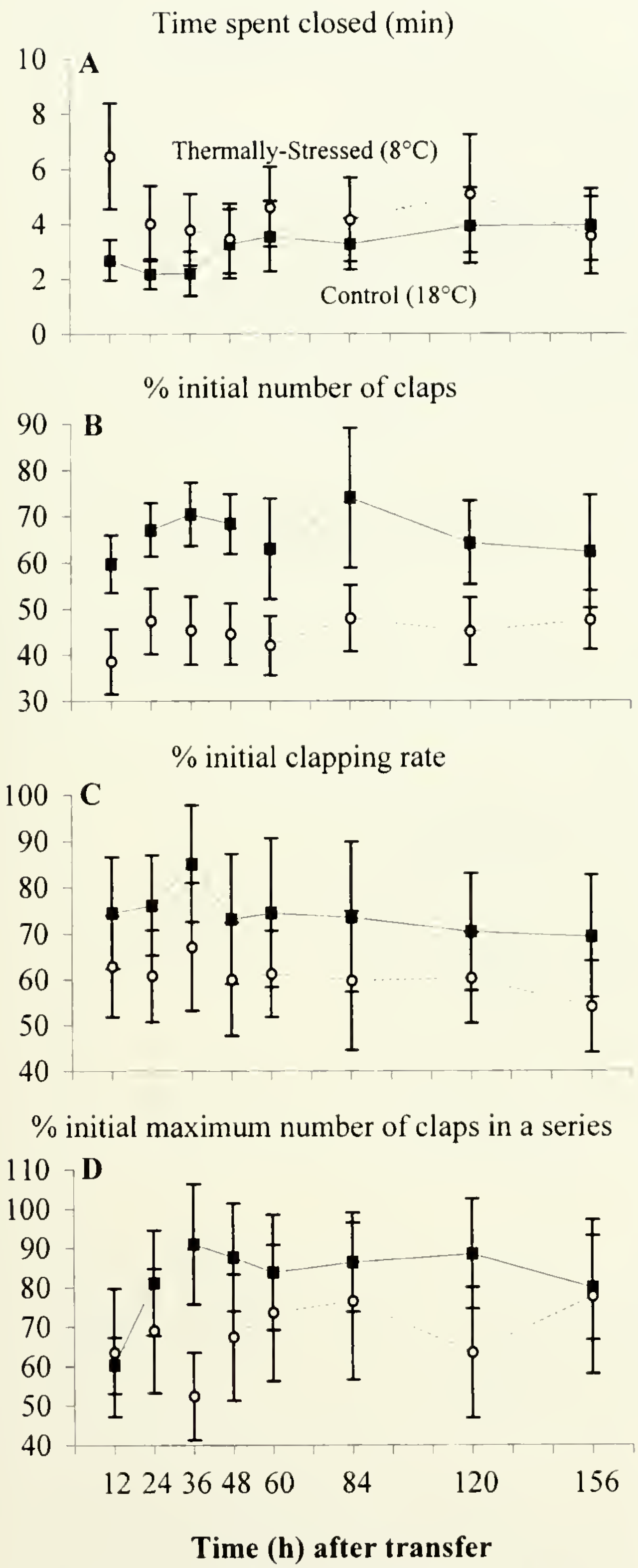

Figure 4. Effect of a transfer from 18°C to 8°C (thermal stress) vs. maintenance at 18°C (control) in seawater on (A) the time spent closed after initial exhaustive stimulation and (B, C, D) performance after 15 min of recuperation relative to initial response for the main escape behaviors of juvenile sea scallops, *Placopecten magellanicus*. Means are shown for all parameters. Vertical bars indicate 95% confidence limits and n = 24 for each treatment.

to 11 mg · g^{-1} wet tissue for exercised scallops. Muscle carbohydrate concentrations did not vary with cold stress. The mean muscle protein content was about 140 mg · g^{-1} wet tissue (Table 4); it did not differ between control and cold-stressed scallops or between the scallops that were forced to swim and those left undisturbed.

DISCUSSION

During seeding operations, juvenile scallops are often subjected to a cold stress. Although sea scallops can live at low temperatures, a rapid decrease in temperature will reduce the capacity to escape predators or colonize suitable habitats. Our results indicate that scallops cannot acclimate their clapping rate to such a cold stress within 6 days and thus may be more vulnerable to starfish predation. Indeed, the transfer of juvenile scallops from 18°C to 8°C markedly slowed their rate of clapping and the maximum number of claps in a series, but did not significantly alter the total number of claps carried out during an escape response. Nonetheless, after 6 days at 8°C, scallops reduced the time spent closed after exhausting escape responses and became similar to control scallops. Over the 6 days of study, cold-stressed scallops reduced their clapping time during an initial stimulation and increased their initial clapping rate whereas control scallops remained quite constant. Nevertheless, as the cold-stressed scallops did not attain the rates typical of control scallops, only partial thermal compensation occurred. Effectively, rate processes usually take considerable time to acclimate when an organism is moved into a colder environment (Bennett 1990). Dickie (1958) stated that the loss of the acclimation to warm temperatures may require up to 3 mo in nature and that sea scallops are extremely slow to recover their normal behavior when exposed to a sudden decrease in temperature.

A marked impact of cold stress was apparent in the recuperation from escape responses. Cold-stressed scallops were less able to recover their total claps and clapping rate than control scallops. Fifteen minutes after exhaustion, scallops transferred to cold water only recuperated 40–50% of their total claps relative to 60–70% for the control scallops. In the Îles-de-la-Madeleine, predators aggregate within three days after seeding of juvenile scallops (Cliche et al. 1994). Since seed scallops are unlikely to have recuperated from cold stress in such a short time, this may place them at a disadvantage when faced with predators that elicit active escape responses.

Temperature is likely to be a major determinant of predation rate since it involves many behaviors (location, attack, capture, and ingestion of prey) that are sensitive to temperature. Up to a certain point, predator activity is known to increase with temperature. For example, predation by starfish on 5–9 mm juvenile scallops shows a Q_{10} of 6.9 (between 4–15 °C) (Barbeau & Scheibling 1994). On the other hand, scallop escape reactions also benefit from higher temperatures (Barbeau & Scheibling 1994). Valve contraction rate increases with temperature (Dadswell & Weihs 1990, Manuel & Dadswell 1991, Olson & Marsh 1993). Sea scallops (5–35 mm in shell height) increase their clapping rate between 9 and 14.5°C (Q_{10} of 1.9) (Manuel & Dadswell 1991). The clapping rates of our 35–45 mm sea scallops gave Q_{10} values of 2.4 (8–18°C) and 1.6 (8–15.5°C) in our first and second experiments, respectively.

The thermal sensitivity of clapping rate may reside in any of its components: latency period, time to peak force and time to relaxation (Olson & Marsh 1993). Thus, a greater clapping rate may come from more rapid adductions, more rapid opening of the

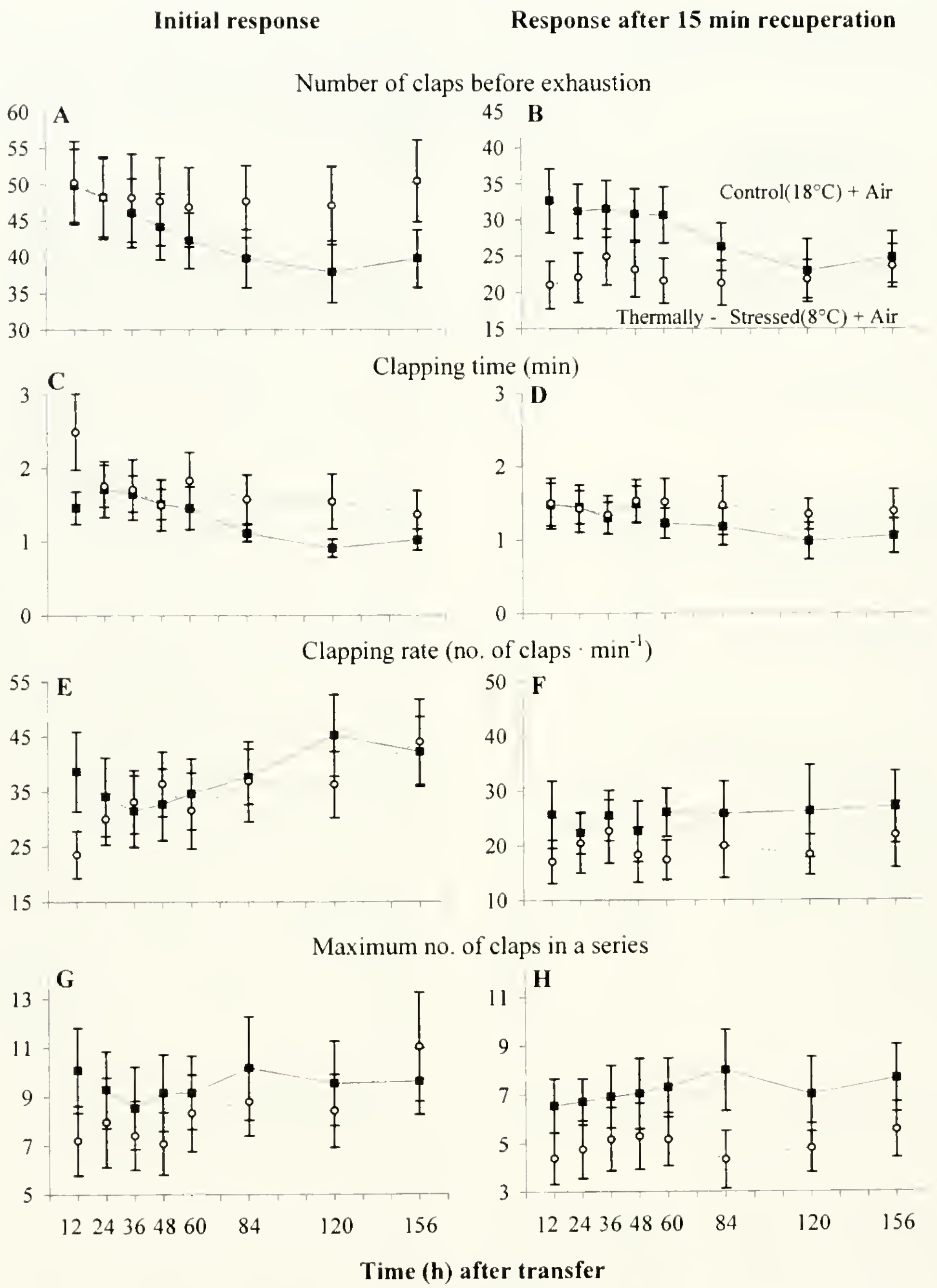

Figure 5. Escape response behaviors of juvenile sea scallops, *Placopecten magellanicus*, after 4 h air exposure and transfer to seawater at 8°C (thermal stress + air) or at 18°C (control + air) measured before and after 15 min of recuperation from exhaustive stimulation. Means are shown for all parameters. Vertical bars indicate 95% confidence limits and *n* = 24 for each treatment.

valves (due to an increase of the hinge elasticity or to greater power in the adduction), or a combination of these factors (reviewed by Manuel & Dadswell 1993). In some interactions between scallops and gastropod or starfish predators, the scallop's response time was greater at a low temperature (Ordzie & Garofalo 1980). Since the liberation of active substances (e.g., saponins (Mackie et al. 1968)) by predators and chemical sensitivity of the scallop's tentacles may decrease with temperature, delayed predator detection may account for this longer latency period. Although the sudden cooling reduced the scallops' escape responses, it did not completely inhibit them. Hence, we did not observe the debility described by Dickie and Medcof (1963). In Dickie's work (1958) with larger *Placopecten magellanicus* (80–100 mm), a drop of 4°C to 7°C was enough to cause virtual immobility for prolonged periods, with scallops keeping their valves almost closed with the tentacles only slightly extended for periods as long as 40 d. The smaller sea scallops we worked with were probably more active. Effectively, swimming is more frequent among younger and medium-sized scallops and rarely seen in >70 mm scallops (Dadswell & Weihs 1990). Further research should assess the resistance of different size classes to fluctuating temperatures (Dickie & Medcof 1963).

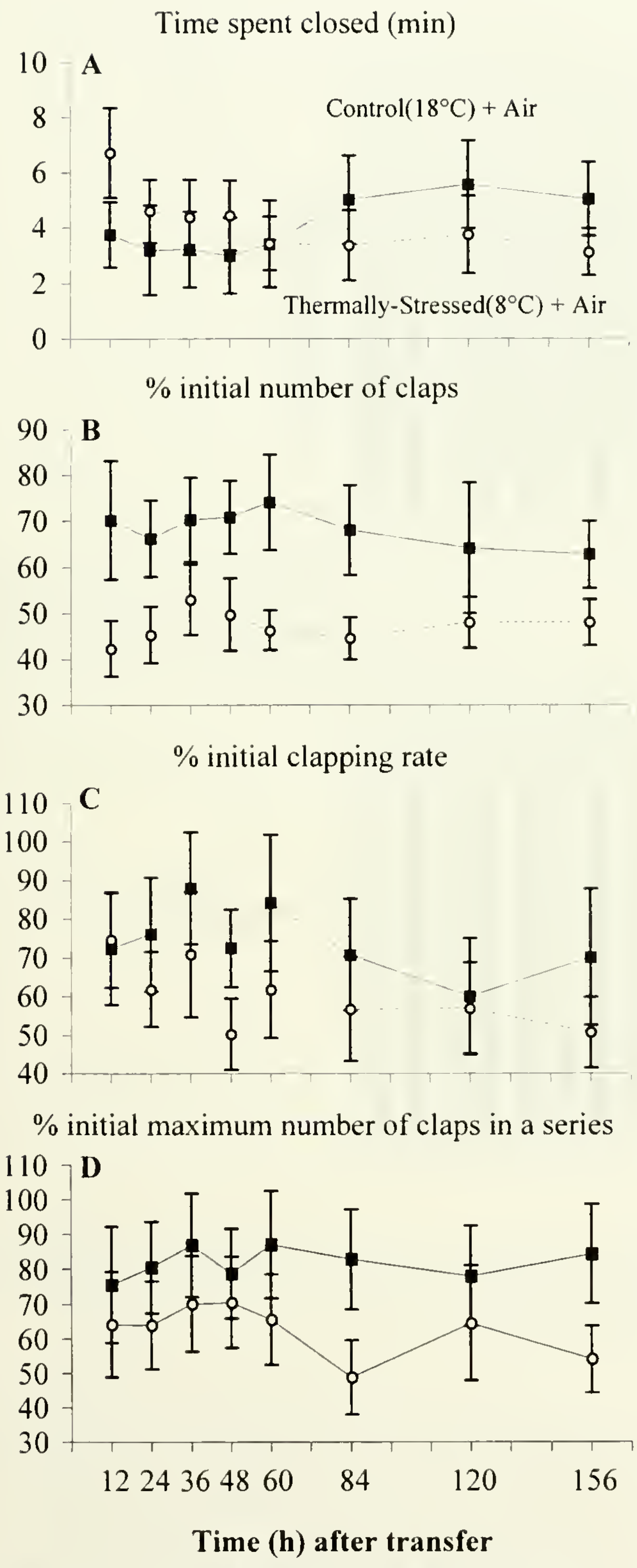

Figure 6. Escape response behaviors of juvenile sea scallops, *Placopecten magellanicus*, after 4 h air exposure and transfer to seawater at 8°C (thermal stress + air) or 18°C (control + air). (A) Time spent closed after initial exhaustive stimulation and (B, C, D) performance after 15 min of recuperation relative to initial response for the main escape behaviors. Means are shown for all parameters. Vertical bars indicate 95% confidence limits and $n = 24$ for each treatment.

Starfish from two thermal regimes were used to provoke escape responses. They were put in tanks at 8°C or 18°C the day before the first escape test. Although we manipulated the predator arms to stimulate the scallops, the amount of chemical cues emitted by cold-stressed and control starfish may have modified escape responses. However, no differences were detected in the response times of scallops at 8°C and 18°C.

In the Cold Shock with Air Exposure Experiment, the differences between control and cold-stressed scallops were smaller than those in the Cold Shock Experiment. The drop of water temperature in the lagoon from 18°C to 15.5°C (September 23–26; see Fig. 2) may have initiated acclimatization to cold water in the scallops remaining in pearl nets. Thus, a transfer to 8°C would have been less of a stress during the second than the first experiment. This suggests that seeding juvenile scallops later in fall when thermal shock is reduced could reduce losses from predation.

The 4 h air exposure seemingly had no effects on the escape parameters of the two groups of control scallops. This is consistent with previous studies of *Pecten maximus* showing no significant differences between 0 and 3–4 h of air emersion on various assessments of scallop's quality (Maguire et al. 1999b, Christophersen 2000, Minchin et al. 2000). Also, our preliminary investigations showed that the righting time of juvenile sea scallops was not significantly prolonged by 4 h emersion (unpublished data). The 4 h emersion period of juvenile scallops during their transfer from the lagoon to open sea seems not to be an issue in terms of vulnerability to predation.

Air exposure for 4 h led the scallops to react more quickly to contact with the starfish. The occurrence of a stress such as air exposure, even if it may not be injurious, may increase the general excitability (Wilkens 1981). Scallops held in laboratory conditions react more rapidly to any perturbation during the first days of their captivity (unpublished data). As contact of a starfish near the region of the dorsal ears often triggers a swimming response (Wilkens 1981), we consistently stimulated in this region, making it unlikely that, the greater occurrence of jumps in cold-stressed scallops was due to differences in our method of stimulation.

Total tissue mass of the control scallops was similar to that of cold-stressed scallops in the air exposure experiment. This variable was not affected by thermal regimen when juvenile sea scallops were subjected to constant (10°C) or fluctuating temperatures (6–15°C, 8 d temperature cycle) during 48 d (Pilditch & Grant 1999). The muscle carbohydrate content of resting scallops from the Cold Shock with Air Exposure Experiment did not differ from that obtained for cultured scallops of the same size, same origin and under similar grow-out conditions (Lafrance et al., submitted). Thus, a 4-h air exposure did not influence muscle carbohydrate levels. The muscle of scallops that performed escape responses contained carbohydrate levels 40% lower than that of resting scallops. Thus, repeated exhausting escapes markedly reduce the scallop's energy reserves in an environment with no food. Extrapolating results from laboratory to field is risky since laboratory conditions can lead to abnormally high levels of stress (Pilditch & Grant 1999). Nonetheless, repeated attacks by predators on the sea bottom, even if not successful, could make scallops more vulnerable to other stresses (sustained food shortage, diseases, etc.). Hence, predator elimination (Ventilla 1982) on seeding grounds should be considered to increase their chance of survival.

The metabolism of *Placopecten magellanicus* is tightly coupled to thermal fluctuations, as shown by Pilditch and Grant (1999) who observed that metabolic rates did not acclimate to thermal oscil-

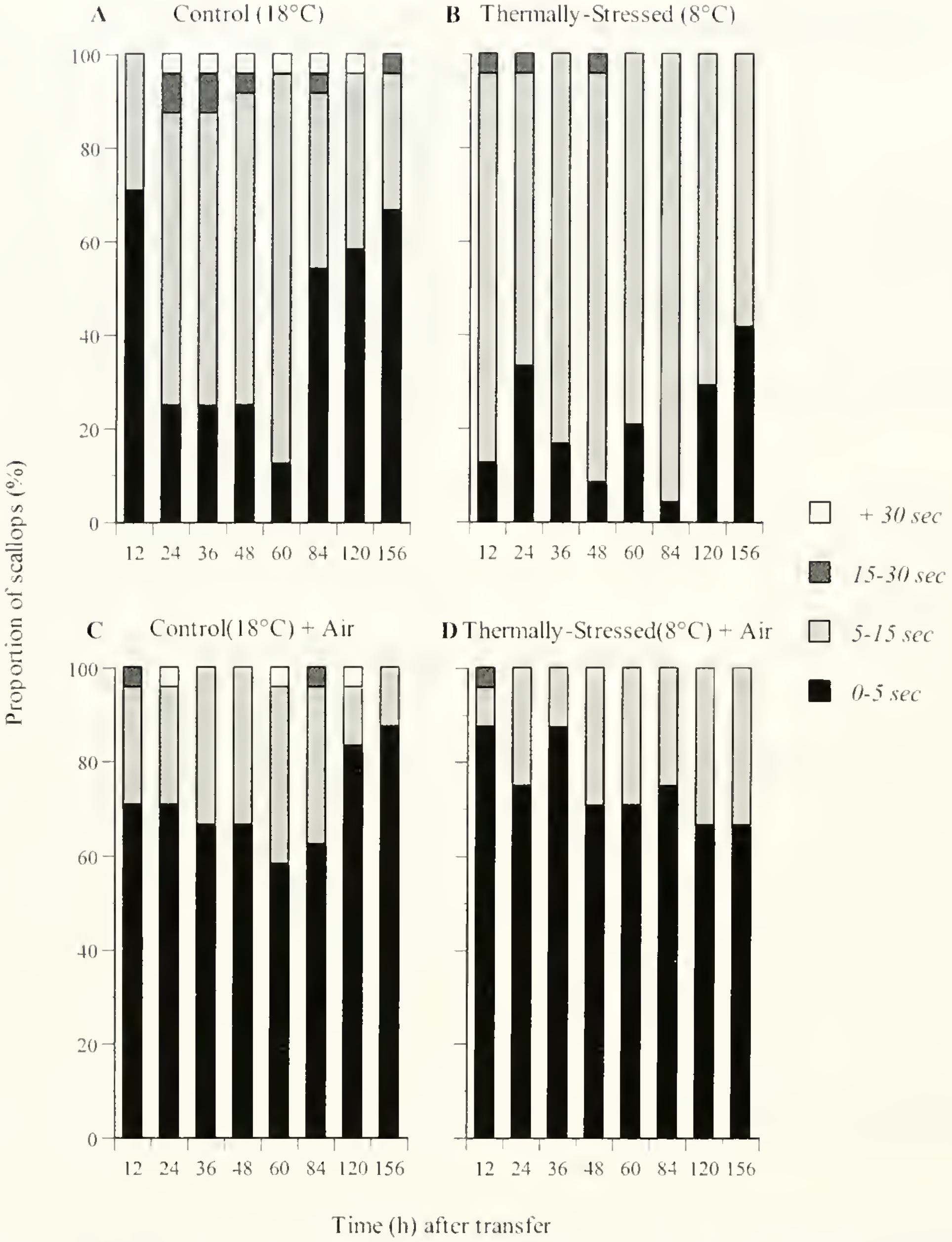

Figure 7. Effect of a thermal stress (transfer from 18°C to 8°C) and a 4 h air exposure on the delay before initiation of escape reaction of juvenile sea scallops stimulated by contact with a starfish. One-hundred percent of scallops corresponds to $n = 24$ for each treatment from (A, B) Cold Shock Experiment and (C,D) Cold Shock with Air Exposure Experiment.

lations between 6°C and 15°C during 6 × 8 d cycles. On the other hand in our study, temperature only decreased at the start of our observations. During long term exposure to low temperatures, scallops are likely to acclimate and at least partially compensate for the decrease in temperature, as shown by the changes in membrane fluidity during thermal acclimation of *P. magellanicus* (Napolitano et al. 1992). In a study of the time course of changes in membrane fluidity during transfer of *P. magellanicus* from 15°C to 5°C, compensation of membrane fluidity through increases in the proportion of polyunsaturated fatty acids required 15 to 21 days (Hall 1999).

This study provides information that should help planning of a crucial step in bottom culture of sea scallops. A 4-h air exposure does not enhance the impact of cold shock on escape responses of *Placopecten magellanicus*. While it has been argued that seeding of juvenile scallops should be performed at cold temperatures to decrease predation (Barbeau & Scheibling 1994), cold temperatures also reduce the scallop's viability in terms of escape behavior. As the scallop's escape capacities require more than 6 days before complete thermal acclimation, seeded juveniles may benefit from operations minimizing thermal shock or from the use of an acclimation period with no predators at intermediate seawater temperatures prior to seeding operations. This need for an acclimation period agrees with the recommendation of Barbeau et al. (1994). However, since crab attacks overwhelm the anti-predator defenses of juvenile scallops at virtually all temperatures (Barbeau &

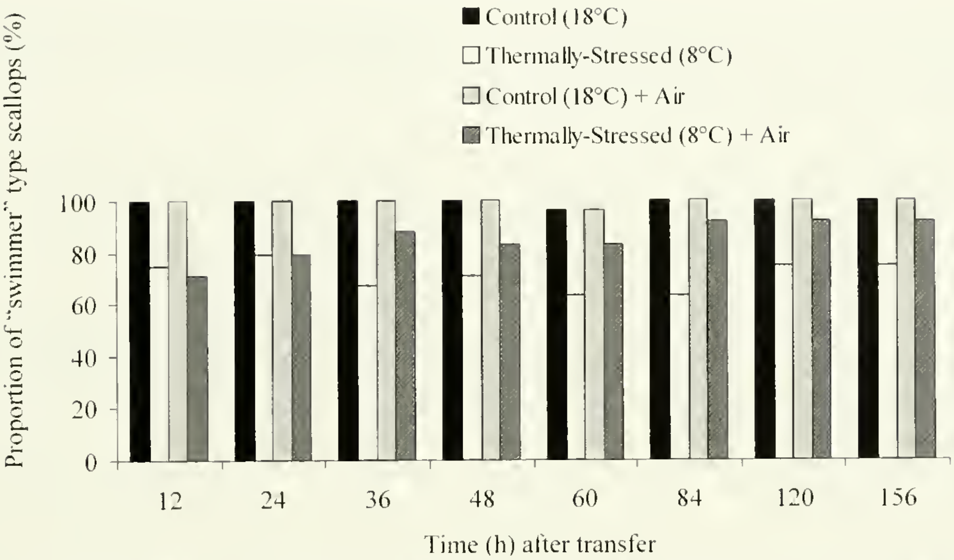

Figure 8. Influence of a thermal stress (transfer from ~18°C to 8°C) and a 4 h air exposure on the proportion (%) of juvenile sea scallops using swimming behavior as a means of escape from a starfish. A "swimmer" was defined as a scallop that made at least 3 claps during most series of claps during the stimulation (evaluated on the response after 15 min recuperation). n = 24 for each treatment from Cold Shock Experiment and Cold Shock with Air Exposure Experiment.

Scheibling 1994, Barbeau et al. 1996), such considerations would not be relevant in an environment dominated by crabs.

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SELECTIVE INGESTION OF PELAGIC VERSUS BENTHIC ALGAE BY THE COCKLE *CERASTODERMA EDULE* (LINNÉ, 1758)

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ABSTRACT The pre-ingestive selection of microphytobenthic algae by the cockle *Cerastoderma edule* was studied in comparison with diets containing the pelagic diatom *Phaeodactylum tricornutum*. Treatments with the different diets covered a range of seston concentrations and organic content similar to field conditions. Rejection rates of *C. edule* exposed to the different treatments were significantly correlated with the concentration of total particulate matter. No significant differences in total rejection rates were found between pelagic and benthic diets. Organic rejection rate was significantly correlated with particulate organic matter of the treatments and no significant differences were found between both diets. Selection efficiency was significantly correlated with particulate organic matter concentration in both diets and no significant differences were found between the diets. Analysis of the pseudofeces composition by flow cytometry from cockles exposed to a mixed diet of microphytobenthic algae and *P. tricornutum*, showed a preferential ingestion of the pelagic diatom. Benthic species, such as small pennates and *Navicula* sp., were preferentially ingested in comparison to larger microphytobenthic species. The largest microphytobenthic species, *Cylindrotheca* sp., was significantly rejected. In general, *C. edule* is an opportunistic filter feeder that takes advantage of both pelagic and benthic algal cells.

KEY WORDS: Benthic diatoms, cockles, flow cytometry, rejection, selection efficiency, *Cerastoderma edule*

INTRODUCTION

Filter feeding bivalves are able to sort particles with their gills and labial palps (Kiørboe & Møhlenberg 1981, Newell & Jordan 1983, Newell et al. 1989, Ward et al. 1993) resulting in preferential ingestion of organic material and rejection of inorganic particles in pseudofeces. This preingestive selection has also been observed within phytoplankton with preferential ingestion of some groups of algal cells, both benthic and pelagic (Shumway et al. 1985, Bougrier et al. 1997, Baker et al. 1998). Preingestive selection of particles may be influenced by their chemical composition or by their size and shape.

Some studies about preingestive selection in bivalves have been carried out by using microscopic techniques to compare water and biodeposits or stomach contents in different species of deposit or filter feeder bivalves (Hummel 1985, Kamermans 1994, Shumway et al. 1987). Flow cytometry offers opportunities for the analysis of algal cells in experiments on the feeding behavior of bivalves (Shumway et al. 1985, Bougrier et al. 1997, Baker et al. 1998). Differential selection of various algal species has been demonstrated, using flow cytometric techniques by Peirson (1983) in the scallop *Argopecten irradians* (Lamarck 1819) and by Shumway et al. (1985) in *Ensis directus* (Conrad 1843), *Ostrea edulis* Linné, 1758, *Placopecten magellanicus* (Gmelin, 1791) and *Arctica islandica* (Linné, 1767). Shumway et al. (1985) showed that the flat oyster *Ostrea edulis* fed with a mixture of three algae of similar size (*Phaeodactylum tricornutum, Prorocentrum minimum,* and *Chroomonas salina*) preferentially ingested the dinoflagellate *Prorocentrum minimum*. In contrast, Cucci et al. (1985) have shown that individuals of *Mytilus edulis* Linné, 1758 fed with a mixed diet of the diatom *Phaeodactylum tricornutum*, the dinoflagellate *Prorocentrum*, and the cryptomonad flagellate *Chroomonas salina*, showed no differential ingestion of cells from suspension. Bougrier et al. (1997) found differences in the preingestive selection of different algal cells (mainly pelagic) by *Mytilus edulis* and *Crassostrea virginica* (Gmelin, 1791) and related to differences in the shape of the algal cells. Recently Loret et al. (2000) have shown preferential ingestion of nanoflagellates and cryptophytes by the bivalve *Pinctada margaritifera* (Linné, 1758), by applying *in situ* HPLC techniques.

There is some controversy about the feeding behavior of bivalves, especially *C. edule*, exposed to benthic algae compared with pelagic algae, or the importance of microphytobenthos as a food source. Kamermans (1994) found microphytobenthic algae in the stomachs of the filter feeding bivalves *Mya arenaria* (Linné, 1758), *C. edule* and *M. edulis*, although the percentage of their abundance compared with pelagic algae, was higher in the deposit feeders *Scrobicularia plana* (Da Costa, 1778) and *Macoma balthica* (Linné, 1758). Recently two studies have been done measuring ratios of C and N isotopes in different primary producers and benthic invertebrates (Kang et al. 1999, Riera et al. 1999). These studies concluded that *C. edule* may prefer to ingest microphytobenthos and phytoplankton compared with detritus from fragments of benthic macro-algae and seagrasses or detritus from other origins. However, Herman et al. (2000), using also stable isotopes ratios, suggested that suspension feeders, such as *C. edule* and *M. arenaria*, depend almost exclusively on pelagic rather than microphytobenthic algae.

The aim of this study is to quantify the pre-ingestive selection of microphytobenthic algae in comparison with a pelagic species by the cockle *Cerastoderma edule*. A mixture of benthic and pelagic micro-algae occurs in the tidal flat environment of this filter feeder bivalve. The response of cockles exposed to the different diets was studied separately and also when both diets were mixed in the water column. In the latter case analysis were done by flow cytometry to analyze differential algal cell acceptance or rejection.

MATERIALS AND METHODS

Animals

In total 187 cockles (*Cerastoderma edule*) were used in the different experiments. Cockles had a shell length of 29.78 mm ±

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1.27 (mean ± standard error) in experiments with microphytobenthos and 29.75 mm ± 0.46 in experiments with *Phaeodactylum tricornutum*); ash free dry weight was 255.75 mg ± 30.15 in experiments with microphytobenthos and 259.87 mg ± 20.55 in experiments with *P. tricornutum*. No significant differences were found in shell length or ash free dry weight of the animals used in both experimental treatments. All individuals were collected from an intertidal mudflat situated in the Oosterschelde estuary (SW Netherlands). Experiments were performed in spring 1997 and summer 1999, in a field laboratory at Jacobahaven (Oosterschelde estuary—SW Netherlands).

Shell length, dry tissue weight (DW) and ash-free dry tissue weight for each animal were determined. After measuring the shell length, animals were immersed in boiling water until the shell was opened. Soft tissues were excised, dried at 70°C for minimum 48 h in a drying oven and weighed (= dry weight). Ash-free dry weight is determined as the weight loss after ignition in a furnace at 520°C for 3 h.

Diets and Treatments

Two different diets were used in the different experiments: microphytobenthic algae (Benthic) and cells of *Phaeodactylum tricornutum* (Pelagic). Microphytobenthic algae were collected in the Oosterschelde estuary. The top layer of sediment (2–3 mm) was sampled and after sampling, coarse sediment and microphytobenthos were separated by mixing them in filtered seawater. After sedimentation of the large particles the water containing mainly benthic algae (based on observations with microscopy) was used for the experimental treatment. The microphytobenthic species were benthic diatoms, including species from the genus *Nitzschia* (length 61 ± 11 μm; width 7 ± 1 μm), *Navicula* (length 46 ± 11 μm; width 22 ± 3 μm), *Cylindrotheca* (length 81 ± 5 μm; width 23 ± 2 μm), and small pennate cells (length 30 ± 2 μm; width 6 ± 1 μm). The pelagic diatom *P. tricornutum* was cultured outdoors in 100-l reservoir tanks.

Pelagic and Benthic algae were added separately to filtered seawater in different concentrations (treatments) for both diets (Table 1). Each treatment was completed by adding silt particles collected in the same location as cockles and microphytobenthos. This silt was dried and burned at 520°C during 4 h for removing the organic content. A coulter counter was used to determine particle concentration, which was monitored during the experiment for differences of the particle concentration from the experimental treatments. Total particulate matter in the different experiments ranged from 40.2 mg.l^{-1} to 161.2 mg.l^{-1} and the organic content ranged from 9.08%–58.91% (see Table 1).

Analysis of Particulate Matter

Water samples of the different treatments were collected from the control chamber (without animal) at the start of the experiment and before collection of the biodeposits. Total particulate matter (TPM: mg.l^{-1}) was determined by filtering one liter sample onto pre-weighed and pre-ashed Whatman GF/C filters, rinsing with ammonium formate and drying at 70°C (minimum 24 h). Particulate inorganic matter (PIM: mg.l^{-1}) was estimated from the same filters as the weight after ignition during 3 h at 450°C. Particulate organic matter (POM) resulted from the difference of TPM and PIM. In all weight measurements an electronic balance was used which determined the nearest 0.01 mg. Dietary composition was characterized in terms of organic content by weight (%OM = POM × 100/TPM), POM and TPM.

TABLE 1.

Characteristics of the different experiments for each diet and treatment. Concentrations of total particulate matter (TPM: mg · l^{-1}), particulate organic matter (POM: mg · l^{-1}), particulate inorganic matter (PIM: mg · l^{-1}) and percentage of organic matter from the seston (% OM).

Treatment code	TPM (mg · l^{-1})	POM (mg · l^{-1})	PIM (mg · l^{-1})	OM (%)
Diet with *Phaeodactylum tricornutum*				
1p	48.90	4.44	44.46	9.08
2p	55.32	29.56	25.76	53.43
3p	58.82	8.18	50.64	13.91
4p	83.64	24.88	58.76	29.75
5p	92.40	17.00	75.40	18.40
6p	95.28	32.32	62.96	33.92
7p	125.84	15.04	110.80	11.95
8p	152.34	17.66	134.68	11.59
Diet with Microphytobenthos				
1m	48.68	28.68	20.00	58.92
2m	53.92	10.10	43.82	18.73
3m	72.52	13.76	58.76	18.97
4m	76.44	30.72	45.72	40.19
5m	80.96	28.04	52.92	34.63
6m	87.48	34.24	53.24	39.14
7m	98.40	20.98	77.42	21.32
8m	161.20	37.92	123.28	23.52
Mixed diet				
1mx	51.56	20.80	30.76	40.34
2mx	59.52	17.42	42.10	29.27
3mx	28.05	5.87	22.18	20.92

Experiments with Separate Diets

A flow-through system was used in all experiments. The water containing the different treatments was pumped to the experimental chambers from a diet-reservoir (300 L) in which a mixer and an air pump promoted the resuspension of particles. Peristaltic pumps (Watson Marlow) were used for pumping the water to the individual experimental chambers. The animals were placed individually in chambers of 300-ml volume and one chamber was used as a control for sedimentation of particles. The flow in all chambers during the experiments was between 3 and 4 l/h, and the concentration of particles in the outflow of the chambers containing animals was always more than 70% compared with the control chamber (without animals).

Collection of pseudofeces started after an acclimation period of 3 h to each treatment. Pseudofeces rejected during 3-h periods were collected 2 to 3 times separately for each individual. Pseudofeces samples were filtered on preweighed Whatman GF/C filters and measured according to the same procedure as described for samples of the diets. Rejection rates (mg.h^{-1}) of total (RR), organic (ORR) and inorganic (IRR) particulate matter were calculated. Rates were standardized to an equivalent 500 mg ash free dry tissue cockle by calculating the expression $Ys = Ye\ (0.5/We)^b$, where Ys: rate of standard-sized cockle; Ye: uncorrected physiologic rate; We: measured ash free dry weight of experimental animal; b: allometric coefficient for clearance rate of cockles (b = 0.57; Urrutia 1997, Smaal et al. 1997).

Selection efficiency (SE) was calculated using the values for organic fraction of seston (f = POM/TPM) and pseudofeces (p = ORR/RR) from the different experiments. Values were calculated as: SE = $(1-(p/f)) \times 100$.

Experiments with a Mixed Diet

In these series of experiments, cockles were exposed to a mixed diet and the same flow through system described in the previous paragraph was used. The diet contained a mixture of microphytobenthic algae and the diatom *P. tricornutum* (size 17 ± 2 μm). The different size of this pelagic species, and its fluorescence characteristics make it clearly distinguishable from the microphytobenthic species used (sizes between 30 ± 2 μm and 81 ± 11 μm). Individuals were acclimated 3 h to the mixed diet and pseudofeces produced by each animal were collected during periods of 1 h. Sample analysis by flow cytometry was done directly after the experiment. Another series of samples of pseudofeces was collected in the experiment for selection efficiency measurements.

Algal composition of diet and pseudofeces was analyzed using a flow cytometer (EurOPA: European Optical Plankton Analyser). Standard beads (1.07 μm, Duke Scientific, USA) were used for calibration and optical adjustments of the EurOPA instrument. A 529 nm and 633 nm laser were used for excitation. Laser light is scattered when a particle traverses the laser beam and is measured in forward and perpendicular (PLS) direction. Laser light irradiating autotrophic phytoplankton is partly emitted as fluorescence. Fluorescence emission excited by the green laser is measured in the red (FGR) and orange (FGO) bandwidth. The number of particles processed in the flow cytometric analysis was 5,000 and 20,000. Only data derived by FGR-triggering (i.e., fluorescent particles) were used to distinguish between groups of particles with different optical characteristics. The grouping or clustering of data was calculated by using the software program Matlab version 1.0.

The algal species studied are easy to distinguish in the scatter plots of the graph representing FGR and PLS data. The star-shaped form of *Phaeodactylum tricornutum* occupied a large area with values of PLS, between 1,500 and 2,200, and values of fluorescence FGR in general below 2,200 and above 1,900. Microphytobenthos were composed with mainly four subgroups (related to the most abundant species). The benthic diatom species with lower FGR and lower PLS was *Navicula* sp., together with a group of small pennate cells, which could not be identified with values of PLS (1,800–2,300) and FGR (1,900–2,200). *Nitzchia* species had a higher PLS value (2,400–2,500) and higher FGR (2,500–2,700). *Cylindrotheca* species displayed the highest PLS (almost 2,500) and the highest FGR (almost 3,000).

To examine the degree of acceptance or rejection of particle types (pelagic or benthic algae), we calculated an electivity index (EI) (Jacobs et al. 1974), modified by Baker et al. (1998), as follows:

$$EI = -[(P - S) / ((P + S) - (2 * P * S))]$$

where P is the particle ratio in the pseudofeces and S is the particle ratio in the suspension. Electivity index can range from −1.0 to 1.0. A positive EI for a given particle type indicates preferential ingestion (depletion of the particle type in the pseudofeces compared with the suspension), and a negative EI indicates rejection (enrichment of a particle type in the pseudofeces compared with the suspension).

Subsamples of the diet and pseudofeces were also fixed in Lugol's and the algal composition was determined using microscopic techniques for determination in addition to the flow cytometer results.

Statistics

All statistical tests were performed using the program SYSTAT for PC version 9.0. Multiple stepwise regression analyses were used to test for significant relationships between physiologic rates and parameters of the treatments. Analysis of covariance was used for comparing the rejection rates and selection efficiencies of cockles with the different diets. Electivity indices were compared with zero using a one-sample, two-tailed, nonparametric Wilcoxon signed-rank test. These analyses test the null hypothesis that selectivity of a particular type is equal to zero (no sorting). A t-student test was used to compare concentrations of the different algal species in samples of the diet and pseudofeces.

RESULTS

Response to Diets

Data about quality and quantity of the experimental treatments are shown in Table 1. All treatments were above pseudofeces threshold and the obtained physiologic values with the different diets and treatments have been listed in Table 2. Multiple stepwise regression analyses indicated a significant positive relationship between rejection rate (RR) and total particulate matter (TPM) from the treatments of both diets (Fig. 1; Table 3). The model rejected parameters POM and %OM. Analysis of covariance in-

TABLE 2.

Physiological response of cockles with the different diets and treatments: Selection efficiency (SE: %), rejection rate of pseudofeces (RR: $mg \cdot h^{-1}$), organic rejection rate (ORR: $mg \cdot h^{-1}$) and inorganic rejection rate (IRR: $mg \cdot h^{-1}$). Mean value ± standard error. Number of measurements (n).

Treatment code	SE (%)	RR ($mg \cdot h^{-1}$)	ORR ($mg \cdot h^{-1}$)	IRR ($mg \cdot h^{-1}$)	n
Diet with *Phaeodactylum tricornutum*					
1p	4.9 ± 6.5	29.3 ± 1.7	2.6 ± 0.3	26.8 ± 1.4	6
2p	46.1 ± 4.3	38.3 ± 7.2	10.8 ± 2.0	27.5 ± 5.5	10
3p	13.9 ± 9.4	22.3 ± 6.2	2.4 ± 0.7	19.9 ± 5.5	6
4p	36.8 ± 9.3	24.3 ± 5.0	5.9 ± 1.9	18.4 ± 3.1	12
5p	9.4 ± 2.5	54.3 ± 5.5	8.9 ± 0.7	45.3 ± 4.9	16
6p	41.9 ± 2.2	45.8 ± 2.7	9.1 ± 0.6	36.7 ± 2.3	12
7p	26.1 ± 3.4	96.4 ± 8.7	8.4 ± 0.6	88.0 ± 8.1	6
8p	14.5 ± 2.6	80.3 ± 5.5	8.0 ± 0.7	72.3 ± 4.9	10
Diet with Microphytobenthos					
1m	31.9 ± 4.1	34.7 ± 2.9	13.8 ± 1.1	20.9 ± 2.2	12
2m	18.7 ± 9.4	17.2 ± 3.1	2.6 ± 0.3	14.6 ± 2.9	6
3m	13.1 ± 9.6	27.4 ± 4.9	4.3 ± 0.6	23.2 ± 4.4	6
4m	34.9 ± 2.4	42.7 ± 1.6	11.1 ± 0.4	31.6 ± 1.5	15
5m	34.3 ± 2.5	70.9 ± 4.5	16.2 ± 1.1	54.7 ± 3.7	12
6m	43.1 ± 3.5	47.9 ± 6.4	10.1 ± 0.9	37.7 ± 5.7	10
7m	22.5 ± 1.9	50.8 ± 6.8	8.2 ± 1.0	42.7 ± 5.8	16
8m	37.5 ± 7.7	91.0 ± 13.2	13.6 ± 0.2	77.4 ± 13.0	6
Mixed diet					
1mx	25.1 ± 4.4	–	–	–	10
2mx	15.6 ± 3.5	–	–	–	6
3mx	6.6 ± 1.6	–	–	–	8

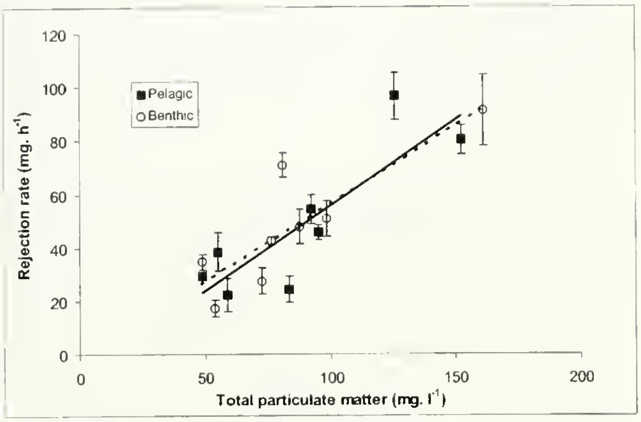

Figure 1. Rejection rate ($mg \cdot h^{-1}$) of pseudofeces as a function of the seston concentration in both diets. Pelagic: Results for treatments with *Phaeodactylum tricornutum* (line). Benthic: Results for treatments with microphytobenthos (dotted line). Vertical bars indicate standard error. See Table 2 for number of measurements.

dicated that there were no significant differences between the diets ($/t/ = 0.256$; $P > 0.05$).

Organic rejection rates (ORR) were only significantly correlated to the particulate organic matter (POM) (Fig. 2, see Table 3), with TPM and %OM as nonsignificant parameters related to ORR. According to the regressions obtained in each case no significant differences were found between both diets ($/t/ = 1.136$; $P > 0.05$).

Multiple stepwise regression analysis indicated a significant positive relationship between selection efficiency (SE) and particulate organic matter (POM) (after log transformation) in both diets (Fig. 3 see Table 3). Although lower values of SE were obtained with the benthic diet, analysis of covariance indicated that there were no significant differences between diets ($/t/ = 0.791$; $P > 0.05$).

Response to a Mixed Diet

Selection efficiency values as a function of POM concentration (see Fig. 3) were generally lower for the mixed diet of pelagic and benthic cells. Higher values of SE were registered at higher values of particulate organic matter.

Flow cytometer results showed significantly lower cell concentrations (expressed as %-age) in pseudofeces in comparison to the diet composition (Fig. 4), and therefore significant positive electivity indices (EI) (acceptance). For both diets, differences of algal concentration in pseudofeces and diet were significant using a t-student test (Pelagic: DF = 12, t = 14.09, $P < 0.001$; Benthic: DF = 12, t = 4.86, $P < 0.01$). However, the EI of cockles was significantly higher for pelagic ($EI_{Pelagic} = 0.31 \pm 0.01$, mean ± standard error) than for benthic cells ($EI_{Benthic} = 0.20 \pm 0.03$, mean ± standard error), indicating a preferential acceptance of the Pelagic rather than the Benthic component of the mixed diet. A lower EI for the benthic species was due to differences in the acceptance or rejection of the different benthic species within the Benthic group (Fig. 5). The smallest benthic species, such as *Navicula* sp. and the group of small pennates, were significantly ingested, displaying higher EI values than larger species such as *Nitzchia* sp. However, *Cylindrotheca* sp., the largest species, was significantly rejected, resulting in a negative EI value.

DISCUSSION

Production of pseudofeces of cockles was significantly related to the seston concentration in both diets. This relationship between

TABLE 3.

Stepwise multiple regression analyses of physiological parameters of *Cerastoderma edule* with total particulate matter (TPM: $mg \cdot l^{-1}$), particulate organic matter (POM: $mg \cdot l^{-1}$), particulate inorganic matter (PIM: $mg \cdot l^{-1}$) and percentage of organic matter from the different diets (% OM). se: standard error.

	Pelagic diet				Benthic diet			
	Coefficient	se	r^2	*P*	Coefficient	se	r^2	*P*
Rejection rate (RR)								
Terms retained			0.73	<0.01			0.72	<0.01
Constant	7.97			<0.01	−1.67			<0.01
TPM	0.64	0.16		<0.01	0.58	0.14		<0.01
Terms rejected								
POM								
% OM								
TPM × POM								
Organic rejection rate (ORR)								
Terms retained			0.63	<0.05			0.68	<0.01
Constant	2.45			<0.01	−0.27			<0.01
POM	0.24	0.08		<0.05	0.41	0.11		<0.01
Terms rejected								
TPM								
% OM								
TPM × POM								
Selection efficiency (SE)								
Terms retained			0.67	<0.01			0.82	<0.005
Constant	−28.85			<0.01	−33.49			<0.005
Log POM	19.92	5.46		<0.01	19.94	3.85		<0.005
Terms rejected								
Log TPM								
Log % OM								

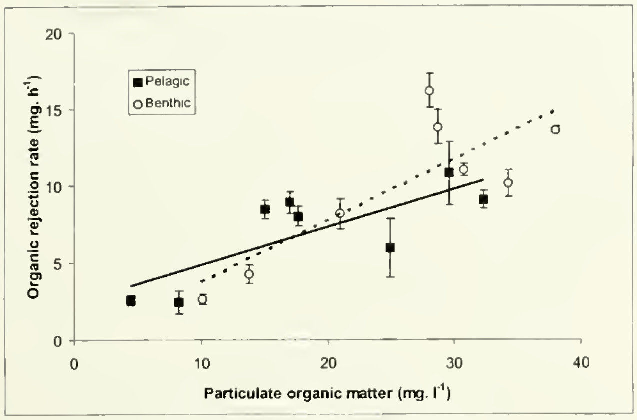

Figure 2. Organic rejection rate of pseudofeces (mg · h^{-1}) as a function of the organic seston concentration in both diets. Pelagic: Results for treatments with *Phaeodactylum tricornutum* (line). Benthic: Results for treatments with microphytobenthos (dotted line). Vertical bars indicate standard error. See Table 2 for number of measurements.

RR and the seston concentration has been observed in several studies and rates were comparable with our outcomes (Iglesias et al. 1992, Navarro & Widdows 1997, Urrutia 1997). Similarly, the rejection rate of organic matter increased with organic content of seston. At a constant clearance rate, filtration rate increases at increasing seston concentrations and the digestive system reaches maximum capacity, hence pseudofeces production increases.

Together with the production of pseudofeces, selection of organic material occurs prior to ingestion. Selection efficiency (SE) values found in our experiments were very similar to values documented for *C. edule* under comparable conditions of food quality (Iglesias et al. 1992, Urrutia 1997). The response by the cockle to the pelagic diet did not differ significantly from the benthic diet. The SE maximizes in *C. edule* as a response of an increment in the organic content in the diet (Iglesias et al. 1992, Urrutia 1997) reaching a maximum value in our study of 40%. This maximum value of gross SE (measurements based on the organic content of pseudofeces) for the cockle is similar to those obtained in previous studies using pelagic algae as a food source (Iglesias et al. 1992, Urrutia 1997). The percentage of organic matter in the different treatments covered a range between 10%–60%. Several authors (Iglesias et al. 1992, Urrutia 1997) have described a decrease of the SE in *C. edule* exposed to treatments above 50% organic content. The decrease of SE values obtained in treatments containing Benthic algae could be related to a lower acceptance or the preferential rejection of some benthic species as it has been found with *Nitzchia* sp. and *Cylindrotheca* sp.

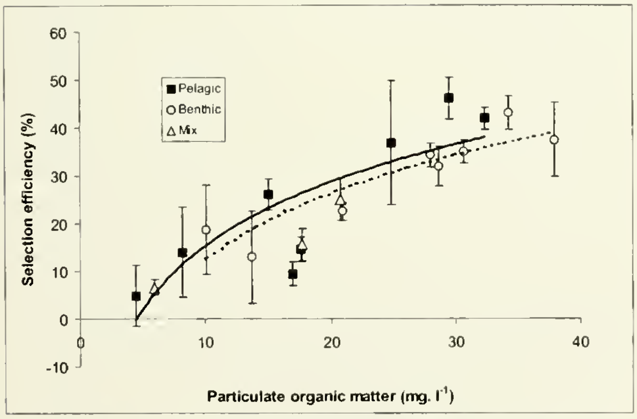

Figure 3. Mean values of selection efficiency (%) as a function of the organic seston concentration in each dietary condition. Pelagic: Results for treatments with *Phaeodactylum tricornutum* (line). Benthic: Results for treatments with microphytobenthos (dotted line). Mix: Results for treatments with a mixed diet. Vertical bars indicate standard error. See Table 2 for number of measurements.

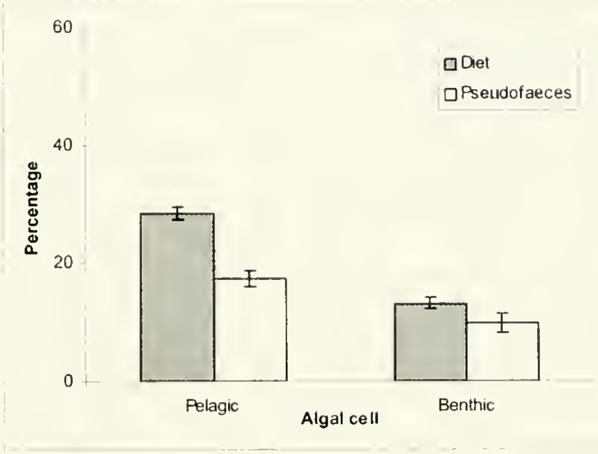

Figure 4. Percentages of the different algal cells, from the total number of particles, both in the diet and the pseudofeces. Pelagic: *Phaeodactylum tricornutum*. Benthic: Microphytobenthic algae Vertical bars indicate standard error. In all measurements $n = 14$.

In this study, we have documented the selection and acceptance of microphytobenthos as a benthic food source by the cockle. However, pelagic algae were accepted preferentially when mixed with microphytobenthic species. Moreover, only some microphytobenthic species were preferentially accepted by *C. edule*. Micro-

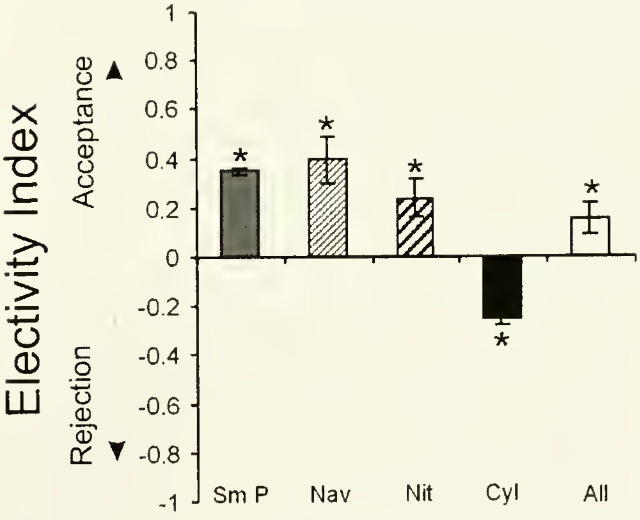

Figure 5. Electivity indices (EI) for the different microphytobenthic species, as separate species or as a group, relative to the total concentration of particles. The studied species are small pennate cells (SmP), *Navicula* sp. (Nav), *Nitzchia* sp. (Nit), *Cylindrotheca* sp. (Cyl) and the benthic group (All), which includes all microphytobenthic species. Vertical bars indicate standard error. * Indices significantly different than zero ($P < 0.05$). In all measurements $n = 14$.

phytobenthos seems to represent a food source in several species of bivalves and its importance for filter feeding bivalves has been addressed previously. Kamermans (1994) found relations between algal composition of the water column (30% were resuspended benthic diatoms) and the stomach contents of the bivalves *C. edule*, *Mya arenaria* and *Mytilus edulis* in the western Wadden Sea. She concluded that selection of algae by the bivalves was absent based on comparison of water and stomach samples. Recently two studies measured ratios of C and N isotopes in different primary producers and benthic invertebrates (Kang et al. 1999, Riera et al. 1999). These studies concluded that *C. edule* preferentially ingested microphytobenthos and phytoplankton compared with detritus from benthic macro-algae or sea grasses fragments. However, Herman et al. (2000) indicated that *C. edule* depends basically on pelagic algae as a food source and benthic algae hardly contribute to the metabolism. Our experiment showed a differential pre-ingestive selection by *C. edule* of the different benthic species. This may explain the actual controversy about microphytobenthos as a food source for suspension feeders. More detailed information about acceptance and/or rejection of single microphytobenthic algal species is needed to clarify the actual controversy.

Results obtained in this study, using flow cytometry techniques, showed a preferential ingestion of Pelagic over Benthic species and a differential selection of the benthic species. Within the Benthic group, some species such as *Cylindrotheca* sp. was rejected in significantly higher concentrations than small pennates or *Navicula* sp. The mixed composition of the benthic diet is a feature that resembles the natural conditions where *C. edule* lives and a preferential selection of some species may occur also in the field. Studies on sorting of food in bivalves using flow cytometry to determine the preferences within mixed samples are scarce (Cucci et al. 1985, Shumway et al. 1985, Chrétiennot–Dinet et al. 1991, Bougrier et al. 1997, Baker et al. 1998). Cucci et al. (1985) did not observe preingestive selection in mussels fed with a diet of a diatom, a dinoflagellate and a cryptomonad. However, Bougrier et al. (1997) observed in the oyster *Crassostrea gigas* (Thunberg, 1793) that a preferential ingestion of flagellates species occurred compared with diatom species. Moreover, Baker et al. (1998) examined the ability of zebra mussels *Dreissena polymorpha* (Pallas, 1771) to preferentially ingest or reject various phytoplankton species in the Hudson River (New York). In their study, zebra mussels selected more efficiently small algal cells, such as cyanobacteria, explaining the changes in the Hudson River phytoplankton community in the last decade.

Accepted benthic species by *C. edule* had smaller and similar sizes (*Navicula* sp.: measured size 46 ± 11 μm; small pennates: measured size 30 ± 2 μm) than the significantly rejected species such as *Cylindrotheca* sp. (measured size 81 ± 5 μm). Therefore, a possible relation may be found with a preferential ingestion or rejection of those algae and their size. Nevertheless, preingestive selection of algae in bivalves could also be related to chemical characteristics (e.g., taste of the particles) or to morphologic characteristics (e.g., shape of the particles). In this context, Shumway et al. (1990) discussed that not only size is an important factor in the preingestive selection of particles but also mucoid trapping and chemoreceptors. Chrétiennot–Dinet et al. (1991) reported that the relative ingestion or rejection was not dependent on the size of the algae in *C. gigas* and *M. edulis*. Bougrier et al. (1997) reported similar results on those bivalves and no relation was found between preingestive selections of algal species with their size. MacDonald and Ward (1994) showed that rejection is not dependent on particle size in the scallop *Placopecten magellanicus*.

The cockle, *C. edule*, preferentially accepted small benthic diatoms such as small pennates and *Navicula* sp., both also with an elliptical smooth shape, and rejected others such as *Cylindrotheca* sp., which has a larger size and large lateral spicules. The actual mechanisms whereby particles are selected on the gills and the labial palps have been studied using endoscopy (Ward et al. 1991, Ward et al. 1993) but not much information is known about their relation with morphologic characteristics of the particles. Bougrier et al. (1997) indicated that a differential rejection or ingestion of diatom and flagellate species might depend on their shape and flexibility, with a preferential rejection of species with inflexible spicules or the presence of a non-flexible membrane (i.e., silicate frustule), which may hamper the pass through the gill filter. Some authors (Shumway et al. 1985, Bougrier et al. 1997) have also detected a preferential ingestion of flagellates in comparison to diatoms and have speculated that the presence of the silicate frustules from the diatoms may appear as mineral particles to sensory receptors of the labial palps. According to this hypothesis the preferential selection of the pelagic diatom *P. tricornutum* could be influenced because benthic species are, generally, attached to silt particles and it may be possible that some species will have more chance to be rejected as inorganic particles by *C. edule*.

In this study, similar values of selection efficiency were registered when *C. edule* was exposed to Pelagic and Benthic treatments, and lower values obtained in mixed treatments. Riera and Richard (1995) have reported in *C. gigas* a preferential ingestion of benthic diatoms and detritus compared with phytoplankton in shallow estuarine waters. But they also reported that *C. gigas* had a preferential ingestion of phytoplankton, when compared with benthic diatoms and detritus, in deeper waters from the same bay. Shumway et al. (1987) found a preferential ingestion of benthic algae by *P. magellanicus* collected from deep-water populations, however individuals from shallow water populations seemed to ingest more pelagic than benthic algae. Gut contents of *P. magellanicus* generally reflected available organisms in the immediate habitat. The preingestive selection of algae in some filter feeding bivalves, such as *C. edule*, could be adapted to the composition and the percentages of the different species available in the water column. The preferential ingestion of benthic species in comparison with pelagic species has been observed in the mussel *M. edulis* in conditions with low concentrations of pelagic algae in the water column (Prou et al. 1994). In our experiments, microphytobenthos is selected at the same efficiency as *P. tricornutum* in nonmixed treatments. In a mixture of pelagic and benthic species, the rejection of microphytobenthos is higher and this could be related to the dominance of the pelagic algae in the diet. Preingestive selection of benthic compared with pelagic species has been studied previously in the oyster *C. gigas* by using the flow cytometry technique (Bougrier et al. 1997). In those experiments oysters preferentially ingested pelagic species than benthic species, in a dominant pelagic species treatment. Baker et al. (1998) observed that the selectivity of different phytoplankton species by the zebra mussel *Dreissena polymorpha* differed depending on the complexity of the offered suspension.

Summarizing, the pre-ingestive selection of benthic algae by *C. edule* is similar and nonsignificantly lower than the pre-ingestive selection of pelagic species, when benthic and pelagic species are not mixed. This situation of maximum presence of benthic diatoms in the seston could be similar to environmental characteristics of tidal flat areas with high resuspension rates of benthic diatoms, or seasons with low pelagic productivity (e.g., autumn and winter). However, acceptance of benthic algae is lower, compared with pelagic, and not equal for the different components of the benthic group when mixed in a pelagic dominant treatment. These conditions may be present during warm periods of the year (e.g., spring and summer) when phytoplankton blooms occur in their natural environment. *C. edule* may be considered as an opportunistic filter feeder that may take advantage of certain algal species, both pelagic and benthic, in relation to their availability in the field.

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REPRODUCTIVE CYCLE OF THE BIVALVE CLAMS *SEMELE SOLIDA* (GRAY, 1828) (SEMELIDAE) AND *GARI SOLIDA* (GRAY, 1828) (PSAMMOBIIDAE) FROM CHILE

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ABSTRACT Commercial clam landings reached an average of almost 91,000 tons annually in Chile over the last decade. In spite of the high value of this resource, few efforts have been made to understand the basic biology of the exploited species, data that might in the future be needed to aid in their protection or even artificial culture. This study is a contribution to the knowledge of the reproductive cycles of two valuable species, *Semele solida* (Gray) and *Gari solida* (Gray). Representative samples of these species were collected at two widely separated localities in Chile and examined histologically to determine their seasonal reproductive cycles. It was found that the species were of separate sexes, and had annual gonadal cycles. In *S. solida* from northern Chile, the reproductive period extended from June 1991 to February 1992. In *G. solida* from southern-central Chile, the reproductive period was relatively short, from October 1991 to February 1992. In both species, most specimens have empty gonads by March. The data obtained are useful in developing management plans related to their reproductive periods. Relevant to culture strategies, *S. solida* has the comparative advantage of a lengthy reproductive period, wherein mature individuals may be frequently encountered in nature for spawning inductions. *G. solida*, with its shorter annual reproductive cycle may have the advantage of being induced to mature in artificial conditioning systems over relatively short periods of time.

KEY WORDS: clam reproduction, reproductive cycle, bivalves, *Semele solida*, *Gari solida*, Chile

INTRODUCTION

Chilean coastal waters host very productive and diverse clam fisheries due to the rich coastal upwelling and favorable water temperatures. The largest clam populations occur in the protected bays and fjords of southern Chile. Over a number of years official fisheries records in Chile (SERNAPESCA 1990–1999) considered all clam species as one generic group ("clams") among which were included the venerids *Protothaca thaca* (Molina), *Venus antiqua* (King and Broderip), *Eurhomalea exalbida* (Chemnitz), *E. lenticularis* (Sowerby), *E. rufa* (Lamarck), and the mactrid *Mulinia edulis*. *Semele solida* (Gray) and *Gari solida* (Gray) belonging to the Semelidae and the Psammobiidae respectively, and objects of this study are also included in this group. They are primarily exploited in artisanal fisheries, and commercialized mostly in canned form. *G. solida* is highly valued from the culinary standpoint. In 1994, the first year of its listing as an individual species, 4613 tons of *S. solida* were harvested, declining to 2071 tons by 1999; *G. solida*, recorded separately beginning in 1990, was registered at 31,373 tons, which declined to 9931 tons by 1999 (SERNAPESCA 1990–1999). The only regulation for the fishery of these clams is a minimum size limit of 55 mm for *S. solida* and 60 mm for *G. solida* (Subsecretaría de Pesca 1996).

Despite their great economic value, not much research has been done on reproduction in Chilean clams, particularly in *S. solida* and *G. solida*. One recent report (Jeréz et al. 1999) suggested that *G. solida* in southern Chile had a continuous reproductive cycle throughout the year, a pattern apparently common among the heavily commercialized clams such as *V. antiqua* (Lozada & Bustos 1984) and *P. thaca* (Henríquez et al. 1981). This also was true for *E. lenticularis* (Campos & Brown 1997, Campos et al. 1999) and *M. edulis* (Jaramillo et al. 1998).

Semele solida (Fig. 1A), locally termed "tumbao", occurs partially buried in sand and gravel bottoms from the intertidal (Osorio et al. 1979) to the subtidal zone (Urban 1994). Its geographic distribution ranges from 12°S to 47°S. (Viviani 1979). *Gari solida* (Fig. 1B), locally termed "culengue", occurs completely buried in bottom sands and gravels, usually at greater depths than *S. solida* from the intertidal to 15-m depth (Urban 1994). Its range of distribution along the Pacific Coast, as given by Viviani (1979) and later by Guzmán et al. (1998), was between 12°S and 47°S.

Biologic data for species of economic importance is fundamental for proposing regulatory recommendations for sustainable harvest of these resources over time. The obvious declines in harvest over the years enhances the need for more information on the reproduction and survival of these species to support efforts directed towards their artificial culture, repopulation, or management as a renewable resource in over-exploited beds.

In this study, we describe the reproductive cycles of *G. solida* and *S. solida* by means of histologic observations of gametogenesis during different seasons of the year. Patterns in reproductive cycles, including gametogenesis and resting gonadal periods were studied in a population of *S. solida* from northern Chile and in a *G. solida* population from central-southern Chile, representing the first study of this nature for these two clam species in their respective regions.

MATERIALS AND METHODS

Adults of each species were obtained by diving at monthly intervals from June 1991 to July 1992. *S. solida* was collected in La Herradura Bay (29°58′S) and *G. solida* from Coliumo Bay (36°32′S) (Fig. 2). Maximum anterior-posterior length of the shell was measured on each specimen, to the nearest 0.1 mm. Maturational status of the gonad was determined monthly on around 30 animals of each species. Tissue samples 5 mm in thick-

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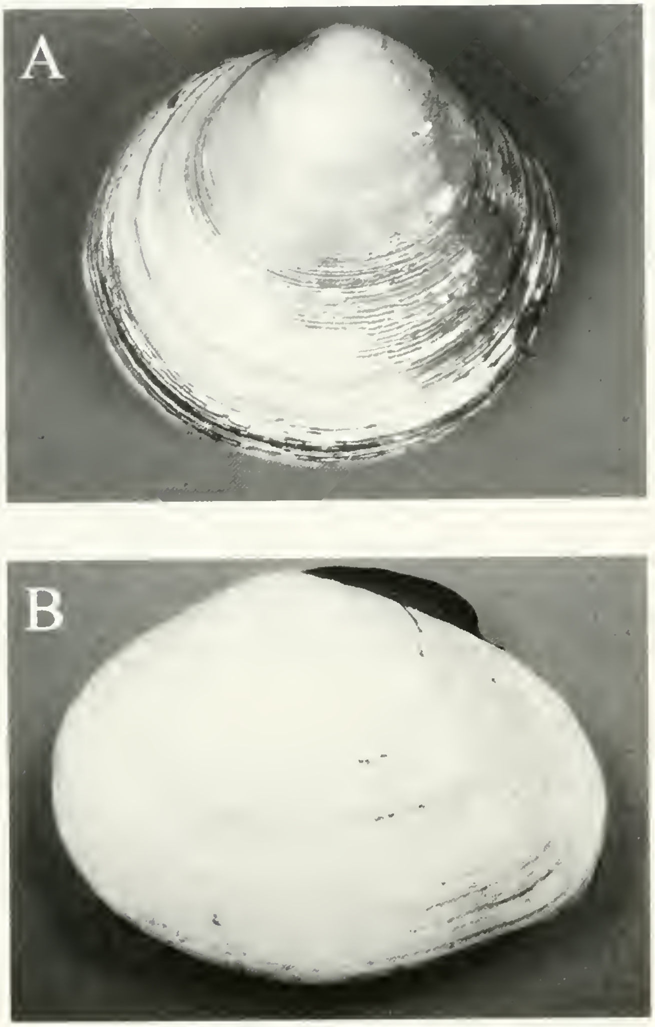

Figure 1. Species under study. A: *Semele solida*, 78 mm total length (maximum anterior-posterior shell dimension); B: *Gari solida*, 89 mm total length.

ness were obtained and fixed 24 h in Bouin's fluid and prepared by routine histologic procedures as follows: dehydration with graded series of ethanol, clearing in xylol and embedding in Paraplast. Five micrometers histologic sections obtained from three levels of each gonad separated 500 μm, were stained with hematoxylin and yellowish eosin, dehydrated in graded series of ethanol, cleared in xylol and permanently mounted with Canadian balsam (Gabe 1968).

The gametogenic cycles of the two clams were followed by describing the histologic appearance of the gonadal sections and classifying them into different stages of maturity using a modification of the scale proposed by Lucas (1965). Each individual was assigned to one of the following stages based on the degree of morphologic development of its germ cells: (d1) = initial development or maturation; (d2) = advanced development or maturation; (d3) = complete development or maturation; (r1) = initial regression or evacuation; and (r2) = total regression or evacuation. The results were expressed as percentage frequency histograms of: (1) males in each gonadal stage; (2) females in each gonadal stage; and (3) males plus females in each stage, separately for *S. solida* and *G. solida* during the sampling period from June 1991 through July 1992.

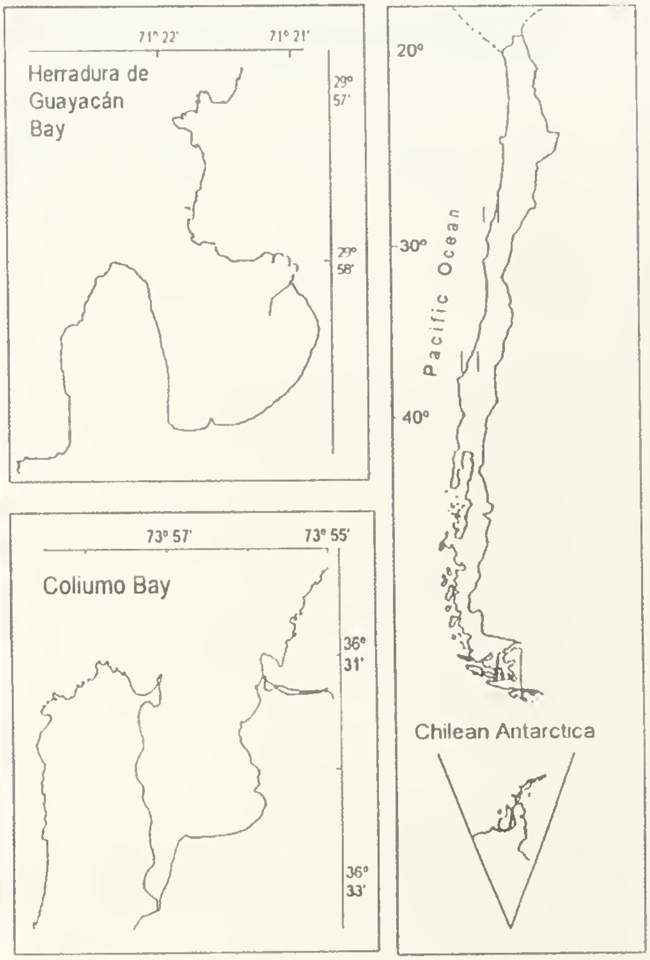

Figure 2. Location of collection sites for A: *S. solida* and B: *G. Solida* on the Chilean coast.

RESULTS

Semele solida specimens measured from 38.9 to 86.0 mm and *Gari solida* from 41.4 to 88.0 mm. The two clam species were of separate sexes, with no hermaphroditism and no sexual dimorphism evident. Histologic analysis of the gonad in both species showed a multilobulate organization of the acini connected to evacuation tubes covered by simple ciliated epithelium similar to that observed in other bivalves (Sastry1979). The acini consisted of a basal lamina of variable thickness depending on the stage of gonadal maturity. Its relative thickness was greatest in specimens initiating gametogenesis, and in those that had spawned. In these specimens an intra-acinar reticulum consisting of vesicular somatic cells and an intra-acinar space containing groups of amebocytes may be found (Figs. 3A and 4F).

The cells of the male germinal line that characterize spermatogenesis may be restricted to two zones of the gonadal acinus: (1) a basal region representing the early germinal line that includes spermatogonia and spermatocytes that form a band of circular voluminous nuclei, and recently formed round spermatids that also form a band of small circular nuclei (Figs. 3A, 3B and 4A, 4B), that is evident in *G. solida*; and (2) a lumen region, representing an advanced germinal line with spermatids undergoing cytodifferentiation with heavy stained elongated nuclei, gathered by their heads in double columns, giving a "feathered" appearance (Figs. 3B, 3C and 4B, 4C).

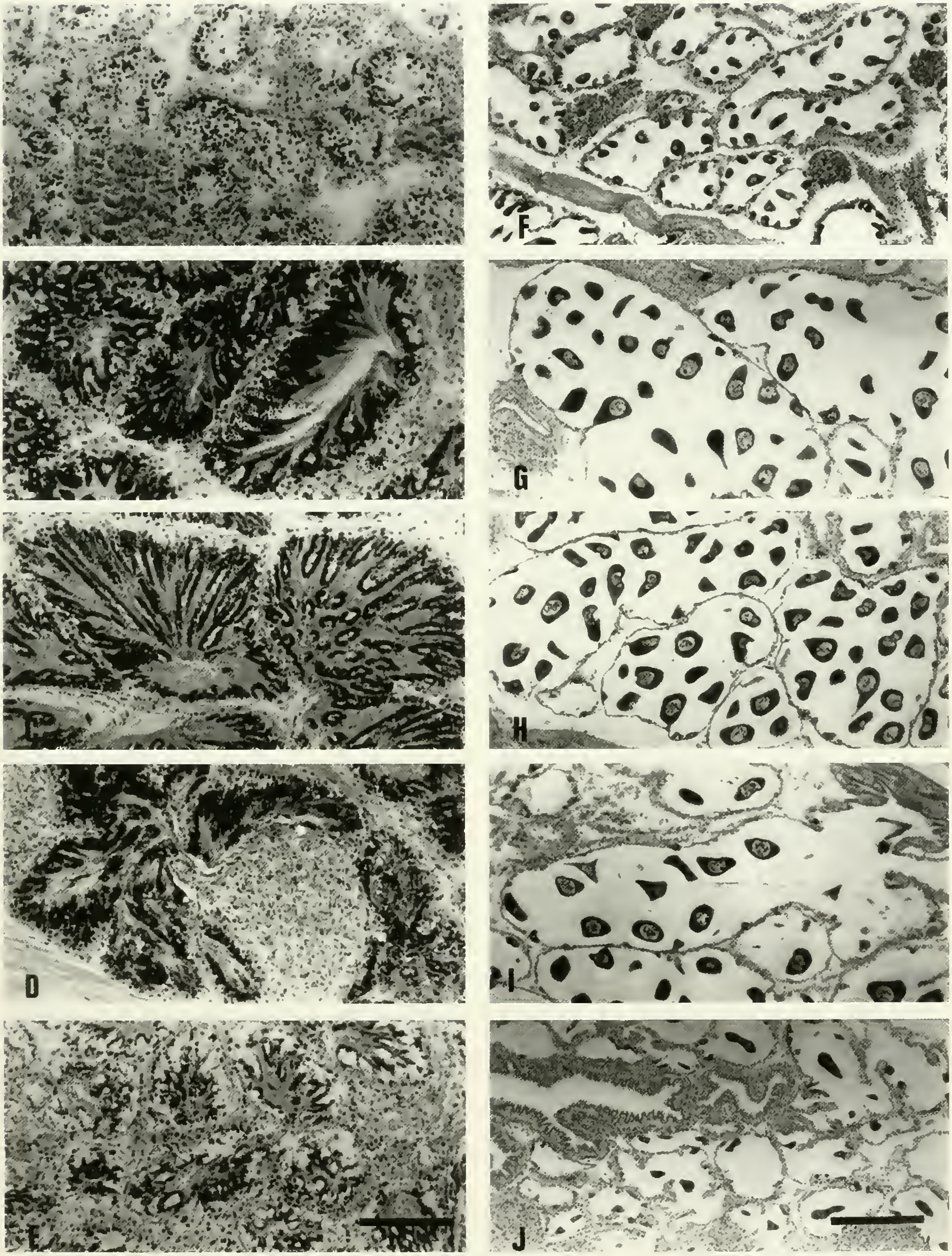

Figure 3. A–E: Light photomicrographs of different histological stages of male and F–J: female gonadal acini of *S. solida* collected in La Herradura Bay from June 1991 to July 1992. A–E bar = 100 µm; F–J bar = 200 µm; A and F = initial development of maturation (d1); B and G = advanced development of maturation (d2); C and H = total development or maturation (d3); D and I = initial regression or evacuation (r1); E and J = total regression or evacuation (r2).

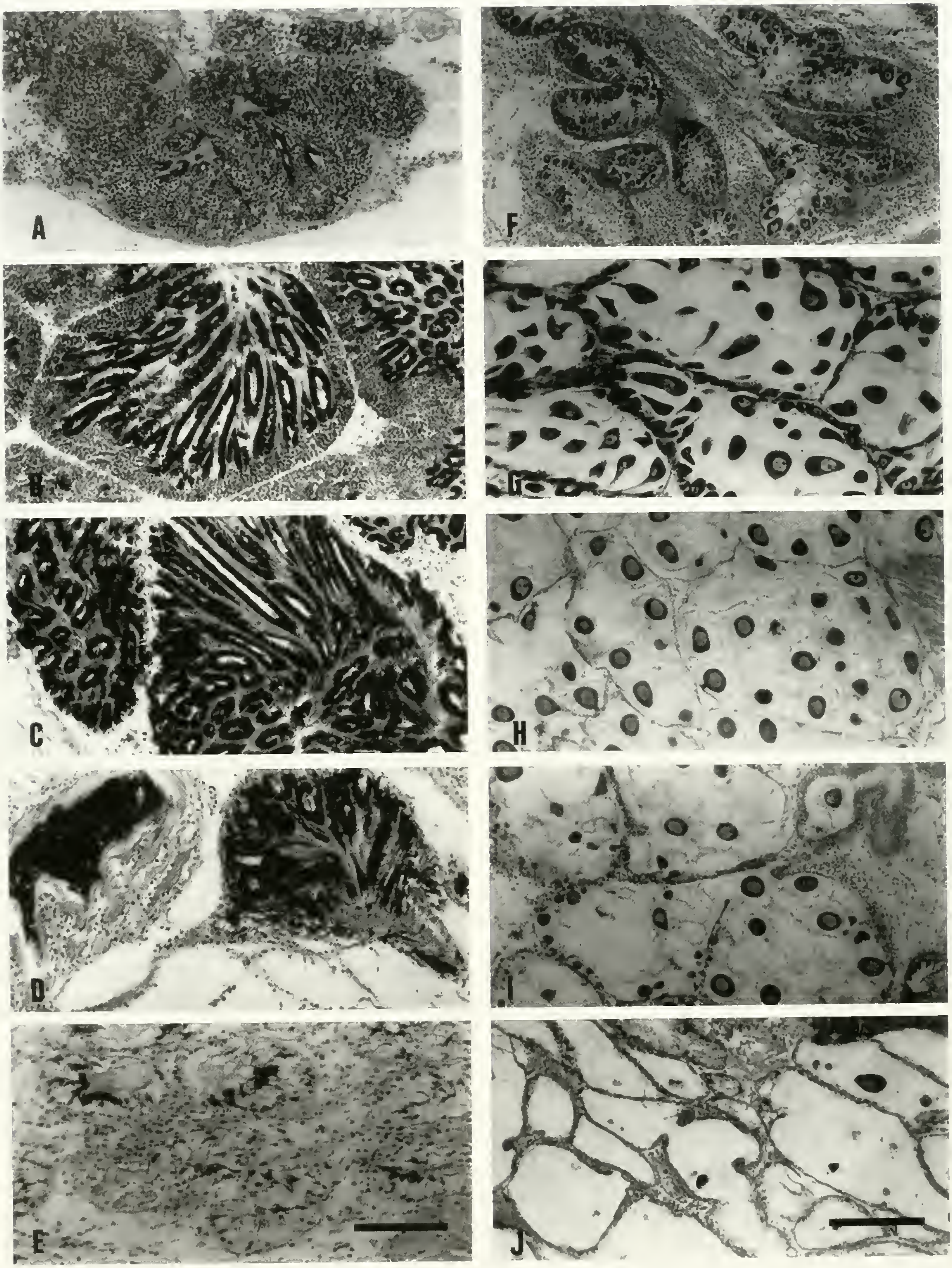

Figure 4. A–E: Light photomicrographs of different histological stages of male and F–J: female gonadal acini of *G. solida* collected in Coliumo Bay from June 1991 to July 1992. A–E bar = 100 μm; F–J bar = 200 μm; A and F = initial development or maturation (d1); B and G = advanced development or maturation (d2); C and H = total development or maturation (d3); D and I = initial regression or evacuation (r1); E and J = total regression or evacuation (r2).

The cells of the female germinal line in the basal region are represented by oogonia, previtellogenic and adhered vitellogenic oocytes. In the lumen region they are represented by pedunculate vitellogenic oocytes as well as free oocytes (Figs. 3G, 3H, and 4G, 4H). The histologic stages of the gonads of *S. solida* and *G. solida* females are shown in Figures 3F–3J and 4F–4J, respectively.

The three sampled areas of the gonad from both species all showed the same degree of gametogenic activity or development of the germinal line, indicating synchronic maturation throughout the gonad.

Semele solida

The distribution of percentage frequencies of the different histologic stages in male gonads, female gonads, and in the population as a whole for *S. solida* are given in Figures 5A, 5B and 5C, respectively. This species presented a seasonal pattern of gonadal development in both sexes. Males and females with developed or mature gonads (d3 stage) as well as in initial regression (r1) (Figs. 3D and 3I) were predominant from June 1991 to February 1992. In contrast, from March to June 1992, there was a greater frequency of individuals in total regression (r2) (Figs. 3E and 3J). Although in both sexes the frequency of samples with gonads in initial stages of development (d1) (Figs. 3A and 3F) was observed between April and July 1992, the number of females in this stage was greater, and predominated over those in total regression (Fig. 5A vs. Figure 5B). However, during the first period, there was a small percentage of males in total regression (r2) and in initial development (d1) (Fig. 5A), which was a condition more apparent in females (r2-d1; Figure 5B). Some observations not included in the figures suggested that individuals technically considered to be in regression could show a new wave of initial maturation beginning at the germinal line in the gonadal acini.

The second period was characterized by the total regression stage (r2), where all individuals had gonads with depleted acini in March 1992 (Figs. 5A, 5B). The percentage frequency distribution of the different gonadal stages for the population sample (Fig. 5C) shows this tendency in both males and females.

It is of interest to point out the difference in gonadal conditions between specimens sampled in June to July 1991 compared with those from the same period in 1992. In 1991 a high frequency of both sexes contained elevated numbers of specimens with gonads in advanced and total development (d2–d3), whereas in 1992 this condition was different, with specimens containing gonadal acini in total regression (r2) or without advanced germinal line (Fig. 5).

Gari solida

In this species the distribution of percentage frequencies of the different histologic stages in males, females, and the entire population are given in Figures 6A, 6B, and 6C, respectively. There is a periodicity in both sexes with the same general tendency.

There is a well-marked period in which advanced and totally developed gonads (d2-d3) are observed, as well as those in initial regression (r1) (Figs. 4D and 4I) from October 1991 to February 1992. In females this period is much shorter (November 1991 to January 1992). This condition persisted in some males until April 1992. Some observations not included in the figures showed individuals in regression during this period, which had a new wave of initial maturation beginning at the germinal line, as observed in *S. solida* (see earlier). In a second, more extensive period, the gonads were characterized by the occurrence of total regression and initial development (r2–d1) (Figs. 4E and 4A, respectively) in males from June to September 1991, and from March to July 1992 (Fig. 6A). In females the r2–d1 period (Figs. 4J and 4F, respectively) extended notably until October 1991, and from February to March 1992 (Fig. 6B).

Figure 6C shows the general frequency of the gonadal stages for the general population, with a similar pattern to that presented separately for males and females. From June to August 1991, February 1991, and April to July 1992 the number of specimens in each population sample exceeded the males and females together because included were specimens whose total regression stage was such that there were no cells on the germinal line differentiated enough to permit sex determination. In comparing the gonadal stages of specimens obtained in June to July 1991 with those of the same period in 1992, stages were observed that were near total regression and initial development showing an inverse fluctuation where regression predominated in 1991 and initial development in 1992.

Finally, it was apparent in both species that the stages of advanced or total maturity were observed in water having higher relative temperature, whereas initial stages of development were related to water of relatively low temperature, although our temperature measurements were not extensive (Figs. 5C and 6C).

DISCUSSION

The reproductive cycle is characterized by a series of events that in annual species comprises a reproductive period involving the gametogenic and spawning phases and a resting period in which there is not gametogenic activity.

Present results have shown similar values between the annual reproductive cycles of *S. solida* and *G. solida*, where both showed seasonal gametogenesis and spawning, followed by a resting period without production of gametes.

The reproductive period of *S. solida*, from June 1991 to February 1992 was longer than that of *G. solida*. Most of the specimens showed gametogenic activity and signs of having spawned. The majority of spawning occurred in February, and in March all specimens had empty gonads. This point marked the initiation of the resting period, indicated by a completely regressive condition in the gonad, which was more marked in *G. solida* than in *S. solida*.

We are cautious to consider the possibility of a second spawning phase during the reproductive period of *S. solida* because of the low number of animals (4) sampled and examined in October, notwithstanding that all of them were in advanced development (D2).

In *G. solida* the reproductive period was relatively short, from October 1991 to February 1992; the spawning phase mostly occurs in February. A majority of the specimens had empty gonads in March 1992; then the spawning phase mostly occurs in February. The presence of specimens with gonads in the initial state of development in this period may indicate possible activity in gonial multiplication and generation of cytes without gametogenic activity that leads to massive production of differentiated gametes. The results showed that low temperatures favored the proliferative activity of the early germinal line, while high temperatures aided cytodifferentiation of the advanced germinal line. This condition was most notable in the reproductive cycle of *G. solida* from Coliumo Bay, a more southerly location. These events occurred

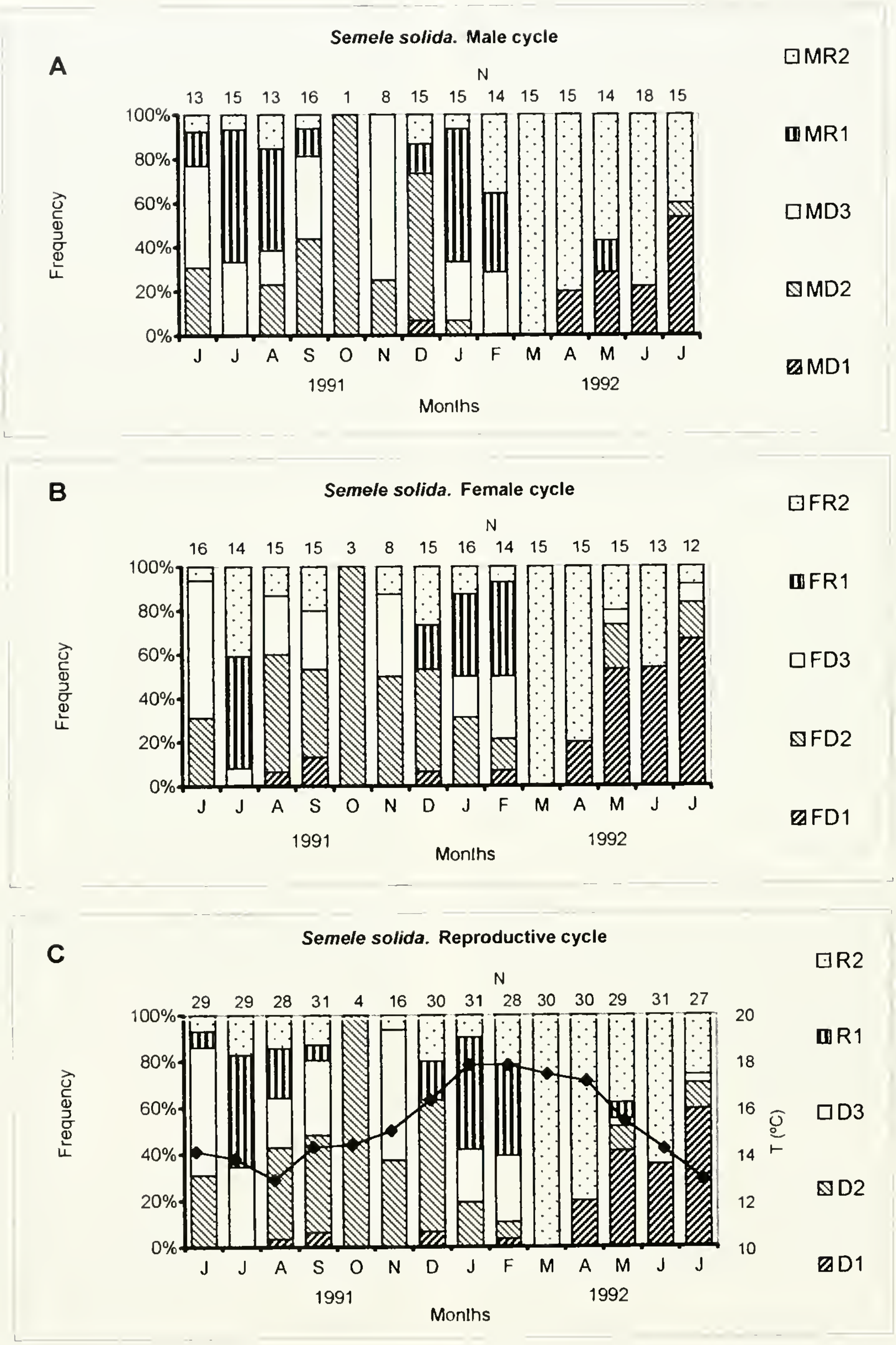

Figure 5. Distribution of different gonadal stages in A. males; B. females; and C. males + females of *S. solida* colleced in La Herradura Bay from June 1991 to July 1992, with sea surface temperature added. The length of each area represents the percentage frequency of specimens in each histologic stage of the gonadal acini. N = number of specimens examined.

simultaneously in both males and females showing (expected) synchrony of the reproductive cycles.

Every monthly sampling during the reproductive period showed a few individuals having gonadal conditions differing from the majority of the specimens, a phenomenon more pronounced in *S. solida*. There was a predominance of advanced stages of gonadal maturity, and also those with complete regression as an evidence that spawning had occurred. These observa-

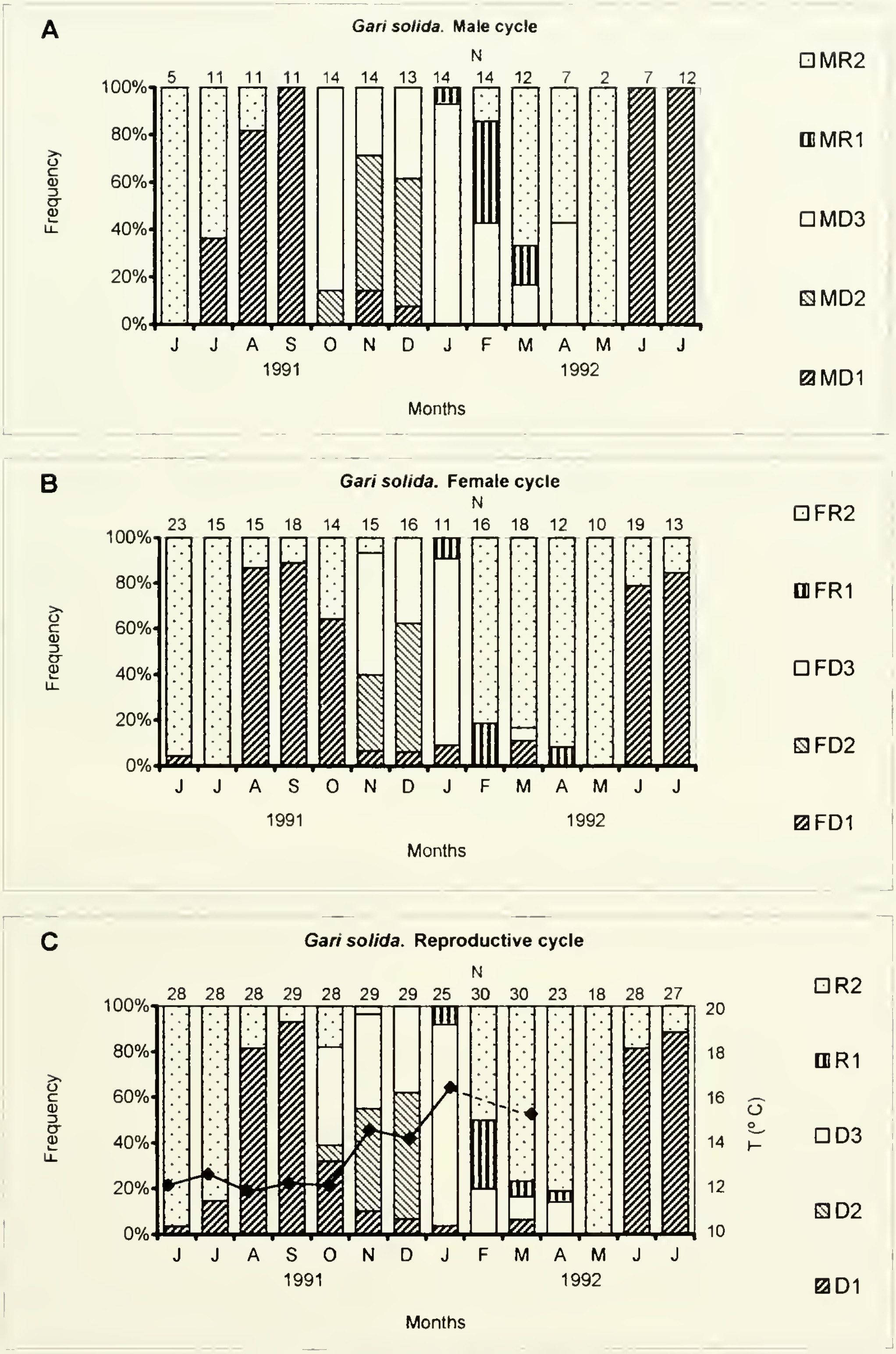

Figure 6. Distribution of different gonadal stages in A. males; B. Females and C. males + females of *G. solida* collected in Coliumo Bay from June 1991 to July 1992, with sea surface temperature added. The length of each area represents the percentage frequency of specimens in each histological stage of the gonadal acini. N = number of specimens examined.

tions suggest intrapopulation asynchrony of gametogenic activity, with partial evacuations of gametes over a longer period. The fact that the specimens showing signs of having spawned showed a new wave of maturation in the germinal layer of their acini, confirmed this asynchrony and strengthened the hypothesis of continuous gametogenesis with various cycles of gametogenic activity and spawning by each individual during the reproductive period.

A difference was observed in the degree of maturity of the population samples of *S. solida* between June and July 1991 where advanced and complete maturity were well represented; in the same period of 1992, on the contrary, maturity was just beginning. This difference may be explained by normal adaptation to environmental conditions such as temperature and food availability, which may vary within a limited range from year to year.

Although Urban and Campos (1994) suggested that the reproductive cycles of *S. solida* and *G. solida* were influenced by temperature, Jeréz et al. (1999) working on a *G. solida* population from the south of Chile found the annual reproductive cycle to be continuous without a marked resting period. Further studies are required to evaluate seasonal variations in gonadal cycles of these species with latitude, as they are distributed over a broad latitudinal range from Callao, Peru (12°S) to Chile's Chonos Archipelago (44°S). The hypothesis here is that the reproductive cycles of these clams become shorter in populations the farther south they occur on their distributional gradient. Some data available on other clam species with extensive distributions support this hypothesis. Populations of *Protothaca thaca* (Henríquez et al. 1981), *Tagelus dombeii* (Acuña et al. 1994) and *Eurhomalea lenticularis* (Campos & Brown 1997) from central and north-central Chile exhibit continuous gonadal activity with various important spawning peaks throughout the year. Nevertheless, *T. dombeii* from south-central Chile showed a period of gonadal regression in the fall (Jaramillo et al. 1998). This phenomenon is not clear across other clam species inhabiting the south-central zone of Chile, such as *Venus antiqua*, *Tawera gayi*, *Mulinia edulis* and *Ensis macha* that show continuous reproductive cycles without resting periods (Lozada & Bustos 1984, Jeréz et al. 1999).

From the practical point of view, regulation of harvesting these clams should be based on considerations of their reproductive cycles by limiting their harvest during the major spawning season. Consideration of the reproductive cycles is also important in obtaining broodstock for aquaculture. Experimental studies should prove this a feasible alternative for production or protection of the resource. In studying resource management of these clams, *S. solida* shows a comparative advantage in having a more extensive reproductive period, as mature individuals may be encountered over an extended period. This implies that mature broodstock would be available in nature for artificially induced spawning (e.g., in aquaculture experimentation) over comparatively long periods. Although *G. solida*, in contrast, has a more restricted reproductive period, it may be a species amenable to artificial conditioning in aquaculture systems, given that its gonads almost always contain high numbers of immature gametogenic cells.

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GROWTH OF NORTHERN [*MERCENARIA MERCENARIA* (L.)] AND SOUTHERN [*M. CAMPECHIENSIS* (GMELIN)] QUAHOGS: INFLUENCE OF SEAGRASSES AND LATITUDE

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ABSTRACT To better understand how seagrasses influence the growth of northern and southern quahogs (*Mercenaria mercenaria* and *Mercenaria campechiensis*), we collected and compared the growth rates of clams from seagrasses and adjacent unvegetated locations along the Atlantic (Massachusetts and New Jersey) and Gulf of Mexico coasts (Alabama and Florida) using identical methods. In particular, we sought to test hypotheses that clam growth is influenced not only by the presence or absence of seagrass (*Thalassia testudinum, Halodule wrightii*, and *Zostera marina*), but also by location within seagrass beds. Walford plots constructed using annual shell-growth band analyses showed that *Mercenaria* spp. growth was significantly affected by the presence of seagrass habitat, although the magnitude of this effect varied with clam age (size) and latitude. Specifically, first-year growth was significantly greater in unvegetated than in adjacent vegetated sites, whereas a measure of lifelong growth (Ford's growth coefficient) was not significantly different between adjacent vegetated and unvegetated sites. We hypothesize that these conflicting results may be due, in part, to differing patterns of energy resource allocation in *Mercenaria* at various life cycle stages. During the first 1.5 y or so, a clam's energy resources are allocated primarily to somatic growth, whereas in subsequent years energy is also allocated to reproduction. Therefore, factors affecting *Mercenaria* growth, such as the presence or absence of submerged vegetation, should be most easily detectable in analyses examining the first year's growth. Even though our data showed greater first-year growth at lower latitudes (Gulf of Mexico sites versus New Jersey and Massachusetts), overall lifelong growth rates were greatest at the Massachusetts sites. We attribute this pattern to the fact that higher first-year growth rates quickly become asymptotic in the warm waters of the Gulf of Mexico, whereas slower but more constant growth rates are typical of clams from colder water sites. We found little evidence for the effects of location (as measured as distance from bed edge) on growth of individuals collected from grassbeds, except where patterns of water movement showed consistent directionality (e.g., Gulf of Mexico sites). Given the multitude of variables that can interact to influence clam growth rates and the unresolved variability in results from single-site studies performed in different locations, some complex, carefully controlled experiments are still needed to elucidate the dominant factors that regulate both short and long-term clam growth.

KEY WORDS: hardclams, *Mercenaria*, seagrass, latitude, growth, quahog

INTRODUCTION

Growth and survival[1] of suspension-feeding animals may be strongly influenced by the presence of submerged aquatic vegetation (SAV), presumably because macrophytes reduce flow and enhance the deposition of suspended materials (e.g., Scoffin 1970, Orth 1977, Tegner & Dayton 1981, Fonseca et al. 1982, Harlin et al. 1982, Eckman 1983, Peterson, et al. 1984, Dayton 1985, Wildish & Kristmanson 1997), thereby altering available food supply (Peterson & Beal 1989, Irlandi & Peterson 1991, Irlandi 1996, but see Judge et al. 1992, Nakaoka 2000). However, studies of the suspension-feeding bivalve *Mercenaria mercenaria* (The Northern Quahog), have continued to produce conflicting results (e.g., Belding 1912, Kerswill 1941, 1949, Haskin 1952; Pratt & Campbell 1956; Ansell 1968, Greene 1979, Hadley & Manzi 1984, Peterson et al. 1984, Arnold et al. 1991, Coen & Heck 1991, Irlandi & Peterson 1991, Judge et al. 1992, Coen et al. in prep.) with regard to the effect of seagrasses. For example, early studies by Kerswill (1941, 1949) found decreasing *M. mercenaria* growth rates as eelgrass (*Zostera marina*) density increased, whereas a later study by Peterson et al. (1984) found just the opposite.

More recent studies (Peterson & Beal 1989; Coen & Heck 1991; Slattery et al. 1991, Nakaoka 2000, Coen et al. in prep.) found that *M. mercenaria* in seagrass beds sometimes grew faster, slower, or at the same rate as *M. mercenaria* in unvegetated habitats. Peterson and Beal (1989) cited two possible explanations for the variability in their results. First, as water velocities decrease, the flux of suspended food particles passing by the clam's siphons will decrease. Alternatively, reductions in velocity could enhance the settlement of suspended materials and increase food concentrations near the bottom. The net effect of decreasing suspended horizontal food flux while increasing the number of food particles settling from the water column will determine whether the impact of vegetation on *Mercenaria* growth is positive or negative (Peterson & Beal 1989). Subsequently, Irlandi and Peterson (1991) have argued that measured variations in food concentration between vegetated and nearby unvegetated areas are insufficient to explain the observed between-habitat differences in clam growth.

There must also be an upper limit to current velocity beyond which bottom instability inhibits feeding, either directly by smothering or indirectly by requiring the clam to expend energy reburrowing to maintain a desired burial depth (Myers 1977, Orth 1977, Turner & Miller 1991, Irlandi & Mehlich 1996, Irlandi 1996) or by decreasing assimilation efficiency (Bricelj & Malouf 1984). Greater bottom stability in vegetated habitats produced by the baffling effect of seagrasses could also lead to greater growth of *Mercenaria* located within seagrass beds (cf. Peterson et al. 1984; Irlandi & Peterson 1991; Irlandi 1996, Coen, unpublished, 2000).

Location within a landscape of habitats and the associated system states (i.e., food, flow, competitors, predators, see Micheli 1996) can also significantly affect bivalve populations. This can result from local food depletion from upstream-feeding individuals (e.g., Okamura 1986, Peterson & Black 1987, Fréchette et al. 1989), within or among habitat conditions (e.g., Irlandi & Peterson

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1991, Kamermans 1993) or the physical activity of neighboring animals interfering with feeding or space acquisition (Virnstein 1977, Peterson 1979, Okamura 1986, Nakaoka 2000). These can manifest themselves under certain situations as density-dependent effects (Grizzle & Morin 1989, Grizzle & Lutz 1989, Grizzle et al. 1992, Kamermans et al. 1992).

The growth of suspension-feeding bivalves may also be influenced by lethal or nonlethal (siphon nipping, reduced foraging) predation by numerous species, such as fishes, gastropods, and crabs (e.g., Coen & Heck 1991, Irlandi & Peterson 1991, Kamermans & Huitema 1994, Peterson & Skilleter 1994, Skilleter & Peterson 1994, Bonsdorff et al. 1995, Nakaoka 2000). Although the siphon tip is easily regenerated, this requires an expenditure of energy (see Peterson and Fegley 1986) that could otherwise be used for shell and soft-tissue growth/repair and reproduction (e.g., Trevallion et al. 1970, Hodgson 1982, Peterson & Quammen 1982, Festa 1975, De Vlas 1985, Zwarts 1986, Coen & Heck 1991, Skilleter & Peterson 1994, but see Irlandi 1994, 1996, and Irlandi & Mehlich 1996 for counter examples). In addition, the mere presence of potential disturbers or predators in the vicinity of *Mercenaria* may cause individuals to cease feeding and thereby lead to reduced growth rates where predators are abundant (e.g., Blundon & Kennedy 1982, Irlandi & Peterson 1991, Nakaoka 2000).

This study was designed to investigate how the presence of vegetation and location within a bed can influence the growth of selected Atlantic and Gulf of Mexico (U.S.) populations of the northern and southern quahogs, *M. mercenaria* and *M. campechiensis*. We were especially interested in determining whether 1) generalizations could be made that would apply to populations of both the northern and southern quahog species regarding the effect of seagrasses on growth; and 2) whether the distance of individual clams from the bed's edge could be an indirect measure of habitat quality as a result of potential differences in food supply, and therefore help explain some of the conflicting evidence regarding the effects of seagrasses on quahog growth rates.

MATERIALS AND METHODS

Study Locations

M. mercenaria and *M. campechiensis* populations were sampled at six different locations in four states (Massachusetts, New Jersey, Florida, Alabama) along a latitudinal gradient from about 42° to 30°N. The Massachusetts sampling area was located on Cape Cod in the Nauset Marsh system (Nauset Harbor and Salt Pond Bay; 42°N, 70°W), whereas *M. mercenaria* were collected from both vegetated ($n = 51$ clams) and unvegetated (sand) substrates ($n = 52$ clams) in May 1987. Vegetation consisted of pure stands of *Z. marina*, and water depth at low tide was less than 1.0 m.

The New Jersey sampling site was in southern Barnegat Bay, near Marshelder Island (39°N, 72°W), and *M. mercenaria* were also collected from both vegetated ($n = 51$ clams) and adjacent unvegetated (sand) substrates ($n = 84$ clams) in May 1987. Vegetation was dominated by eelgrass (*Z. marina*), with lesser amounts of widgeon grass (*Ruppia maritima*). Water depth (at low tide) was less than 1.0 m.

Collections were also made in the Perdido Bay system (30°N, 87°W), with one site at the western end (see Fig. 1 in Coen & Heck 1991; Perdido Pass, Alabama), and one site at the eastern end of the Bay (in the Gulf Islands National Seashore, Big Lagoon, Florida, see Fig. 2 in Spitzer et al. 2000). Native *M. campechiensis* were collected at the Perdido Pass site in April, May, and June 1987 ($n = 32$ clams) and at the Gulf Islands National Seashore site (30°N, 87°W), in June 1987 ($n = 21$ clams). All clams collected from these two sites were taken from vegetated habitats because there were no hard clams found in the unvegetated sand adjacent to the seagrass beds. Submerged vegetation at the two sites consisted of shoal grass, *Halodule wrightii* (Alabama) and mixed stands of *H. wrightii* and turtle grass, *Thalassia testudinum* (Florida). Water depth (at low tide) at both sites was less than 1.5 m.

Additional details on vegetation composition and physiochemical conditions at these study sites can be found in Heck et al. (1989, Massachusetts); Wilson et al. (1990, New Jersey); and Coen and Heck (1991), Judge et al. (1992, 1993), and Wilson (1991) for the Gulf of Mexico sites.

Clams were collected by hand after being located by treading at each sampling location. Clam location within grassbeds relative to the edge of the bed was estimated as the minimum distance from collection point to the nearest grass bed edge along one of the four compass directions. All *Mercenaria* were frozen after collection and later were thawed to allow removal of the soft tissue without damaging the shell. Shells were then washed and allowed to air dry. Valves of each clam were separated, with one valve used for age and growth estimates and the other archived (see also Wilson 1991). We assumed that all specimens collected from Atlantic locations are *Mercenaria mercenaria* (L.), and all individuals collected from Gulf sites are *Mercenaria campechiensis* (Gmelin).

Analysis of Growth Increments

For age and growth determinations, one valve from each clam was embedded in epoxy resin before sectioning with a Buehler Isomet™ low-speed saw. Sections were made through the umbo to the ventral margin, along the axis of maximum growth (Kennish et al. 1980). A second cut was made approximately 10 µm from the first to produce a thin section. To facilitate examination of annual growth increments, each thin section was ground and polished on a Buehler Ecomet III™ grinder/polisher with appropriate polishing compounds. During the polishing procedure, visual inspection of the thin section under a microscope determined when the thin section was readable (see Wilson 1991 for more detail).

When thin sections were viewed under the microscope, growth increments appeared as alternating translucent narrow dark and wide white (opaque) regions corresponding to periods of slow (narrow microgrowth increments) and rapid (wide microgrowth increments) growth, respectively (Barker 1964, Rhoads & Pannella 1970, Kennish 1980, Fritz & Haven 1983, Peterson et al. 1983, 1985, Grizzle & Lutz 1988, Arnold et al. 1991, 1996, Bert & Arnold 1995). One annual growth cycle, therefore, consists of two opaque regions (spring and fall) and two translucent regions (summer and winter). In older *Mercenaria*, there is often only one opaque region, with the fall band being faint or absent. Annual growth was marked at the end of each successive winter band for Atlantic sites (Pannella & MacClintock 1968, Rhoads & Pannella 1970, Kennish & Olsson 1975, Fritz & Haven 1983), and at the end of each successive summer band for the Gulf of Mexico sites (Clark 1979, Fritz & Haven 1983). Thus, one year's growth was measured as the linear distance between consecutively marked annual growth bands.

To obtain a quantitative measurement of annual growth, the distance between consecutive bands was traced on paper at 60×

with a camera lucida. Tracing followed the boundary between the outer, prismatic and middle, homogeneous shell layers with the end points located at the point where each successive annual band crossed this boundary (rather than the point where each successive annual band reached the outer edge of the shell), serving to delineate each year's growth. Distances were measured with a calibrated ocular micrometer.

Visual examination of the thin sections indicated that annual growth increments corresponding to ages >12 y could not be resolved with certainty (Wilson 1991, Lowell Fritz, Rutgers University, personal communication). In older *Mercenaria*, annual growth increments become narrower and more difficult to distinguish owing to decreased lateral shell growth (Hopkins 1941, Fritz & Haven 1983). This slowing of lateral growth may be accompanied by an increase in shell thickness, especially along the shell's margin. This pattern of shell growth was evident in most of the larger (i.e., older) *Mercenaria* specimens in this study. Visual examination of the thin sections identified clear annual growth increments corresponding to ages <11 y. Therefore, only the first 10 annual growth increments from each individual specimen were used for growth rate determinations.

Walford plots (see Ricker 1975) of yearly clam growth in length were constructed for individual *M. mercenaria* and *M. campechiensis* (see above assumption) for each site by plotting total length in a given year (designated G_{t+1}) versus total length in the previous year (designated G_t). Total length was calculated directly from the summation of annual measured growth increments measured from thin sections. In all cases, the first data point represents length at year two versus length at year one. "Population" Walford plots for each site, constructed by plotting G_{t+1} versus G_t values across all ages and clams from a given site, contained a minimum of 63 and a maximum of 756 data points (Wilson 1991).

A linear regression of G_{t+1} versus G_t yielded two values of interest: the slope of the regression line and its y-intercept. The y-intercept is the growth for the first year (McCuaig & Green 1983, hereafter referred to as the first year growth). The slope (also called Ford's growth coefficient or simply growth coefficient) is the fraction of total growth still to be attained after the first year (Walford 1946, McCuaig & Green 1983). The slope represents the decrease in growth increments over age classes (Ricker 1975) and has been used to characterize clam growth rates after the first year. The point at which the regression line for the Walford plot intersects the line of slope = 1, indicates the point at which growth stops and maximum size is reached.

Walford plots were constructed for each site to determine the site-specific population growth coefficient and first year growth. SAS (Version 5) regression procedure (Proc Reg) and its "Test" option (Freund & Littell 1986) were used then to simultaneously test for differences in growth coefficient (slope), first year growth (intercept), and coincidence of the regression lines among pairs of sites. Pairwise comparisons of *M. mercenaria* between vegetated and unvegetated habitats were only analyzed for sites in Massachusetts and New Jersey. Between-habitat (within site) comparisons were not made at the two Gulf sites because no *M. campechiensis* were found in any of the surrounding unvegetated habitats in either Alabama or Florida.

Walford plots were also constructed for individual *M. mercenaria* and *M. campechiensis* to determine growth coefficients and first year growth for each clam. Ford's growth coefficient and first year growth for each clam were then tested for the significance of correlations between these parameters and the linear distance to the grassbed edge (see above). Because proper construction of the Walford plots requires a minimum of three sets of data points (i.e., three G_{t+1}, G_t pairs), only those individuals having four or more annual growth bands were used to calculate the growth coefficient and first year growth for individual *Mercenaria* and sites (see Wilson 1991 for more details).

RESULTS

Year 1 and Overall Growth

First year growth, calculated for each site (Fig. 1; Table 1) ranged from a high of 2.11 cm for the Gulf Islands National Seashore vegetated site in Florida (mixed *H. wrightii* and *T. testudinum*) to a low of 1.10 cm for the vegetated site in Massachusetts (*Z. marina*). First-year growth was significantly greater in sand (=unvegetated) than in seagrass at both the Massachusetts and New Jersey sites (see Table 2).

There was also significantly greater first year growth at the vegetated sites in New Jersey, Alabama, and Florida than at the

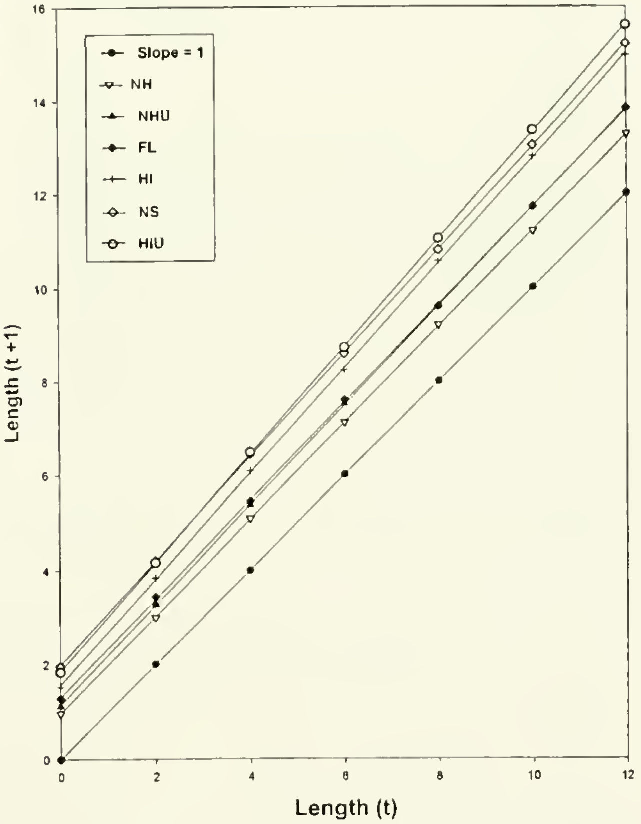

Figure 1. Walford plots for all six sites constructed by plotting length at year t_{+1} versus length at year t for all ages of all clams combined. Only the regression lines fitted to each site sampled and a line of slope = 1 (solid circle) are displayed. The six lines displayed correspond to the following sites: (1) unshaded diamond ◇ line = vegetated Gulf Islands National Seashore, Florida site (mixed *Thalassia testudinum* and some *Halodule wrightii*), (2) open circle line ○ = unvegetated New Jersey site (sand), (3) solid diamond line ◆ = vegetated Perdido Pass, Alabama site (*Halodule*), (4) large ✚ line = vegetated New Jersey site (*Zostera marina* and some *Ruppia maritima*), (5) large shaded triangle line ▲ = unvegetated Massachusetts site (mud/sand), and (6) large unshaded triangle line ▽ = vegetated Massachusetts site (*Z. marina*).

TABLE 1.

Growth parameters for Walford plots calculated from clams collected at each study site.

Study Sites	n	Intercept (First-Year Growth) cm	Slope (Ford's Growth Coefficient)	r^2
Massachusetts, vegetated (*Zostera*)	51	1.10	0.91	0.96
Massachusetts, unvegetated	84	1.28	0.88	0.96
New Jersey, vegetated (*Zostera*)	51	1.64	0.82	0.95
New Jersey, unvegetated	52	1.88	0.80	0.94
Gulf Islands National Seashore, vegetated (*Thalassia*)	21	2.11	0.82	0.94
Perdido Pass, vegetated (*Halodule*)	32	1.73	0.81	0.87

Intercept represents the first year's growth and the slope represents Ford's growth coefficient.

Massachusetts site (Table 2), and first-year growth was significantly greater in Florida (*Thalassia/Hadodule*) than New Jersey (*Zostera*) grassbeds. However, no significant difference in growth was observed between clams from New Jersey and Alabama (Perdido Pass, *Halodule*) grassbeds.

Growth rates of the 291 clams measured, while not presented individually here, varied over about an order of magnitude. For example, first-year growth ranged from a low of 0.67 cm for a clam at the vegetated site in Massachusetts (in *Z. marina*) to a high of 6.31 cm for an individual at the Perdido Pass vegetated site (taken from *H. wrightii*).

Ford's growth coefficients for individual clams ranged from a low of 0.14 for a clam at the vegetated site in Perdido Pass (*H. wrightii*) to a high of 0.97 for an individual at the vegetated site in Massachusetts (*Z. marina*). Overall, site-specific Ford's growth coefficients ranged from a high of 0.91 for the vegetated site Massachusetts (*Z. marina*) to a low of 0.80 for the unvegetated site in New Jersey (Table 1; Fig. 1). The only significant differences in the growth coefficients were between both the Massachusetts sites and all other sites in New Jersey, Alabama, and Florida. These significantly higher growth coefficients at the Massachusetts sites were unexpected because first-year growth was lowest at these sites.

Growth and Location in the Grassbed

There were two significant positive relationships between first-year growth and distance to the edge of the grassbed, which were found at the vegetated sites in Massachusetts (*Z. marina*) and Perdido Pass, Alabama (*H. wrightii*; r = 0.315 and 0.432; $P < 0.05$ and 0.01, respectively), indicating greater growth in year 1 with increasing distance from the edge of the bed at these two sites (Table 3). The only significant correlation between Ford's growth coefficient for individual clams and distance from the grassbed edge was found at the vegetated site at Perdido Pass (*H. wrightii*; Table 3, $r = -0.435$; $P < 0.05$), a result that indicates greater lifelong growth near the edge of the bed.

DISCUSSION

The effects of habitat (seagrass versus unvegetated substrate) on clam growth did not vary consistently when examined by either clam age or geographical location. With respect to age, we found significantly greater growth during year one in clams collected from the unvegetated habitats at both Massachusetts and New Jersey (Table 2). We attribute this to the fact that *M. mercenaria* growth is greatest during the first 1.5 y of life, with decreasing growth thereafter as sexual maturity is reached (Haskin 1952, Gustafson 1955, Walker 1985, Eversole et al. 1986). This suggests to us that habitat effects would be most evident in analyses of the rapid growth attained in the first year. However, this finding conflicts with that of Irlandi and Peterson (1991), who found faster growth of juvenile clams (mean size of 2.6–2.87 cm in length) planted for up to 6 months in seagrass (vs. sand), and who concluded that ". . . results should dispel any remaining doubts about

TABLE 2.

Pairwise comparisons of first-year growth and Ford's growth coefficient among study locations.

Pair-Wise Comparisons	N Site 1/Site 2	First-Year Growth F Value	First-Year Growth Significance Level ($P <$)	Ford's Growth Coefficient F Value	Ford's Growth Coefficient Significance Level ($P <$)
Massachusetts, vegetated vs. unvegetated	51/84	7.90	0.01	3.10	Not significant
New Jersey, vegetated vs. unvegetated	51/52	9.61	0.01	3.72	Not significant
Massachusetts, unvegetated vs. New Jersey, unvegetated	84/52	78.80	0.01	50.71	0.01
Massachusetts, vegetated vs. New Jersey, vegetated	51/51	55.47	0.01	37.28	0.01
Gulf Islands National Seashore vs. Perdido Pass	21/32	5.92	0.05	0.29	Not significant
Massachusetts, vegetated vs. Gulf Islands National Seashore	51/21	89.21	0.01	22.66	0.01
Massachusetts, vegetated vs. Perdido Pass	51/32	38.03	0.01	21.10	0.01
New Jersey, vegetated vs. Gulf Islands National Seashore	51/21	19.98	0.01	0.00	Not significant
New Jersey, vegetated vs. Perdido Pass	51/32	0.83	Not significant	0.62	Not significant
All four vegetated subsites (GINS, MA, NJ, and PP)	21/51/51/32	35.38	0.01	13.00	0.01

Comparisons were performed on the regression equations calculated for the Walford plots at each location using the test option in the regression procedure of SAS version 5.

TABLE 3.

Pearson product moment correlations between first-year growth and Ford's growth coefficient and proximity of clams to the edge of the grassbeds at each study site.

		First-Year Growth		Ford's Growth Coefficient	
Study Sites	*n*	Correlation Coefficient	Significance Level ($P <$)	Correlation Coefficient	Significance Level ($P <$)
Massachusetts (*Zostera*)	51	0.32	0.02	−0.14	0.34
New Jersey (*Zostera*)	51	−0.05	0.72	0.01	0.95
Gulf Islands National Seashore (*Thalassia*)	21	−0.16	0.50	0.15	0.53
Perdido Pass (*Halodule*)	32	0.43	0.01	−0.43	0.01

the potential for seagrass in relatively high-energy environments to promote faster growth of suspension feeder living buried in the sediments." Perhaps the key to resolving the differences in our results and those of Irlandi and Peterson (1991) is differences in flow regimes among sites examined. These differences could also reflect some inherent latitudinal differences between conditions in North Carolina and those conditions further north (see results of Ansell 1968, or life-long growth reported by Slattery et al. 1991). For example, at our two more northern sites, the winter dieback of seagrasses may impact clam growth seasonally to a greater extent, than that which more typically occurs in lower latitudes.

Pair-wise comparisons of first year growth between Massachusetts, New Jersey, and Gulf of Mexico (Florida and Alabama) sites revealed significant differences in all but the vegetated New Jersey versus Perdido Pass comparison. The observed pattern of generally increasing first-year growth with decreasing latitude was expected, and is consistent with patterns reported more than 30 years ago by Ansell (1968).

Significantly higher values of Ford's growth coefficient were found for all comparisons of the vegetated site in Massachusetts with the other vegetated sites (Table 2) and in the comparison of the Massachusetts versus the unvegetated New Jersey sites. We attribute these differences to slower, but steady lifelong growth in Massachusetts, with clams eventually attaining a larger final size, in contrast to the more rapid year 1 growth that quickly becomes asymptotic in New Jersey, Alabama, and Florida. This is consistent with a previous study of both *Mercenaria* species (Jones et al. 1990), which found rapid, but variable growth in Gulf of Mexico *M. campechiensis* populations that was often, but not always, greater than rates measured for Atlantic coast populations of *M. mercenaria*. Jones et al. (1990) also found life spans to be typically shorter in Florida than elsewhere. Based on our finding of rapid early growth, and the results of Jones et al. (1990), it appears that Gulf of Mexico *M. campechiensis* typically have high but variable growth rates and often shorter life spans than their Atlantic coast congener.

We found no significant differences in life-long growth at either the Massachusetts or New Jersey sites (Table 2) when comparing clams in seagrass with those in adjacent unvegetated areas, although as noted above, we could only consider growth in the first 10 years of life. This result agrees with those of Slattery et al. (1991), who found no significant differences in lifelong growth between vegetated and unvegetated habitats at the same study sites in Massachusetts and New Jersey. However, Slattery et al. (1991) found that life-long clam growth in North Carolina was greater in vegetation than adjacent sand, just as Peterson and colleagues had previously reported (Peterson et al. 1984, Peterson & Beal 1989, Irlandi & Peterson 1991, but see Nakaoka 2000 for short duration results).

It is unfortunate that we could not find clams in unvegetated areas at our Gulf of Mexico sites to better determine whether there are generally observable latitudinal trends in the effects of vegetation on clam growth, or whether North Carolina conditions are unique for *Mercenaria* (e.g., Peterson & Beal 1989, Nakaoka 2000). Later studies at the same sites (Coen et al., unpublished data) also did not find clams in sand and recorded similar low densities within grassbeds. Some studies involving experimental planting of juvenile clams in the northern Gulf of Mexico, which controlled for location in the grassbed, genetic differences, filter feeder abundance, and sublethal predation, have reported shifts in growth rates between vegetated and unvegetated substrates depending on season and year. For example, Coen and Heck (1991) found greatest growth at the same Alabama site in sand than at the grass edge or bed center, whereas Coen et al. (manuscript in preparation) found that growth effects varied with location within the bed, although the relative importance of different effects sometimes varied with season at the same two Alabama and Florida sites.

Correlations between the growth coefficient and first-year growth and the distance of clams relative to the grassbed edge were significant only at the Perdido Pass *Thalassia-Halodule* site and for first year growth only at the Massachusetts *Zostera* site (Table 3). As Irlandi and Peterson (1991) note, until recently these results would have been unexpected because the prevailing view has been that bivalve growth is positively correlated with flow rates or food flux (Belding 1912, Kerswill 1949, Haskin 1952, Pratt & Campbell 1956, Greene 1979, Hadley & Manzi 1984, Bricelj et al. 1984, Arnold et al. 1991, Bock & Miller 1994, Wildish & Kristmanson 1997, but see Irlandi & Peterson 1991, Judge et al. 1992, Lenihan et al. 1996), and slower current velocities with increasing distance into the seagrass bed should lead to reduced growth (e.g., Fonseca et al. 1982). However, Peterson et al. (1984), Irlandi and Peterson (1991), and Judge et al. (1993) have all indicated that food supply may actually be greater in vegetated areas primarily resulting from the availability of suspended benthic diatoms and epiphytes. The latter work, along with Coen and Heck (1991), and Coen et al. (manuscript in preparation), also evaluated position in the grass bed and its affect on growth. An additional explanation for variable growth between vegetated and adjacent unvegetated areas was suggested by Irlandi and Peterson (1991), who found differential growth on alternate edges of seagrass patches, possibly as a consequence of variation in sediment stability.

Because current velocity and direction were not measured, verification of whether distance to the grassbed edge as recorded corresponded to distance from the source of prevailing currents was not possible. However, it did not appear that currents flowed in a consistent pattern across the beds in Massachusetts, New Jersey, and Florida but rather varied greatly in their point of origin. The Perdido Pass site had the clearest directionality of currents and the "leading (flood tide) edge" of the bed was easily determined (Judge et al. 1992, 1993), perhaps helping to explain the significant relationship found there between growth and proximity to the grassbed edge (Table 3). In addition, any shifts in seagrass bed boundaries over a clam's lifetime, or over a significant portion of the growing season because of winter dieoffs, could also affect our one time distance estimates from the point of collection to the edge of the bed and weaken the calculated correlations. Given the numerous articles that discuss seagrass patch dynamics, this potential problem is likely (e.g., Irlandi 1997, Robbins & Bell 2000). Our previous work has also suggested that siphon nipping can significantly impact clam growth (e.g., Coen & Heck 1991, Coen et al., manuscript in preparation)

An additional factor whose importance is difficult to assess here or in previous studies is the impact that harvesting may have. Both the New Jersey and Massachusetts populations are subject to recreational harvesting, and commercial harvesting was observed at the New Jersey site. In contrast, there is no recreational or commercial harvesting at either of the Alabama or Florida sites, resulting in a large part from, very low natural densities. To the extent that harvesting is biased toward different-sized individuals or to the extent that desirable sized clams are found and collected in specific locations within the beds, results from harvested populations could bias our conclusions. For example, we typically observed, as have others, how aggregated hard clams often are, with similar age-classes within a high-density patch. In addition, harvesting changes the number of filter feeders and potential competitors, which is another variable of potential significance that could not be controlled.

CONCLUSIONS

Our results clearly showed significantly greater first-year, but not life-long, growth in sand than in seagrass in Massachusetts and New Jersey habitats. Therefore, whether one concludes that the presence of seagrass significantly affects clam growth at our study sites depended on clam age and the amount of time considered. As reported previously by others, we found strong evidence for greater first-year growth in *M. campechiensis*, but we also found higher life-long growth rates in our most northern *M. mercenaria* population, and we found little evidence that location (i.e., distance to nearest bed margin) within the seagrass bed was consistently related to either short or long-term growth rates.

Although we attempted to account for one potentially confounding factor, location within a seagrass bed, we were unable to control for siphon nipping, a factor that seems to produce significant effects on clam growth only some of the time (Coen & Heck 1991, Irlandi & Peterson 1991, and Coen et al., manuscript in preparation vs. Kamermans & Huitema 1994, Irlandi & Mehlich 1996, Irlandi 1996, and Nakaoka 2000), nor could we control for the effects of harvesting on filter feeder abundance. Given the complexity of factors that interact to affect clam growth rates over *Mercenaria*'s broad geographic range, and past conflicting results, it is clear that additional, extensive field experiments using the same design are still needed. At present, we are still unable to make accurate predictions about the effects of seagrasses on bivalve growth rates and related post-settlement survival.

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REPRODUCTION OF *CALLISTA CHIONE* L., 1758 (BIVALVIA: VENERIDAE) IN THE LITTORAL OF MÁLAGA (SOUTHERN SPAIN)

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ABSTRACT The reproductive cycle of *Callista chione* Linnaeus, 1758 was studied using histology and changes in flesh dry weight, in the littoral of Málaga (southern Spain), from June 1999 to May 2000. Histologic study of the gonads showed a long reproductive period, with spawning throughout the year. Three peaks of spawning have been observed through histology as well as flesh dry weight variation. The first one lasts from February to March and is accompanied by the highest decrease of weight; the second one is in spring, with the highest percentage of population in spawning but with a lower decrease of weight, and the third one is in summer. The latter peak represents a new activation of the gonads from postactive stages, without passing through a resting period. The absence of a resting period in the reproductive cycle of the studied population could be influenced by the mild seawater temperatures and high levels of chlorophyll *a* (attributable to the occurrence of upwellings) in the littoral of Málaga. The decrease of the average size in the population of Málaga from 1981 (70 mm) to 1999–2000 (54 mm length), points out an overexploitation of this resource. The above data, together with the removal of the close of season since 1990 in Málaga province, make it necessary to regulate a close of season in this area. We propose a close of season from February to March, months during which there was the most intense release of gametes in the population.

KEY WORDS: *Callista chione*, histology, biomass, reproductive cycle, fishery

INTRODUCTION

Callista chione Linnaeus, 1758 is an Atlantic-Mediterranean species ranging in the Atlantic from the southwestern British Isles to Morocco, and also in the Canary, Madeira, and Azores islands (Tebble 1966, Poppe & Goto 1993), and in the Mediterranean. It lives in fine and clean sand, from low tide down to 180 m (Poppe & Goto 1993).

Most of the studies on this species regard growth (Hall et al. 1974, Cano 1981, Forster 1981, Valli et al. 1983–1984, Strada & Zocco 1985, Valli et al. 1994). Because *C. chione* is a commercial species, some studies in Mediterranean waters were conducted in relation with the monitoring of infections by protozoans (Bravo et al. 1990, Canestri-Trotti et al. 1998, Canestri-Trotti et al. 2000) and heavy metals (Belmonte & Grasso 1986, Valli et al. 1994). Others were related with the influence of the dredge design on the size of the individuals captured and on the damage caused to the associated macrofauna (Gaspar et al. 1999), and with the fishery of this species in the Gulf of Trieste (Italy) (Del Piero 1994). Some few studies have addressed physiologic aspects. Charles et al. (1999) analyzed the selective utilization of bacteria and microalgae by *C. chione*. Cano (1983) analyzed different indexes of condition over a year in Málaga.

Although *C. chione* is a target-species of local fisheries in the whole Mediterranean area, there are few studies on reproduction. Valli et al. (1983–1984) presented preliminary data on the reproduction of *C. chione* in the Gulf of Trieste (northern Adriatic) later completed in Valli et al. (1994). Nicotra and Zappata (1991) analyzed the ultrastructure of the mature sperm and spermatogenesis from an Italian population.

In southern Spain, *C. chione*, locally known as "concha fina," is a very popular shellfish with a considerable consumer market and an average of about 599 tons/year from 1985–1996, according to the data of regional authorities. Most of 90% of this amount was obtained and consumed in the littoral of Málaga. Moreover, there is an significant illegal market and a quantity of catches that could be of the same order of magnitude as the official catch, but is not taken into account by official statistics. The law in Andalucía (an autonomous region including eight southern provinces of Spain) rules that the fishing season for *C. chione* must be closed from 1 February to 30 September (order of Consejería de Agricultura y Pesca, November 12, 1984). However, the provincial delegations are authorized to change it within this interval; in the case of Málaga, the close of season has been removed since 1990. The latter decision has been justified by the absence of biologic studies on the reproductive cycle of the species in the area, together with the fact that Málaga is a tourist area, which implies greater demand for shellfish species during almost all year, particularly in summer.

The absence of previous studies on the reproductive cycle of *C. chione* in southern Spain together with the overexploitation of this resource because of the absence of close of season, led the regional authorities of fishery (Consejería de Agricultura y Pesca) to promote this research. This is part of a project on the reproductive cycles of the most important commercial bivalves of Andalucía supported by the Consejería de Agricultura y Pesca (Department of Fishery) and entrusted to D.A.P. enterprise (Tirado & de la Rúa 2000).

MATERIALS AND METHODS

A total of 3,882 specimens of *C. chione* were examined and measured for shell length, ranging from 23–89 mm. The samples were collected from June 1999 to May 2000, with monthly frequency from October to February and with fortnightly frequency in the other months. The specimens were captured using a dredge with a toothed aperture, teeth length of 26 cm, and 6.7 cm of mesh, usual among the fishermen of the area. The samples were taken in Fuengirola (36°28′N, 4°43′W) (Fig. 1), at 20 m depth, in a sandy bottom.

To evaluate the possible influence of environmental factors on the cycle, the temperature of the seawater at 20 m depth was measured. Samples of water (2 L) were taken from the bottom for

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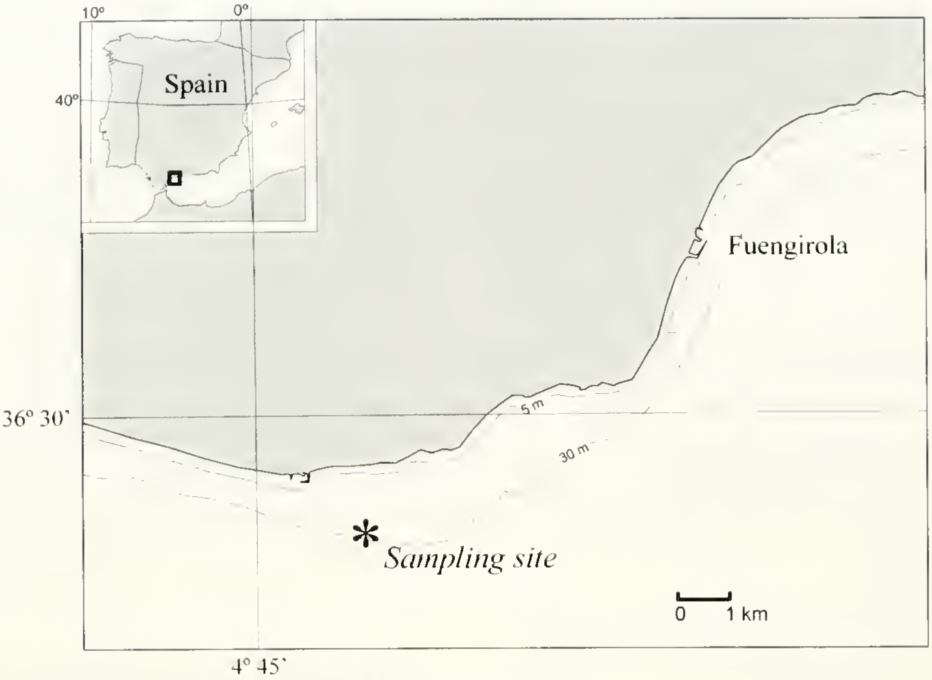

Figure 1. Sampling area.

determination of chlorophyll *a*. Pigment analyses were carried out by filtering the water through Whatman GF/C glass filters. The pigments of the retained cells were then extracted with acetone for twelve hours in cool, dark conditions, following the recommendations of Lorenzen and Jeffrey (1980). Concentrations of chlorophyll *a* were calculated using the trichromatic equations of Jeffrey and Humphrey (1975).

A total of 3,371 specimens were used for the analysis of flesh dry weight variation (about 200 specimens/sample). The length of every specimen was measured, and the soft parts were then pulled out of the shell, placed in the drying stove at 100°C for 24 h, and weighed to the nearest milligram. Two different indexes of condition were applied, the flesh dry weight/L^3 variation, and that proposed by Crosby and Gale (1990) Condition Index (CI) flesh dry weight × 1,000/volume of the internal cavity of the shell.

The regression of flesh dry weight on the length was calculated for each sample to estimate the variation in biomass of a standard individual, based on the logarithmic transformation of Ricker's function $W = aL^b$ (Ricker 1975), where W is the weight, L is the length, a is the ordinate at origin, and b is the slope.

The histologic study was performed on 511 specimens (usually 30 per sample), with shell length ranging between 23–85 mm. For the histologic processing, specimens were anesthetized with $MgCl_2$, fixed in 10% formaldehyde, embedded in paraffin, sectioned at 10 μm, and stained with hematoxylin of Carazzi and eosin, and a trichromic staining (V.O.F according to Gutiérrez 1967) of hematoxylin of Carazzi, light green, orange G, and acid fuchsine. The stages of gonad development were scored according to the scale proposed by De Villiers (1975) for *Donax serra* Röding 1798 in South Africa: cytolized, preactive, active, spawning, and postactive [the equivalent stages from Seed (1969) and Boyden (1971) are provided in Table 1].

The test of Kolmogorov–Smirnov and Kendal and Pearson's rank correlations included in the program SPSS 8.0, were used to check the distribution of the data. Cross correlation between both condition indexes and percentage of spawning with seawater temperature and chlorophyll *a* levels were calculated to assess the influence of the environmental factors on the reproductive cycle.

RESULTS

Sex Ratio

The sex of the specimens of *C. chione* cannot be distinguished macroscopically by the color of the gonads. Therefore, sex determination must be made microscopically. A total of 511 specimens were microscopically examined, but it was impossible to determine the sex of some individuals in several months. These samples were not considered for the sex ratio estimation. From the remain-

TABLE 1.

Different scales of gonad condition.

Authors	Scales of Gonad (G) Condition				
De Villiers (1975)	Cytolized	Preactive	Active Spawning	Postactive	
Seed (1969)	Resting G. (0)	Developing G (1,2,3)	Ripe G. (5)	Spawning G. (4,3)	Spawning G (2,1)
Boyden (1971)	Indeterminated G (I)	Developing G.	Ripe G (III)	Spawning (IV)	Resting G. (V)

G = gonad.

ing 272 individuals, 125 (45.96%) were males and 147 (54.04%) females. The sex ratio for all them can be considered as 1:1 (χ^2 = 0.012, $P > 0.95$) (Fig. 2).

Sexual Cycle

Biomass Analysis

The variation of flesh dry weight/L^3 ratio during the annual cycle is shown in Figure 3. The mean values of both variables, flesh dry weight and size (L^3), were considered. The standard deviations were between 10.59–48.32%. A broad size range (Fig. 4) can be observed in the samples, which explains, in part, these differences. Also, a broad weight range can be observed in most of the samples (Fig. 5), which is related, in part, with the presence of different stages of development of the gonads.

From June to October, *C. chione* shows two decreases of the above ratio (Fig. 3), with a small increase in August. During the autumn months, there is another increase, followed by a decrease from January to March. During the spring, there is a continuous slight increase, before the first spring decrease.

The other index (CI) shows less pronounced increases and decreases (Fig. 6). Two decreases can be detected, one of them from late August to December and the other one from January to March.

To minimize the bias introduced by the somatic growth of individuals during the cycle and by the variation in the size of the specimens between successive samples, the variation of flesh dry weight was estimated for a standard individual of 54-mm length, taking into account the regression lines for every sample (Table 2). This size is close to the mean size of the population.

Figure 7 shows a major decrease in the flesh dry weight from January to March, although there are also drops from June to September. It is interesting to observe the difference in flesh dry weight between the first half of June 1999 and second half of May 2000, which could indicate that the sexual cycles are out of phase between contiguous years. In autumn, the values remain more or less stable. The two major increments of the flesh dry weight of the standard individual were registered between March and April and between the two samples of June.

Gametogenic Cycle

Data from the histologic study are presented in Table 3, which shows the total number of specimens analyzed and their development stages, according to the scale of De Villiers (1975). The studied population of *C. chione* shows continuous spawning throughout the year, with values higher than 30% of the population in 12 of the 17 samples examined. The whole population was spawning in May, while the lowest percentage of spawning (30% of the sample) was registered in the first half of September (Fig. 8).

If we considered the absence of any individual in cytolized or postactive stage, the main period of sexual activity lasts from February to June. In February, the presence of active individuals points to the beginning of the active period (Table 3). There is not, however, a true resting period because of the existence of spawning in the population at any time of the year. The regression of the gonads begins in June and July, with the occurrence of individuals in postactive stage, which were predominant from the second half of September to December, together with the presence of some individuals in cytolized stage.

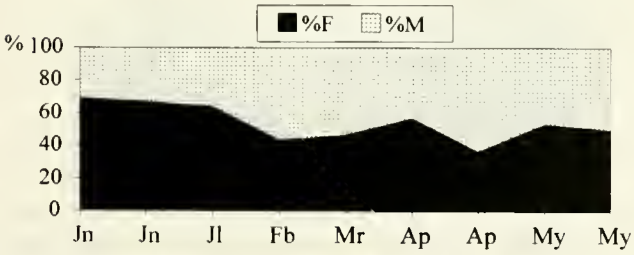

Figure 2. Relative frequency (%) of sexes during the year of study.

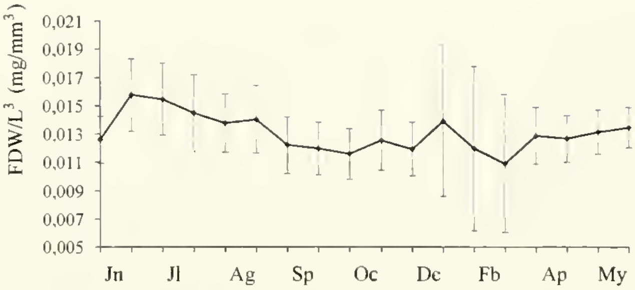

Figure 3. Flesh dry weight (FDW)/Length (L^3) ratio throughout the year of study. Bars show standard deviation.

The gametogenic cycle is asynchrononic in the population, which is evidenced by the presence of at least two developmental stages in nearly all the samples. An asynchrony is also detected in the individuals attributable to the coexistence of areas with different stages in the same gonad.

Several cohorts of ovocytes can be detected throughout the year, together with the direct step from postactive to active stage, without a previous cytolized and preactive phases.

Environmental Factors

The maximum temperature (Fig. 9) was registered in the second half of August (22°C) and the minimum (13.8°C) in January. The maximum of chlorophyll *a* (Fig. 9) levels occurs in the first half of August 2000 and the second peak in the first half of May 1999. Between these extremes, we observed several minor peaks, at the end of summer (first half of September), in autumn (October), and at the end of winter (March).

Coefficients of correlation of Pearson were estimated between temperature and FDW/L^3 and temperature and CI, because the variables showed a normal distribution (according to the test of Kolmogorov–Smirnov). However, between percentage of population in spawning and temperature, chlorophyll *a* levels, and the different condition indexes, the coefficients of correlation of Ken-

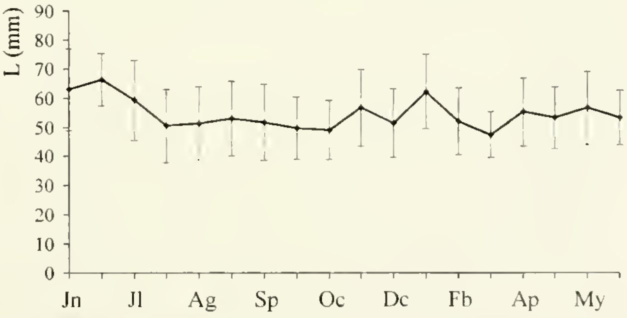

Figure 4. Monthly average length (L) throughout the year of study. Bars show standard deviation.

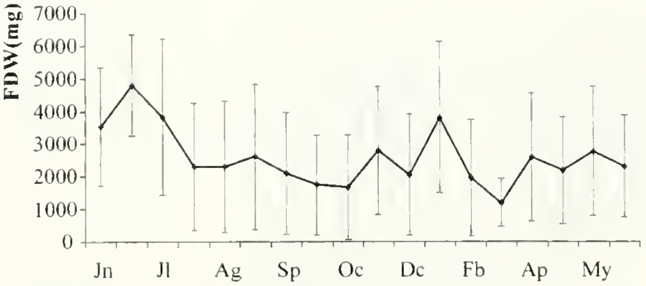

Figure 5. Monthly average flesh dry weight (FDW) throughout the year of study. Bars show standard deviation.

dal were estimated, because the percentage of spawning dataset showed a non-normal distribution.

These coefficients of correlation have been calculated simultaneously and with one and two months of delay. The temperature was directly correlated, with one month of delay, with CI ($r = 0.629$, $P < 0.05$). In the same way, the temperature was also directly correlated, with a delay of two months, with the FDW/L^3 of the population ($r = 0.573$, $P < 0.05$). A significant coefficient of Kendal's correlation has been obtained between the temperature and the flesh dry weight of the standard individual, with a month of delay ($\tau = 0.394$, $P < 0.05$). No other correlation was significant.

DISCUSSION

Sex Ratio

Callista chione is a species that does not show sexual dimorphism, and the sex ratio is 1:1. Our data regarding the sex ratio are similar to those of Valli et al. (1994) in the Gulf of Trieste, who obtained percentages of 46.25% males and 53.75% females. Also, we have not found any hermaphrodites in the studied population.

Reproductive Cycle

According to the histologic data, *C. chione* from the littoral of Málaga had individuals in spawning stage throughout the year (Fig. 8), while in the Gulf of Trieste (northern Adriactic) the spawning extends from February to September (Valli et al. 1983–1984), or from March to September (Valli et al. 1994). This difference is easily explained by the much lower winter seawater temperatures (in the order of 6°C) registered in the Northern Adriatic. However, the high amount of phytoplankton and zooplankton of the Gulf of Trieste resulted in the absence of a resting period, because the individuals remain in a preactive stage during autumn and winter months (Valli et al. 1994). In Málaga, most of the individuals remained in postactive and spawning stages during the autumn and winter months (Fig. 8). Cano (1983) studied different condition index in *C. chione* from the littoral of Málaga, detecting only one strong decrease of dry weight (between January and February of 1981). Other species from southern Spain, such as *D. trunculus*, *D. venustus*, and *D. semistriatus*, show very long spawning periods, but in these species, there was at least one month without spawning (Tirado & Salas 1998, Tirado & Salas 1999).

TABLE 2.

Linear regression calculated for each month.

Months	Lm	Regression Lines	R^2	R	*n*	W (L = 54 mm)
June	62.93	$y = 2.927 \times -1.774$	0.967	0.984	128	1983.59
June	66.20	$y = 3.017 \times -1.837$	0.908	0.953	94	2445.84
July	59.23	$y = 3.218 \times -2.204$	0.935	0.967	197	2346.59
July	50.40	$y = 3.205 \times -2.192$	0.958	0.979	200	2286.74
Aug	51.10	$y = 3.297 \times -2.371$	0.969	0.985	202	2194.50
Aug	52.94	$y = 3.294 \times -2.358$	0.966	0.983	200	2229.42
Sept	51.57	$y = 3.261 \times -2.363$	0.958	0.979	199	1934.74
Sept	49.49	$y = 3.345 \times -2.510$	0.945	0.972	201	1928.17
Oct	48.90	$y = 3.533 \times -2.837$	0.952	0.976	200	1917.97
Nov	56.56	$y = 3.423 \times -2.645$	0.974	0.987	152	1930.79
Dec	51.30	$y = 3.524 \times -2.820$	0.979	0.990	200	1928.28
Jan	62.00	$y = 3.246 \times -2.309$	0.900	0.949	203	2057.40
Feb	51.75	$y = 3.115 \times -2.150$	0.998	0.999	198	1760.88
Mar	47.30	$y = 2.265 \times -0.762$	0.511	0.715	200	1451.32
Apr	55.04	$y = 3.449 \times -2.675$	0.938	0.968	199	1997.13
Apr	53.06	$y = 3.168 \times -2.191$	0.919	0.959	200	1982.24
May	56.38	$y = 3.137 \times -2.124$	0.972	0.986	199	2045.41
May	53.30	$y = 3.335 \times -2.451$	0.972	0.986	200	2123.80

LM = average length; R^2 = coefficient of determination; R = coefficient of correlation; *n* = number of observations; W (L = 54 mm) = weight of a standard individual of 54-mm long.

The mild seawater temperature (between 13.8°C–23°C) and the high concentration of phytoplankton (because of the presence of upwellings) in the littoral of Málaga (Fig. 9) would favor, probably, a long reproductive cycle and the absence of a resting period. The temperature is the most influential factor, according to the coefficients of correlation. It is correlated with weight increments, a consequence of development of the gonads.

According to the hypotheses of most authors, the gamete release seems to be controlled by such physical environmental variables as changes in temperature, salinity, or photoperiod (Sastry 1979 and references therein). An alternative hypothesis is that phytoplankton induces spawning (Ruiz et al. 1992). Starr et al.

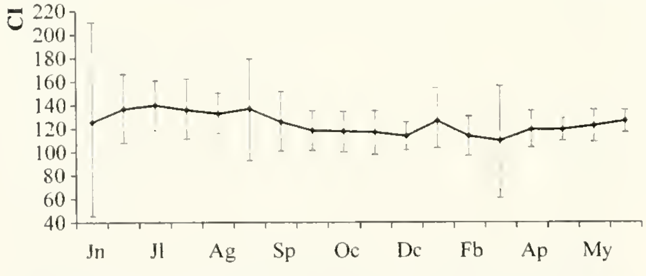

Figure 6. Index of condition of Crosby & Gale (CI): flesh dry weight × 1,000/ volume of the internal cavity of the shell, throughout the year of study.

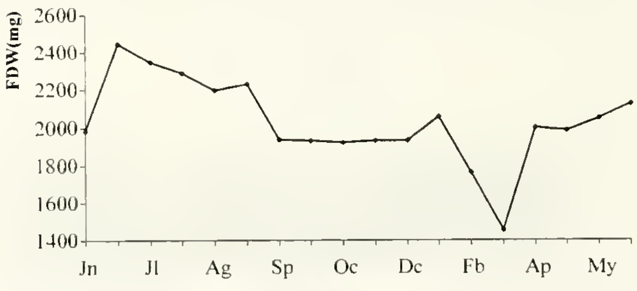

Figure 7. Variations in flesh dry weight in a standard *Callista chione* animal 54-mm long.

TABLE 3.

Developmental stages of the gonad over the year.

Months	C	Pr	EA	A	S	Ps	*n*
June		1			11	20	32
July 1					6	24	30
July 2	1	4	9	1	13	1	29
Aug 1	6	3	9	2	10		30
Aug 2	9	1			11	9	30
Sept 1	4		1		14	11	30
Sept 2	4				4	22	30
Oct	3				6	21	30
Nov	7				5	18	30
Dec	1				9	20	30
Jan		1			14	15	30
Feb			4	7	19		30
Mar		2	3	4	19	2	30
Apr 1				1	29		30
Apr 2				3	27		30
May 1					30		30
May 2	1				29		30

C = cytolized; Pr = preactive; EA = early active; A = active; S = spawning; Ps = postactive; *n* = number of observations.

(1990) showed that blooms of phytoplankton should be sufficient to induce spawning in mussels as well as in urchins. The spawning of *Crassostrea gigas* in El Grove (Galicia, northwestern Spain), with water temperature below 18°C, was correlated with phytoplankton bloom (Ruiz et al. 1992).

Although the chlorophyll *a* levels in the littoral of Málaga during this study did not show significant correlation with any analyzed index or variable, the coincidence of some decreases in weight (Figs. 3, 6) with peaks of chlorophyll *a* (Fig. 9), seem to indicate some influence.

The data of biomass showed three important decreases: between January–March, second half of August–September, and June and July (Figs. 3, 7). The latter was not reflected in CI (Fig. 6). The first drop is coincident with the start of activation of the gonad and with the increase of percentage of population in spawning (Fig. 8). At the first half of summer, there are high percentages of individuals in postactive stage, which begin a new gonadal activation in the second half of July. This direct step from postactive to active stages without a previous cytolized phase, has been found in *Donax trunculus* (L. 1758) from the littoral of Málaga (Tirado & Salas 1998) and seem to be related with the presence of peaks of chlorophyll *a* in this area (Fig. 9). Ansell (1961) reports that in the population of *Chamelea striatula* from Kame Bay (Millport), the ovary passes directly from the spawning condition to an early stage of development, because of the development of young ovocytes in the ovary before the end of spawning.

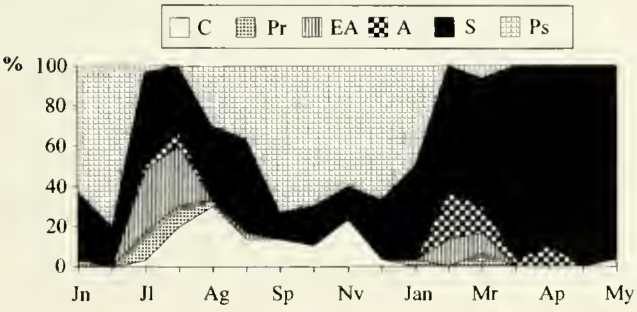

Figure 8. Relative frequency of different stages of development of the gonads in *C. chione*.

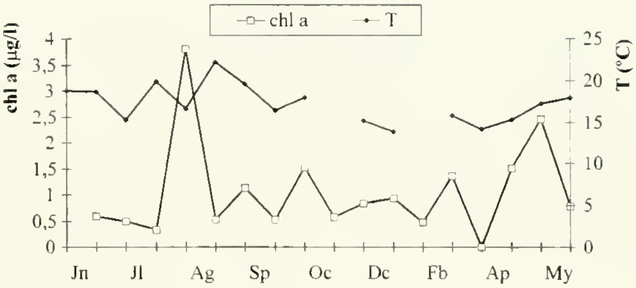

Figure 9. Seawater temperatures and changes in concentration of chlorophyll *a* in seawater throughout the year of study.

Also, according to the data for the standard individual (Fig. 7), the main decrease of flesh dry weight was registered in winter; whereas, those of summer seem to be less intense. Although the percentage of the population spawning in spring was the highest of the year (Fig. 8), the resulting decrease of biomass was less than in winter or summer (Fig. 3). This is consequence of the fact that the emissions from winter were more intense, with implication of the entire gonad; whereas, in spring and summer, there were only partial spawnings. The latter, together with the coexistence of different stages of development in the same gonad, could indicate that the spring/summer spawning is at least the second one for an individual during the cycle. In the field, it is difficult to know if a particular individual has more than two spawnings by reproductive cycle. In the laboratory, the venerid *Chamelea striatula* spawns repeatedly at intervals throughout the spawning season (Ansell 1961). In the littoral of Málaga, two spawning periods per individual were detected in *D. trunculus*; whereas, in other such species as *Donax venustus* and *Donax semistriatus*, only one spawning per individual and cycle was observed (Tirado & Salas 1998, Tirado & Salas 1999).

The asynchronic gametogenic cycle in the population is reflected by the high standard deviations of the flesh dry weights (Fig. 5), the existence of several cohorts of ovocytes, and the coexistence of several stages of development in the same gonad. The coexistence of different stages has been found in many bivalves from temperate areas, among them, *Chamelea striatula* (Ansell 1961), *Donax serra* (De Villiers 1975), *Tapes rhomboides* (Morvan & Ansell 1988), *D. trunculus* (Tirado & Salas 1998), *D. venustus* and *D. semistriatus* (Tirado & Salas 1999).

Although the total of captures of *C. chione* in the littoral of Andalucía is high (about an average of 599 tons/year from 1985–1996), in the last few years (from which statistical data have been published) this volume has decreased to values of about 188 tons in 1995, or about 259 tons in 1996. On the other hand, the average length of the Málaga population has decreased from about 70 mm in 1979–1981 (Cano 1981) to 54 mm in 2000 (present study). All these points indicate an overexploitation of this resource. Moreover, the recorded data on growth indicate a slow growth of *C. chione*. Hall et al. (1974) registered a growth of 2 mm by year in the Gulf of Trieste; Forster (1981) suggested that a specimen of Plymouth reaches 9 cm in 40 y. The above data make it an urgent necessity to provide a close of season that, according to the data of this study, must be February–March period during which there was the most intense release of gametes in the studied population.

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INGESTION, DIGESTION, AND ASSIMILATION OF GELATIN–ACACIA MICROCAPSULES INCORPORATING DEUTERIUM-LABELED ARACHIDONIC ACID BY LARVAE OF THE CLAM *VENERUPIS PULLASTRA*

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ABSTRACT Olive oil gelatin-acacia microcapsules (GAMs) enriched with deuterium-labeled arachidonic acid (*AA) were prepared and fed to *Venerupis pullastra* larvae. In a first experiment, larvae were either starved or fed these microcapsules (*AA-GAM) for 12 h. The *AA-GAM–fed larvae incorporated *AA and oleic acid (OA) in their neutral and polar lipids. The incorporation yield was around 5% for *AA in neutral and polar lipids and 2% and 1% for OA in neutral and polar lipids, respectively. In a second experiment, larvae were fed for 7 days with a mixed algal diet supplemented with or without *AA-GAM. The microcapsules were ingested and digested without any harmful effect on larvae. OA was incorporated preferentially in neutral lipids whereas *AA was equally distributed in neutral and polar lipids. The incorporation yield was higher in the second experiment probably in relation to the presence of algae. However, the incorporation rate of both tracer fatty acids decreased with time in both lipid classes. *AA dropped form 16–2% in both neutral and polar lipids and OA decreased more rapidly in polar (16–3%) than in neutral lipids (33–15%). These changes in the incorporation yield could correspond to the growth slowing down at the approach of the metamorphosis or to a saturation in the levels of fatty acids because the percentage of natural arachidonic acid stayed stable in neutral lipids or tended to decrease in polar lipids. The easy fabrication and use of GAM associated to the direct measurement of a deuterated fatty acid by gas chromatography is a promising tool for studying lipid metabolism in mollusks.

KEY WORDS: clam, *Venerupis pullastra*, nutrition, microcapsules, larva, fatty acids

INTRODUCTION

The production of living microalgae (LMA) as food for the larvae and spat of bivalves in commercial hatcheries accounts for approximately 30% of operating costs (Coutteau & Sorgeloos 1992). Because of the high cost and unpredictability of the algal culture, the development of artificial diets for bivalve mollusks was attempted by several investigators (Jones et al. 1974). Microcapsules of the cross-linked protein-walled type were used to examine aspects of protein (Langdon & Siegfried 1984) and carbohydrate (Kreeger et al. 1996) metabolism of bivalves and gelatin-acacia microcapsules were investigated in lipid nutrition (Langdon & Waldock 1981, Chu et al. 1987, Numaguchi & Nell 1991, Knauer & Southgate 1997a, 1997b). Some potential alternatives, such as dried microalgae, microalgal pastes, lipid emulsions, and microcapsulated or yeast-based artificial diets (for revision, see Coutteau & Sorgeloos 1993; Robert & Trintignac 1997), have shown promising results. The total or partial substitution of microalgae by artificial particles has been proposed, where, ideally, nutritional requirements of bivalves should be satisfied using diets whose composition could be precisely controlled. The gelatin-acacia microcapsules (GAMs) are not a suitable vehicle to deliver complete artificial diets to the larvae of bivalves because only water-insoluble nutrients can be encapsulated using this method. However, they may be a useful tool in studies addressing aspects of the lipid nutrition in addition to being a supplement for mixed microalgal diets poor in some essential fatty acids. GAM could be a useful supplement if sufficient quantities of LMA were unavailable to feed spat (Numaguchi & Nell 1991). GAMs are simple to produce, relatively easy to use, and can be prepared rapidly on demand. However, the potential of GAM as a substitute for LMA under large-scale culture conditions must still be assessed (Knauer & Southgate 1997a). A number of studies have been undertaken to prove GAM ingestion, digestion, and assimilation and have shown that GAM were readily digested by bivalves (Chu et al. 1982, Southgate 1988) and that the lipids supplied were assimilated with high efficiency (Knauer & Southgate 1997c). The data generated in such studies will facilitate the further development of suitable artificial diets for the larvae of marine bivalves. The knowledge of the nutritional requirements of the larvae of cultured bivalves is necessary to improve the efficiency of algal diets used in hatcheries and to design convenient artificial diets. Previous studies pointed out the energetic role of lipids during the larval development of mollusks (Holland 1978), as well as the changes in the composition of the polyunsaturated fatty acids (PUFAs) occurring in neutral and polar lipids during embryogenesis. This nutritional transition suggests that a metabolic control takes place in developing larvae (Marty et al. 1992). It is probable that this control results from a selective incorporation of dietary fatty acids by acyltransferases, indicating a preferential incorporation of long-chain PUFA, especially the 22:6(n-3) in the case of the larval development of *Pecten maximus* (Marty et al. 1992). This study was proposed as an attempt to confirm the assimilation of the fatty acids incorporated in GAM and their bioconversion in endogenous fatty acids of neutral and polar lipids in the larvae of marine bivalves. The data generated could indicate whether GAM could serve as a tool in studies of lipid metabolism in marine bivalves as well as demonstrate their use as a nutritional supplement in mixed microalgal–microcapsulated diets.

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MATERIALS AND METHODS

Chemicals

Lipid Standards and Reagents

Deuterium-labeled arachidonic acid (FA-503 arachidonic acid-5, 6, 8, 9, 11, 12, 14, 15-d_8) was obtained from BIOMOL Research Laboratory, Inc. (Plymouth Meeting, PA). Identification and quantification were based on standard fatty acid methyl ester mixture (Sigma-Aldrich, Chemical Spain). Internal standard, Tricosanoic acid (C23:0), was purchased from Nu-Chek-Prep. Butylated hydroxytoluene (BHT) and fluorescein isothiocyanate (FITC) were supplied by Sigma Chemical Co. (St. Louis, MO) and sodium hydrogen bicarbonate was from Merck (Darmstadt, Germany).

Solvents

Hexane, chloroform, and methanol were high-performance liquid chromatography (HPLC) grade from Merck (Darmstadt, Germany). Boron trifluoride (10%, w/w) in methanol (BF_3) was obtained from Supelco (Bellefonte, PA).

Fabrication of GAMs

Two different sets of GAM were fabricated following a modified method described by Rodriguez et al. (1992).

FITC-GAM

GAMs were prepared with 500 μL of cod liver oil and by later addition of FITC. Briefly, 500 μL of cod liver oil was homogenized with antioxidant BHT (5%, w/v). Then, 500 mL of cod liver oil plus antioxidant was emulsified with a 1:1 mixture of a 2% (w/v) solution of gelatin and a 2% (w/v) solution of acacia, which had been made up separately in distilled water. The emulsion was stirred in a Virtis Tempest IQ^2 blade homogenator programmed at 30,000 rpm for 2 min The pH of the mixture was reduced until the coacervation pH (4.3), by the drop-by-drop addition of dilute HCl. The mixture was stirred for 40 min and pH was then raised to 9.3 by the addition of dilute NaOH. The resulting GAM suspension was poured into 500 mL of cold distilled water and kept in a refrigerator for 2 h. Previously a solution of FITC 0.25% (w/v) in 100 mM sodium hydrogen bicarbonate ($NaHCO_3$) was prepared. Two milliliters of stock microcapsules were incubated at 4°C for 12 h with 0.5 mL of FITC solution diluted with 2.5 mL of 100 mM $NaHCO_3$ solution. The mean diameter of FITC-GAM was 4.0 ± 1.0 μm (n = 100). Two milliliters of FITC-GAM was used to feed a batch of 23-day-old *Venerupis pullastra* larvae and subsequently were observed under fluorescent microscopy.

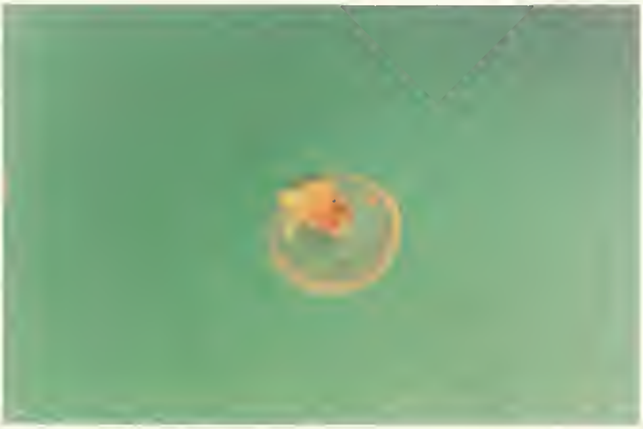

Figure 1. Larva of *V. pullastra* observed under fluorescence microscopy. Accumulation of yellow fluorescence indicates ingestion of microcapsules. (Author: J.-L. Rodríguez)

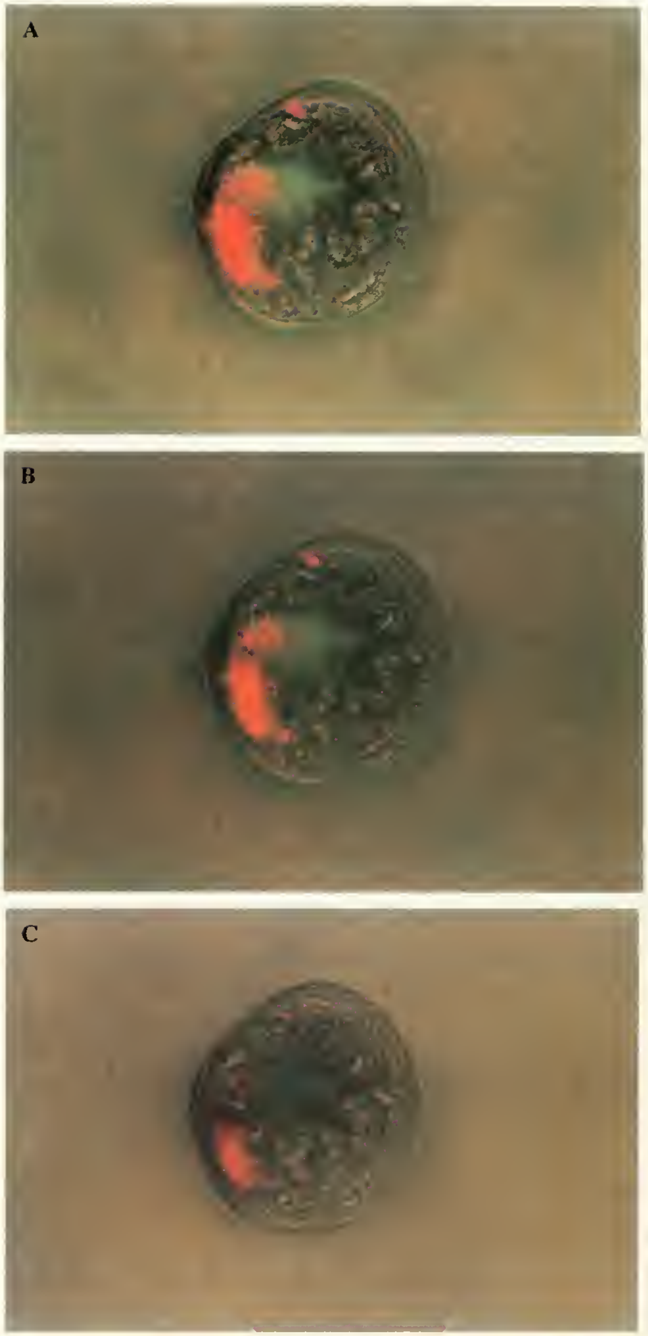

Figure 2. Digestion of microcapsules throughout a period of 20 min. Sequence A–C shows the disappearance of the yellow fluorescence inside a larva of *V. pullastra*. (Author: J.-L. Rodríguez)

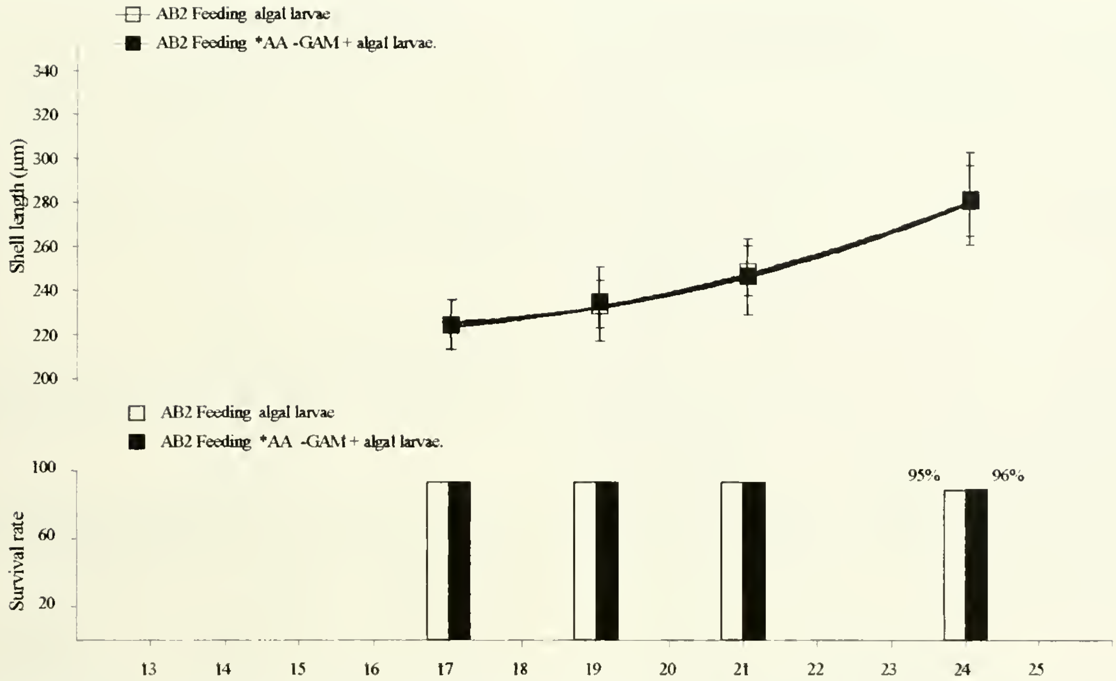

Figure 3. Shell length (µm) of the AB2 batch, fed with algal (open squares) and *AA-GAM + algal (solid squares). Survival rate (%) of the same batch fed with algal (open bars) and with *AA-GAM + algal (solid bars). Results are expressed in µm (%); Mean ±, SD, n = 100.

Deuterated Arachidonic Acid (*AA)-GAM

GAMs were prepared with olive oil supplemented with deuterium-labeled arachidonic acid. Briefly, 50 µL of olive oil was homogenized with 5 mg of deuterium-labeled arachidonic acid and antioxidant BHT (5%, w/v). Deuterated *AA-GAM was obtained following the method previously described. The resulting GAM suspension was poured into 250 mL of cold distilled water and held in a refrigerator for 2 h. The mean diameter of *AA-GAM was 3.5 ± 0.9 µm (n = 100) and the stock suspension of GAM was kept at 4°C and shaken daily.

Feeding Experiments

A population of *V. pullastra* D-larvae termed AB2 was selected by its good growth and survival rates and distributed in 500-L tanks at a density of 5 larvae·mL^{-1}. Larvae were fed daily with a microalgal mixture of *Pavlova lutheri* (Droop), *Isochrysis aff. galbana* Green (clone T-iso; Tahiti *Isochrysis*), *Skeletonema costatum* (Greville), and *Chaetoceros calcitrans* (Tanako) at a density of 15/15/15/15 cells·µL^{-1}.

Experiment 1

Larvae 14 days of age from the AB2 culture were collected and transferred at the same density into two 6-L flasks for a 24-h experiment. One batch was starved whereas the other was fed with 60 µcap·µl^{-1} *AA-GAM. *AA-GAM–fed larvae had seawater renewed 12 h after feeding, and no food was supplied then. Twelve hours later, both fed and starved larvae were collected on a 45-µm mesh.

Experiment 2

Larvae 17 days of age from the same AB2 culture were distributed in two 150-L tanks at a density of 4 larvae·mL^{-1} and fed daily with two different diets for 7 days, either a microalgal diet of *P. lutheri*, *I. aff. galbana*, *S. costatum*, *C. calcitrans*, and *Tetraselmis suecica* (Butcher) at a density of 20/20/15/15/10 cells·µL^{-1} or a diet consisting of the same microalgal mixture supplemented with 5 µcap·µL^{-1} *AA-GAM.

Samples for Fatty Acid Analyses

Larvae from the 24-h experience were collected as indicated above. Larvae from the 7-day experience that were 18, 19, 21, and 24 days of age were collected 4 h after feeding on a 45-µm mesh (n = 1).

All larval samples were crushed and stored in a $CHCl_3$–MeOH mixture (2:1, v/v) under nitrogen at −30°C until fatty acid analysis could be performed. Samples of microalgal mixture (n = 3) and microcapsules (n = 3) were filtered on GF/F (Whatman) glass fiber filters and stored similar to larvae before analysis.

Fatty Acid Analysis

The separation of the polar and neutral lipids was performed by micro-column liquid chromatography as described by Marty et al. (1992). Total lipids were evaporated to dryness and dissolved three times using 500 µL of chloroform/methanol (98:2). Neutral and polar lipids were separated on a silica gel 6% (w/w) hydrated microcolumn (30 × 5 mm) using chloroform/methanol (98:2) and methanol successively as eluting solvents. The fractions were collected under nitrogen in screw-capped flasks containing a known amount of 23:0 as internal standard for quantitative determinations. Fatty acid composition and quantification of polar and neutral lipids were determined using gas chromatography (CG), after purification of fatty acid methyl esters (FAMEs) by HPLC. FAMEs from the neutral and polar lipid fractions were transesterified with 10% (w/w) BF_3 in methanol (Metcalfe and Schmitz 1961) for 10 min at 100°C. After cooling, FAMEs were extracted with hexane. The organic phase was evaporated under nitrogen and dissolved in chloroform/methanol (98:2) for purification by HPLC (Hennion et al. 1983, modified). FAMEs present in each lipid fraction were injected in a CG System HP6890 series equipped with a split/splitless injector, a flame ionization detector, and a DBWAX capillary column (30 m × 0.25 mm ID; 0.2-μm film thickness). The carrier gas was H_2, at an initial pressure of 80 kPa. The oven was programmed to stay at the initial temperature of 60°C for 2 min, rise from 60 to 160°C at a rate of 50°C/min, stay for 2 min and from 160 to 170°C at 1.5°C/min, then 170–185°C at 2°C/min, next 185–240°C at 3°C/min, and finally remain at 240°C for 10 min. Injector and detector temperatures were 230°C and 250°C, respectively. The flow rates of compressed air, hydrogen and make up gas (nitrogen) were 300, 30, and 20 $mL \cdot min^{-1}$, respectively. Fatty acids were identified by comparison of their retention times with those of standards. The C:X (n-Y) notation was adopted, where C was the number of carbons, X the number of double bonds, and n-Y the position of the first double bond from the terminal methyl group.

TABLE 1.

Fatty acid composition of the deuterium-labeled microcapsules (*AA-GAM).

	*AA-GAM (%)		*AA-GAM (ng/larvae)	
Fatty Acid	**Mean**	**SD**	**Mean**	**SD**
14:0	0.061	0.003	0.028	0.002
16:0	10.830	0.066	4.920	0.583
18:0	2.606	0.075	1.182	0.118
20:0	0.390	0.024	0.176	0.011
22:0	0.131	0.007	0.059	0.004
24:0	0.073	0.027	0.032	0.009
16:1 (n-9)	0.171	0.018	0.077	0.002
16:1 (n-7)	0.872	0.036	0.395	0.033
18:1 (n-9)	**65.691**	**0.310**	**29.863**	**3.804**
18:1 (n-7)	2.481	0.493	1.145	0.371
20:1 (n-9)	0.326	0.019	0.147	0.010
18:2 (n-6)	10.056	0.333	4.564	0.497
18:2 (n-4)	0.000	0.000	0.000	0.000
18:3 (n-6)	0.000	0.000	0.000	0.000
18:3 (n-3)	0.749	0.029	0.339	0.028
18:4 (n-3)	0.000	0.000	0.000	0.000
20:2 (n-6)	0.000	0.000	0.000	0.000
20:3 (n-6)	0.000	0.000	0.000	0.000
20:4 (n-6)-d_8	**5.363**	**0.238**	**2.428**	**0.192**
20:4 (n-6)	0.000	0.000	0.000	0.000
20:4 (n-3)	0.000	0.000	0.000	0.000
20:5 (n-3)	0.000	0.000	0.000	0.000
22:4 (n-6)	0.000	0.000	0.000	0.000
22:5 (n-6)	0.000	0.000	0.000	0.000
22:5 (n-3)	0.000	0.000	0.000	0.000
22:6 (n-3)	0.076	0.012	0.034	0.003
TO.SAT.	14.215	0.208	6.453	0.711
TO.MONO	69.541	0.728	31.627	4.210
TO.POLY	16.244	0.520	7.366	0.711
(n-3)/(n-6)	0.053	0.001	0.002	0.000
ng/larvae			45.446	5.613

Values in bold are, respectively, the major fatty acid present in olive oil [18:1 (n-9)] and the deuterium-labeled arachidonic acid added [20:4 (n-6)-d8]. Results are expressed in percentages (%) and in ng supplied to each larva (ng/larva) in experiment 1. Values are means ± SD (n = 3).

Incorporation yield was calculated using the following equation:

$$\text{incorporation } \%: \frac{(\text{AA–GAM}_n\text{–algae}_n)\text{ng FA} \times \text{larvae}^{-1}}{(\text{AA–GAM–algae})\text{ ng dietary FA} \times \text{larvae}^{-1}} \times 100$$

where the numerator is equal to fatty acid difference between larvae fed the microcapsules + algae and larvae starved or fed the algae and the denominator is the fatty acid supplied by the microcapsules.

RESULTS

Ingestion and Digestion of Microcapsules

After feeding on FITC-GAM for 4 h, larvae were observed under fluorescent microscopy. The ingestion of FITC-GAM was

TABLE 2.

Fatty acid composition of the diets supplied in experiment 2: algal diet (mixted microalgae only) and *AA-GAM + algal diet (mixted microalgae supplemented with microcapsules).

	Algal Diet (ng/larvae)		*AA-GAM + Algal Diet (ng/larvae)	
Fatty Acids	**Mean**	**SD**	**Mean**	**SD**
14:0	15.220	1.520	15.223	1.520
16:0	14.105	1.670	14.590	1.721
18:0	0.694	0.290	0.811	0.300
20:0	0.189	0.245	0.207	0.244
22:0	0.109	0.014	0.115	0.014
24:0	0.122	0.046	0.125	0.047
			0.000	
16:1 (n-9)	0.181	0.029	0.188	0.030
16:1 (n-7)	19.401	1.695	19.440	1.698
18:1 (n-9)	**7.766**	**0.648**	**10.710**	**0.997**
18:1 (n-7)	1.289	0.142	1.401	0.175
20:1 (n-9)	0.083	0.012	0.097	0.012
			0.000	
16:2 (n-7)	2.356	0.236	2.356	0.236
16:2 (n-4)	0.692	0.045	0.692	0.045
16:3 (n-6)	0.000	0.000	0.000	0.000
16:3 (n-4)	0.964	0.137	0.964	0.137
18:2 (n-6)	4.876	0.308	5.326	0.345
18:2 (n-4)	0.216	0.029	0.216	0.029
18:3 (n-6)	0.623	0.040	0.623	0.040
18:3 (n-3)	5.152	0.354	5.185	0.357
18:4 (n-3)	6.767	0.575	6.767	0.575
18:5 (n-3)	1.663	0.146	1.663	0.146
20:2 (n-6)	0.048	0.027	0.048	0.027
20:3 (n-6)	0.074	0.007	0.074	0.007
20:4 (n-6)-d_8	**0.000**	**0.000**	**0.239**	**0.019**
20:4 (n-6)	1.407	0.095	1.407	0.095
20:4 (n-3)	0.052	0.007	0.052	0.007
20:5 (n-3)	4.015	0.155	4.015	0.155
22:4 (n-6)	0.043	0.008	0.043	0.008
22:5 (n-6)	0.964	0.007	0.964	0.007
22:5 (n-3)	0.105	0.032	0.105	0.032
22:6 (n-3)	6.047	0.145	6.050	0.145
TO.SAT.	30.975	3.403	31.611	3.461
TO.MONO	29.582	2.470	32.701	2.846
TO.POLY	37.027	1.979	37.753	2.034
ng. Diet/larvae	98.304	8.358	102.785	8.845

Values in bold are, respectively, the major fatty acid present in olive oil [18:1 (n-9)] and the deuterium-labeled arachidonic acid added [20:4 (n-6)-d8]. Data are means ± SD (n = 3).

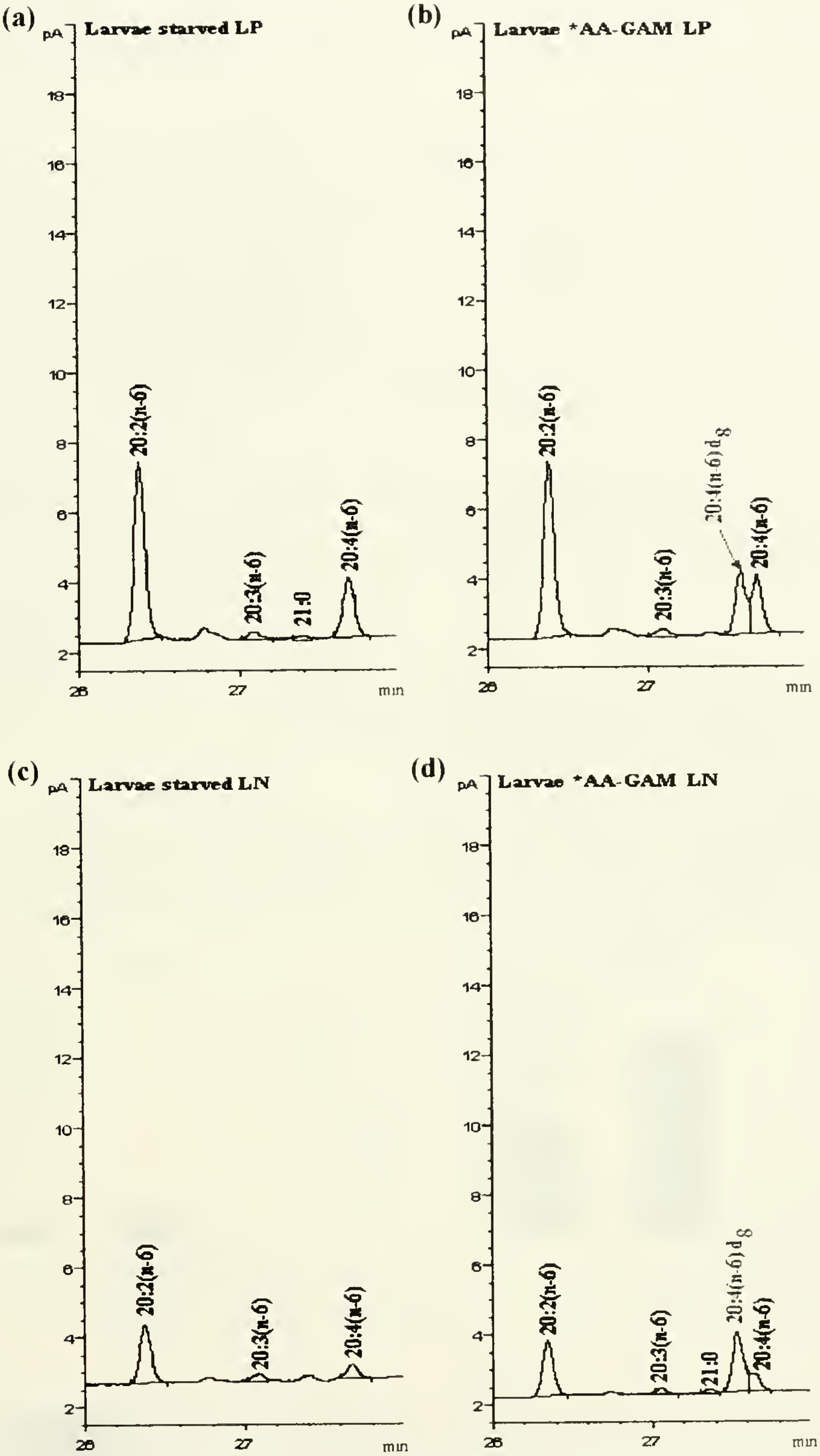

Figure 4. Partial chromatograms of FAMEs prepared from larvae of *V. pullastra* corresponding to retention time between 26 and 28 min. Figure shows the incorporation of 20:4(n-6)d_8 in polar (b) and neutral (d) lipids in larvae fed with microcapsules with respect to starved larvae (a) and (c).

proved by an accumulation of yellow fluorescence (the dye color) inside the larva (Fig. 1). A sequence of photographs taken throughout a period of 20 min showed the decrease of yellow fluorescence in the digestive gland of a larva fed with microcapsules, due to digestion (Fig. 2).

Growth and Survival

Throughout the 7-day experiment, larvae were sampled with the daily water renewal to determine growth (anterior-posterior shell length) and survival. Growth and survival were similar for the batches fed either microalgae supplemented with microcapsules or the microalgal diet (Fig. 3).

*Fatty Acid Composition of *AA-GAM*

The mean fatty acid compositions of *AA-GAM are listed in Table 1. The fatty acid profile of microcapsules fabricated with 50 μL of olive oil and 5 mg of deuterated arachidonic acid 20:4(n-6)-d_8 showed a predominance of 18:1(n-9) (65.7 ± 0.31%), 16:0 (10.8 ± 0.07%), 18:2(n-6) (10.1 ± 0.33%), and 20:4(n-6)-d_8 (5.4 ± 0.24%). A single microcapsule contained 3.8 pg total fatty acids.

Dietary Fatty Acid Supply

The diet consisting of *AA-GAM exclusively was used in the 24-h experiment and supplied 60 GAM·μL^{-1}, which corresponded to 29.86 ng·larva^{-1} for the 18:1(n-9) and 2.43 ng·larva^{-1} for 20:4(n-6)-d_8 (Table 1).

Table 2 shows the fatty acid composition of the two different diets (algal diet and *AA-GAM–supplemented diet) used in the 7-day experiment. The first supplied 7.77 ng·larva^{-1} of 18:1(n-9) and the second supplied 10.71 ng·larva^{-1} of 18:1(n-9) and 0.24 ng·larva^{-1} of 20:4(n-6)-d_8.

Assimilation of Microcapsules in the 24-h Experiment

The FID-CG analysis allowed the separation and the quantification of deuterated *AA in polar and neutral lipis of larvae (Fig. 4). Larvae fed *AA-GAM exclusively showed incorporation of 20:4(n-6)-d_8 in polar and neutral lipid fractions (0.10 and 0.12 ng·larva^{-1}, respectively). The amount of the major fatty acid in olive oil, 18:1(n-9), also increased in neutral and polar lipids when compared with that of starved larvae: 1.08 ng·larva^{-1} versus 0.33 in neutral lipids and 0.66 ng·larva^{-1} versus 0.28 in polar lipids (Fig. 5a). Incorporation yield percentages of 20:4(n-6)-d_8 were nearly equal in both lipid fractions (4.30% for polar lipids and 4.80% for neutral lipids), whereas for 18:1(n-9) they were 2.53% and 1.31% for neutral and polar lipids, respectively (Fig. 5b).

Fatty Acid Composition of Larvae Fed with Different Diets

Larvae fed with *AA-GAM–supplemented diet showed an increase in the incorporation of the two monitored fatty acids [20:4(n-6)d_8 and 18:1(n-9)] in both lipid fractions when compared with those fed the microalgal diet (Fig. 6).

With regard to 20:4(n-6)-d_8, incorporation profiles were similar in both lipid fractions throughout the 7-day experiment (16.3% for neutral lipids and 16.8% for polar lipids between days of culture 18 and 19). Assimilation percentages were higher during the early days of the culture and decreased in the late days from 16.3 to 5.6% in neutral lipids and from 16.7 to 6.1% in polar lipids (Fig. 7).

Incorporation of 18:1(n-9) was better in neutral than in polar lipids. Throughout the experience, the incorporation yield decreased in both fractions, although the phenomenon was more pronounced in polar lipids (Fig. 7).

The lipid content, the percentage of neutral and polar fractions and the fatty acid composition of larvae were similar in both 7-day experiments (Table 3). Supplementation with microcapsules did not affect the incorporation of the essential PUFA provided by microalgae. However, larvae fed microcapsules appeared to have a higher FAME content if compared with larvae fed microalgae only.

DISCUSSION

Numerous artificial particles have been tested to supplement or partially replace the living algal diet for mollusks. These included yeast (Epifanio 1979), flour (Albentosa et al. 1999), dried algae

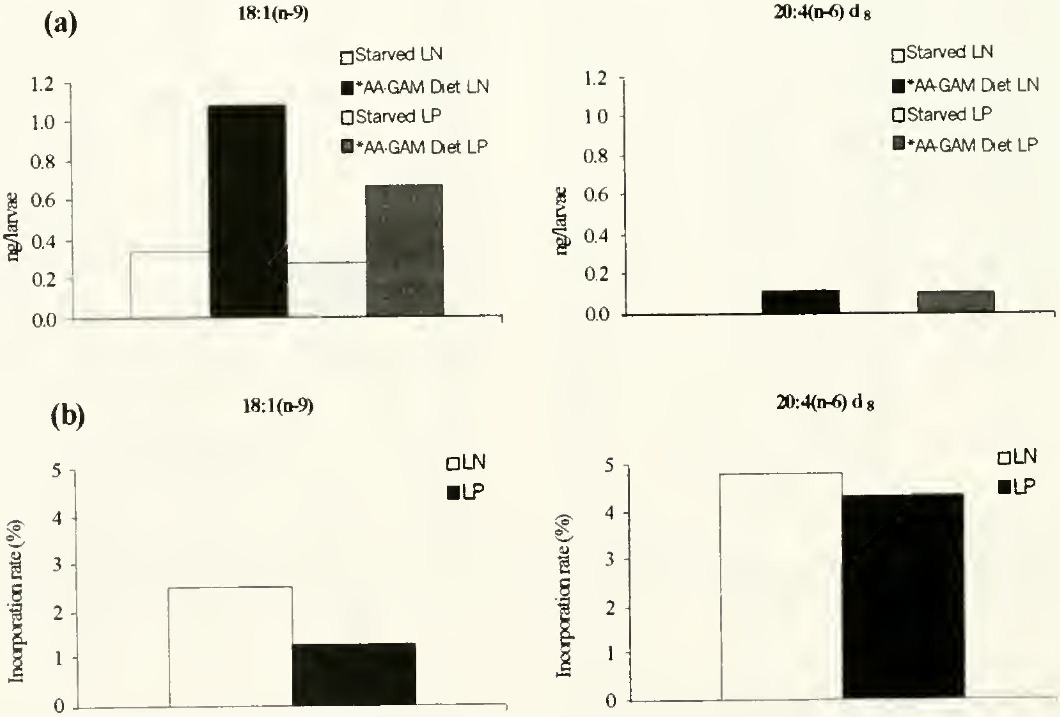

Figure 5. Bar diagrams showing the incorporation of both traced fatty acids in experiment 1. In (a), comparison of their contents as neutral and polar lipids in starved larvae and those fed *AA-GAM, expressed in ng/larva. In (b), incorporation rates as neutral and polar lipids in larvae fed *AA-GAM, expressed in percentages. In all cases, n = 1.

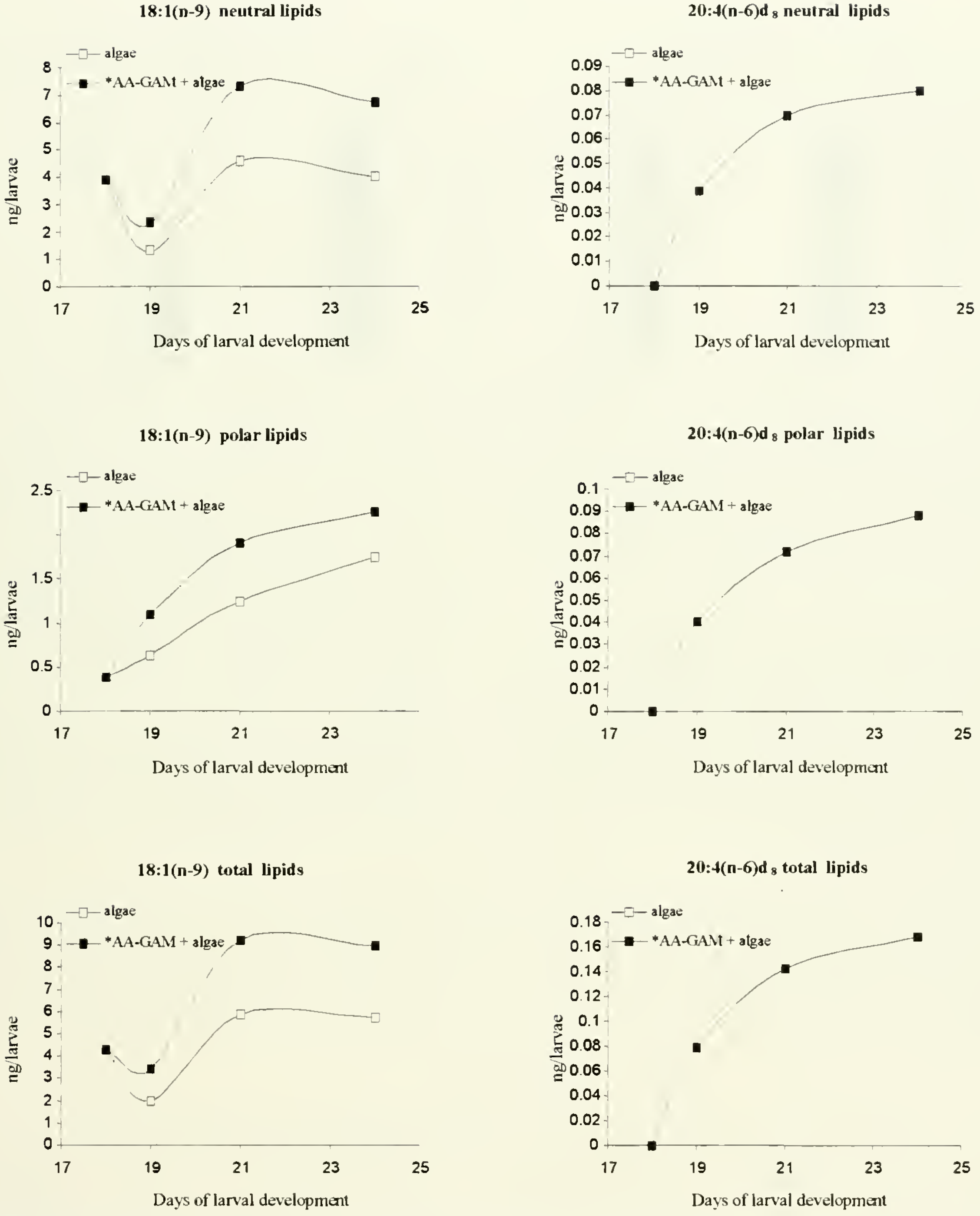

Figure 6. Dynamics of 18:1(n-9) and 20:4(n-6)d_8 in neutral, polar, and total lipids of larvae fed microalgae and *AA-GAM + microalgae during experiment 2.

(Laing & Millican 1991), emulsions, and liposomes (Coutteau et al. 1996, Caers et al. 1999, 2000, Soudant et al. 2000). Development of particles like gelatin-acacia spraybeads (Buchal & Langdon 1998) was mostly devoted to identifying essential molecules and to understanding their metabolism. In this article, we report data on the incorporation efficiency of the essential PUFA AA using GAM. Because mollusks contain AA in their corporal fatty acid, we incorporated deuterated AA (*AA), which can be easily discerned from the natural one by direct GC analysis, allowing a good estimation of its assimilation. This straightforward methodology avoids the use of radioactivity and its additional measurements. Indeed, it may be applied to other essential fatty acids. Although deuterated metabolites of AA were not detected in our study, their determination is possible with this methodology.

Small-scale GAM fabrication is easy and suitable for experimental studies on metabolism, in particular for lipids, which are easily encapsulated. Olive oil was chosen as lipidic support because its composition is very simple. Oleic acid (OA) represents 65% of the total fatty acid present in olive oil. Moreover, olive oil lacks most very long-chain PUFA that are essential for marine mollusks (Trider & Castell 1980, Enright et al. 1986, Soudant et al. 1996). Therefore, the fatty acids from this oil would not interfere

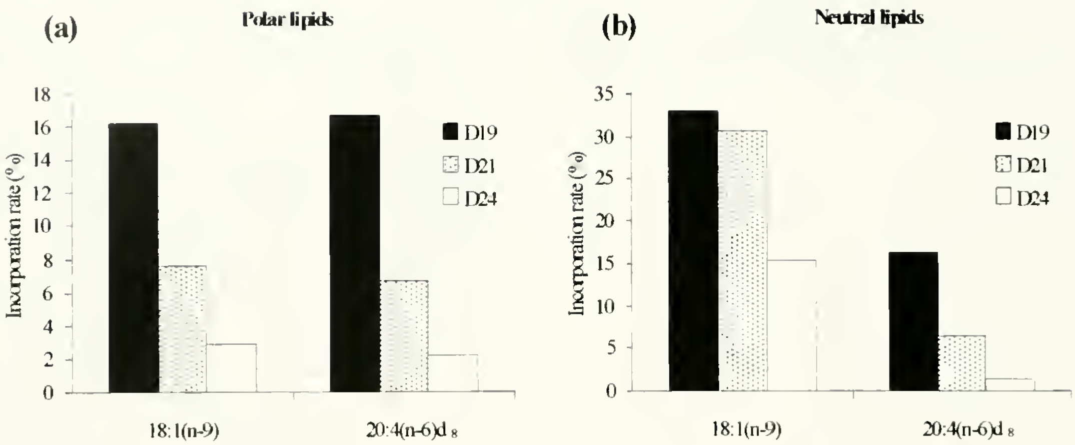

Figure 7. Incorporation rates of 18:1(n-9) and 20:4(n-6)d_8 in polar and neutral lipids of larvae fed with *AA-GAM + microalgae. (n = 1).

with larval development. The size of GAM obtained was compatible with mollusks' ingestion and they did not show harmful effect on larvae. On short-term observation (7 days), no deleterious effect was observed on growth. Growth and larval mortality were similar both in the presence and in the absence of GAM in our experiments.

The gradual disappearance of the fluorescent labeling observed in the digestive system of larvae (Fig. 2) was probably due to the digestion and assimilation of microcapsules by the larvae. In previous studies, Sudan red-stained GAM faded in the stomach and digestive diverticula of *Crassostrea virginica* larvae during the early 24 h after feeding, and disappeared completely within 48 h (Chu et al. 1982). Also, *Crassostrea gigas* spat were demonstrated to assimilate ^{14}C-labeled lipids incorporated in corn oil GAM (Knauer & Southgate 1997c).

Digestion and assimilation were eventually confirmed by tracing the deuterated arachidonic acid supplied by *AA-GAM. Results showed that *V. pullastra* larvae were able to incorporate this fatty acid into their endogenous lipids as both neutral and polar fatty acids. The results obtained in both 24-h and 7-day experiments established incorporation of 20:4(n-6)-d_8, which could only be detected in larvae if *AA-GAM had been assimilated. However, larvae fed with *AA-GAM also showed an increase of OA, the other tracer fatty acid of GAM, in both neutral and polar lipids when compared with those starved or fed algae.

Contrary to neutral lipids, which may correspond to transient storage of ingested food, polar lipids are mostly located in membranes. Thus, incorporation in polar lipids better indicates true assimilation in both experiments. These results confirmed ingestion, digestion and assimilation of the fatty acids furnished by GAM.

With regard to the second experiment, it is noteworthy that essential PUFAs supplied by algae were found to be accumulated at the same level in neutral and polar lipids of larvae regardless of the diet. This shows that the incorporation of PUFA provided by the algae was not modified by the microcapsules, probably meaning that the amount of algae filtered by the larvae did not decrease by the presence of GAM. During the time course of this experiment, the polar lipid content increased continuously, which resulted in a regular growth. The neutral lipid content showed a clear drop between day 18 and 19 and then tended to reach a maximum at day 21. This may suggest the existence of a high energetic demand at this point of the larval development.

Fatty acid incorporation rates were observed to vary depending

TABLE 3.

Dynamics of the essential PUFAs and the total FAMEs in polar and neutral lipids during experiment 2.

	18 Day[a]	19 Day		21 Day		24 Day	
	Algae	Algae	Algae + *AA-GAM	Algae	Algae + *AA-GAM	Algae	Algae + *AA-GAM
Polar lipids							
20:4 (n-6)	0.378	0.453	0.595	0.731	0.922	1.105	0.982
20:5 (n-3)	0.552	1.160	1.349	2.381	2.679	3.506	3.397
22:6 (n-3)	2.744	3.474	4.284	6.942	7.396	9.574	10.109
Total FAME (ng/larvae)	13.939	19.378	24.951	37.501	44.109	51.362	53.533
Neutral lipids							
20:4 (n-6)	0.316	0.298	0.304	0.963	1.095	1.054	1.119
20:5 (n-3)	3.875	1.182	1.124	4.136	4.341	4.792	5.302
22:6 (n-3)	2.437	1.324	1.418	4.232	4.403	4.163	5.073
Total FAME (ng/larvae)	53.935	18.541	21.209	62.576	71.264	56.544	69.745
Total lipids							
Total FAME (ng/larvae)	67.874	37.919	46.16	100.077	115.373	107.906	123.278
Percent neutral lipids	79.463	48.896	45.947	62.528	61.768	52.401	56.575

[a] Data are expressed as mean for three analysis.

on the fatty acid supplied, as well as on the stage of larval development. OA incorporation was mainly observed in neutral lipids whereas *AA was equally distributed in neutral and polar lipids. OA is a monounsaturated fatty acid, thought to play a mainly energetic role in the reserve lipid fraction. The equal distribution of *AA in polar and neutral lipids is more surprising since, like vertebrates, some marine bivalves (*Pecten maximus*) have shown a preferential incorporation of AA in polar lipids, especially in the phosphatidyl inositol class (Soudant et al. 1998). However in *V. pullastra*, the natural AA supplied by the food was found at a similar level in neutral and polar lipids of 24-day-old larvae. The preferential location of arachidonic acid in polar lipids may be species specific. Furthermore, it is known that the fatty acid composition of diet is largely reflected in neutral lipid composition and to a lesser extent in polar lipids of animals. This is because polar lipids are regulated according to the needs of structural maintenance of membranes. Fatty acids supplied by the food would be first incorporated in neutral lipids and then, depending on the needs, transferred to polar lipids. The percentage of AA in polar lipids of mollusks fed with algae tended to decrease in the course of the experiment, perhaps because those needs were satisfied by the algae or, maybe, the needs of the larvae just dropped naturally with development. This would also explain the weak incorporation of *AA in polar lipids in the case of larvae fed with *AA-GAM.

OA and *AA incorporation in both neutral and polar lipids was high during the early days of the experience but decreased toward the later days. These data coincided with the fact that the amount of neutral and polar lipids per larva leveled off beween days 21 and 24, corresponding to a decrease in growth rate and lower biosynthesis needs. Moreover, the decrease of the natural AA percentage in polar fatty acids and its stable percentage in neutral lipids may explain the decrease of incorporation of tracer fatty acids from GAM. It is during this period that larvae are ready to undergo metamorphosis and change into spat.

Comparing the values of incorporation of both fatty acids supplied by GAM between experiments, we observed that incorporation increased in the second experiment. This may be explained by the presence of microalgae in the second experiment, which facilitated the retention of artificial GAM either by favoring the filtration or by adhesion of GAM on algae. Similar results were obtained by Knauer and Southgate (1997b), who reported an efficiency increase from 57–80% when *Dunaliella* was added to GAM.

An appropriate dosage of lipid components incorporated in microcapsules could be useful as a nutritional supplement for lipid-poor microalgal diets. The possibility of using microcapsules containing labeled fatty acids also allows us to trace the molecules and to study their distribution and possible metabolic conversion to structural and reserve lipids. This would contribute to go deeply into both energetic and structural needs for fatty acids in molluskan larval development, and to design nutritionally optimal lipidic diets to obtain spat of good quality.

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ENHANCING HARD CLAM (*MERCENARIA* SPP.) POPULATION DENSITY IN THE INDIAN RIVER LAGOON, FLORIDA: A COMPARISON OF STRATEGIES TO MAINTAIN THE COMMERCIAL FISHERY

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ABSTRACT Hard clams of the genus *Mercenaria* support an important commercial fishery in the Indian River Lagoon on the east central coast of Florida. The fishery is relatively young but has proven to be quite sporadic, with two periods of exceptional landings (mid-1980s and mid-1990s) bounded by periods of almost complete fishery collapse. In response to a request from commercial fishery participants, three strategies for enhancing the abundance of harvestable hard clams in the lagoon were compared. The first strategy involved harvesting adult clams from a low-density population and transplanting them at high density in a concentrated area, in an effort to increase fertilization efficiency and thereby maximize reproductive success. That approach does not appear to be viable in the Indian River Lagoon because mortality of transplanted clams approached 100% and because Indian River hard clams display a remarkably high incidence (>80%) of gonadal neoplasia. Neoplasia reduces the life span of Indian River hard clams relative to their northern congeners and probably reduces fecundity of those clams that do survive. The second strategy involved seeding juvenile clams at very high densities (843–7165 m^{-2} depending upon seed size), again with the intent of maximizing fertilization efficiency but with the additional goal of maximizing residual reproductive value. Various planting treatments were tested in an effort to minimize mortality of seed clams, but losses were still high (generally >70%), and the yield did not appear to justify the cost. The final strategy involved spawning large numbers of hard clams in the laboratory, fertilizing the resultant eggs, and immediately releasing the larvae at a predetermined location in the lagoon. Large numbers of larvae did appear to survive the 8-day larval life span, but it remains to be seen whether those larvae will translate into harvestable hard clams. In any event, enhancement of hard clam populations appears to be feasible only when the density of naturally occurring clams is so low that reproductive viability is compromised. Otherwise, natural reproductive potential will swamp any artificial efforts at population enhancement.

KEY WORDS: *Mercenaria*, Indian River Lagoon, population enhancement, spawner transplant, seeding, larval release

INTRODUCTION

Hard clams of the genus *Mercenaria* support an economically and culturally important fishery in the Indian River Lagoon on the east central coast of Florida. This is a relatively new fishery that developed in the early 1980s (Fig. 1) and was originally centered near the town of Grant in southern Brevard County (Fig. 2). The clam population that supported the original fishery collapsed in the late 1980s, probably in response to decreased salinity caused by the release of St. Johns River floodwaters into the Indian River basin (Barile and Rathjen 1986). Another substantial set of hard clams was detected in the northern Indian River Lagoon, between Cocoa and Titusville, in the early 1990s. That population supported a profitable and productive fishery throughout the mid-1990s (Fig. 1), but the population again collapsed and again the proximate cause appeared to be decreased salinity resulting from above-average rainfall in the watershed. As of the year 2001, the fishery for naturally occurring hard clams in the Indian River Lagoon has remained depressed, although a small number of fishermen continue to pursue the few clams that are available.

The Indian River is a shallow, narrow, bar-built lagoonal system that stretches from Oak Hill to Stuart along the central Atlantic coast of Florida. For our study, we considered only that area of the Indian River Lagoon (IRL) system that is located north of Sebastian Inlet (Fig. 2) and that is composed of the Indian River (IR), the Banana River (BR), and Newfound Harbor. Prehistorically, the IRL was a single continuous basin, and the IR and BR were connected to the south at Dragon Point and to the north through Banana Creek. In recent years, development and bridge construction have resulted in the subdivision of the IRL into a series of basins that are defined by the causeways that span the lagoons. Water exchange between basins is restricted to the areas under the bridges that connect the causeway dikes (Evink & Morgan 1982). Additionally, the pattern of water exchange between the IR and BR has been altered in the last 40 years. Development of the Kennedy Space Center essentially severed the Banana Creek connection between the two lagoons (McCall et al. 1970), and construction of the Canaveral Barge Canal in the early 1960s (Yusof 1987) created a new connection south of Banana Creek.

Reported clam landings from the Indian River Lagoon for the past 20 years suggest that at least under certain conditions, the capacity for production of hard clams in the lagoon is substantial. However, no single basin of the lagoon appears to be consistently productive. Instead, an individual basin may support a dense clam population for several years, after which the population collapses and remains depressed until another major set of juvenile clams occurs either in that basin or in another area of the lagoon. Environmental conditions can vary substantially among basins, creating a potential mismatch between successful spawning events (Hesselman et al. 1989) and the environmental conditions necessary to support the survival and development of that spawn.

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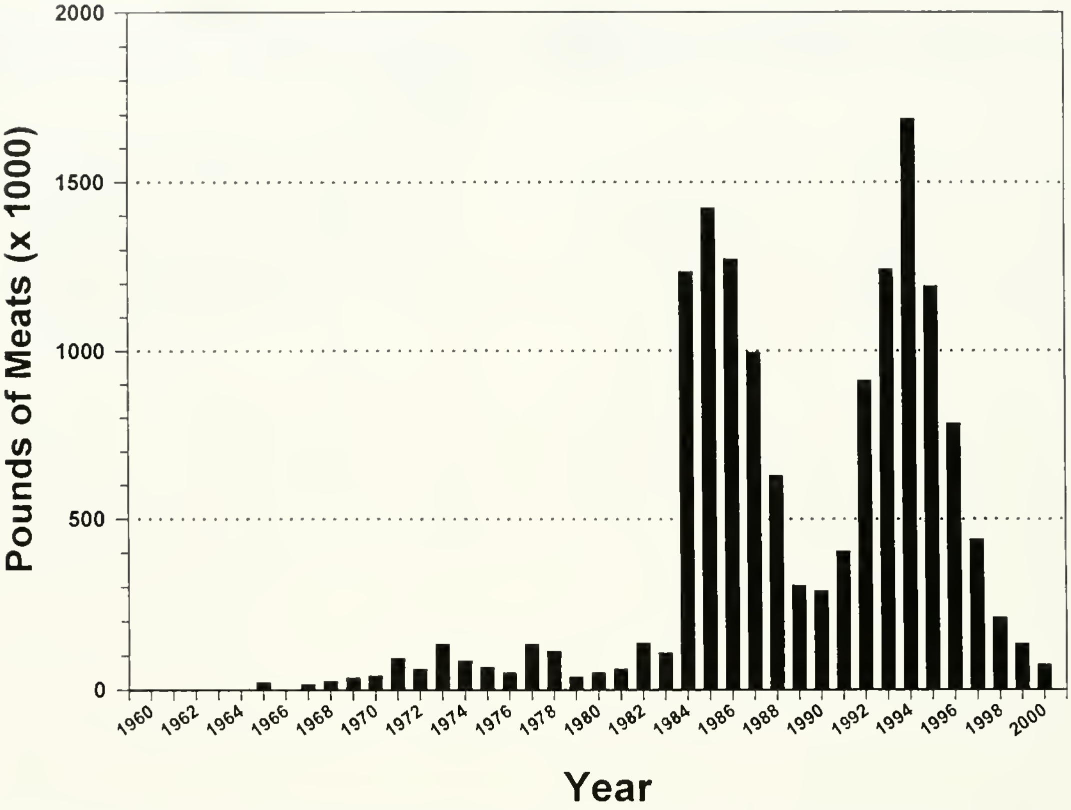
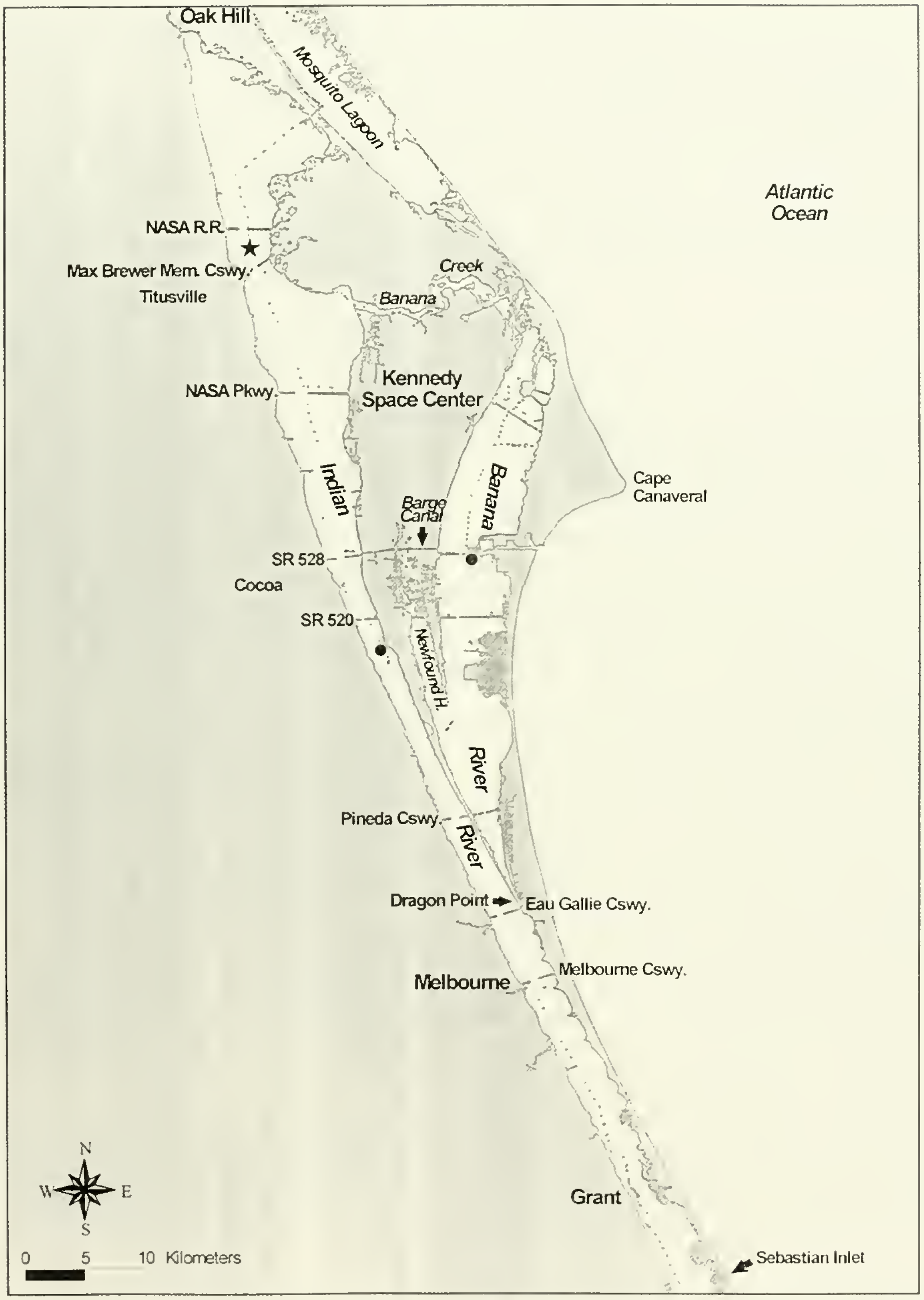


Figure 1. Commercial hard clam (*Mercenaria* spp.) landings from Brevard County, Florida. Data from 1960 through 1985 were provided by the National Marine Fisheries Service. Data from 1986 onward were provided by the Florida Marine Research Institute's Fisheries Dependent Monitoring Program.

Salinity is one of the key environmental variables affecting the success of hard clam populations. Adult clams are not well adapted to salinities below 20 practical salinity units (p.s.u.), and embryonic and juvenile clams tend to be even more sensitive (Castagna & Chanley 1973). In the Indian River Lagoon, salinity may range from less than 15 p.s.u. (Barile & Rathjen 1986) to more than 40 p.s.u. (Young & Young 1977) with extreme conditions causing even those limits to be exceeded. Furthermore, the salinity conditions in one basin of the lagoon may be ideal for clams, whereas those in an adjacent basin may be inimical to clam survival (e.g., McCall et al. 1970, Barile & Rathjen 1986). Thus, a suitable match between environment and biology may be very localized, which is why a commercially successful hard clam spawn is a rare event in the Indian River Lagoon.

The diversity of water quality conditions in the lagoon may create difficulties for the natural clam populations occupying the IRL, but it also may create an opportunity for enhancement of those populations. Although it is difficult to predict when conditions will be suitable for reproduction, recruitment, growth, and survival of hard clams, gross conditions (e.g., salinity, dissolved oxygen, turbidity; Arnold et al. 2000) can be evaluated from the results of frequent water-quality monitoring activities. Such monitoring programs are ongoing in the lagoon, under the auspices of the St. Johns River Water Management District and the Florida Department of Agriculture and Consumer Services. These programs make it possible to identify water-quality conditions suitable for clams, although it is not possible to ensure that those conditions will occur at a time and place coincident with a spawning event.

A variety of options are available to increase clam abundance in the lagoon. Here, we compare three approaches that might be effectively applied in the IRL. The spawner transplant approach involves harvesting mature animals and subsequently replanting them in an area that is either more suitable for survival and reproduction or that will allow larvae to disperse to such areas (e.g., Carter et al. 1984). Generally, animals are harvested from a relatively large area (square kilometers) and replanted into a much smaller area (square meters), thus concentrating potential spawners in an effort to increase reproductive success. This strategy has been used in efforts to increase the population abundance of a variety of organisms, including hard clams (e.g., Carter et al. 1984), bay scallops (Peterson et al. 1996), and abalone (Tegner 1992). The rationale for the seeding approach is similar to that for the spawner transplant approach: the animals are concentrated in a small area in the hope of increasing reproductive success. Seeding differs from spawner transplants in that young, generally prereproductive animals are planted (e.g., Marelli & Arnold 1996). Thus, the residual reproductive value (Ricklefs 1979) of the planted animals should be maximized relative to a spawner transplant operation that may include a variety of age classes of the target organism. Seeding as an enhancement strategy also has a rich history in the population-enhancement literature, including numerous efforts involving hard clams (e.g., Castagna & Kraeuter 1977, Walker 1985, Peterson et al. 1995, Marelli & Arnold 1996). The third approach discussed herein involves the release of recently fertilized eggs directly into the lagoon, thereby circumventing the spawning process entirely. This strategy has been tested with abalone (Preece et al. 1997, Shepherd et al. 2000), but to our knowledge had not been tested with marine bivalves such as the hard clam prior to our study.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Cape Canaveral
Indian
Banana
Barge Canal
SR 528
Cocoa
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Figure 2. Indian River Lagoon, Florida, showing locations of spawner transplant and seeding studies in the Indian River and Banana River lagoons. Star indicates site where adult clams were collected for the spawner transplant study. Closed circles indicate sites of the spawner transplant and seeding studies in the Indian River lagoon and of the spawner transplant study in the Banana River lagoon.

MATERIALS AND METHODS

Spawner Transplants

In the spawner transplant component of this study, we harvested adult clams of a variety of sizes, marked the shells with spray paint to allow for later identification, and then replanted the clams into an area closed to shellfish harvest. The objective of this project was to concentrate reproductively mature clams to maximize the number of successfully fertilized eggs (Levitan 1995). During October 1998 (fall relay), we used professional clam harvesters to gather 5,000 clams from an area of the Indian River

Lagoon north of Titusville (Fig. 2). The harvested clams were returned to shore, where the shells were allowed to dry for several hours and then labeled with yellow spray paint for later identification. The labeled clams were split into two groups of 2,500; the following day, the clams in one group were planted at a site in the Indian River and clams in the other group at a site in the Banana River. To transplant the clams, we poured them over the side of our research vessel as we traversed the extent of a 100-m^2 study plot demarcated at each corner by a crab trap float. Similar transplants were conducted during January (winter relay; blue paint), April (spring relay; green paint), and August 1999 (summer relay; red paint) to assess the best season for conducting transplant operations.

Sampling of the adult relay plots was conducted 2 wk after each relay event and again 3, 6, 9, and 12 mo after each relay event (Tables 1 and 2), thus allowing us to assess reproductive status and mortality during each season of the year for each transplant date. To sample, we thoroughly hand-raked the contents of 20 randomly selected 0.25-m^2 quadrants within each plot, returned all recovered hard clams to the research vessel for identification and counting, and subsampled 15 randomly selected clams from each plot (2-wk samples excepted) for later reproductive analysis. The location of each sample quadrant was marked with a PVC stake to ensure that plots were not resampled. Note that the Indian River spawner transplant plots could not be effectively sampled after the passage of Hurricane Irene in October 1999 because the sample plots were destroyed by that storm. The Banana River spawner transplant plots appeared to be unaffected.

Clam samples for reproductive analysis were processed according to the following procedures. Live animals were returned to the laboratory, where the gonad was excised from each animal and stored for 24 hours in a solution of 5% formaldehyde in seawater. Large gonads were removed from the fixative solution after about four hours, lacerated to ensure penetration of the fixative, and returned to the fixative for the remaining 20 hours. Afterwards, the gonads were thoroughly rinsed in tap water to remove fixative, dehydrated through a series of alcohol concentrations, and infiltrated with JB-4 mounting plastic. Two 3.5-μm sections, separated from each other by at least 50 μm, were then cut from each embedded gonad using a diamond blade microtome and the sections mounted on labeled glass slides. Mounted sections were stained with hematoxylin and eosin, covered, and stored for later microscopic analysis.

Gonad sections were examined under a binocular light microscope and each sample assigned a qualitative ranking of gonad developmental stage as described by Arnold et al. (1997) and summarized in Table 3. This ranking scheme is a composite of gonad development ranking schemes previously used by Loosanoff (1937), Jaramillo et al. (1993), and Walker and Heffernan (1994), with the addition of categories for early spawning and for unreadable samples.

Seeding

On October 13, 1998, we planted three size-classes of seed clams under four protective conditions at our study site in the Indian River lagoon. Seed clams were obtained from the Division of Aquaculture, Harbor Branch Oceanographic Institution, Ft. Pierce, Florida. Seed size-classes were 2 mm mean shell height (SH = maximum distance from umbo to ventral margin), 8 mm mean SH, and 16 mm mean SH. Protective conditions included no cover, oyster shell cover, plastic mesh netting cover, and a combination of oyster shell and plastic mesh netting cover. Oyster shell was purchased from a commercial aggregate company and averaged approximately 5 cm in maximum shell diameter. Fifteen-millimeter mesh plastic netting (Vexar) was purchased from a commercial aquaculture supply company. Two replicates of each size-class by protective-cover combination were deployed, for a total of 24 treatments, each of which was assigned to an individual 1-m^2 plot. We planted approximately 7,165 2 mm SH clams (27 g wet weight), 2,340 8 mm SH clams (259 g wet weight), or 843 16 mm SH clams (119 g wet weight) in each plot, which equated to approximately $30 of clam seed per plot. Before planting, all clams in the 2 and 8 mm SH size-classes were marked with tetracycline (Marelli & Arnold 1996), and the valves of all clams in the 16 mm SH size-class were painted so that we could identify them later.

On October 27 and 28, 1998, we used a hydraulic suction dredge (e.g., Peterson et al. 1983) to sample five 0.0278-m^2 cores from each replicate plot to determine initial planting mortality. On November 11 and 12, 1999, we again collected five 0.0278-m^2 suction dredge samples from each replicate plot to estimate mortality and shell growth after one year. The location of each replicate was determined by using a string grid to ensure that the replicates within each plot did not overlap.

Larval Release

The larval release study was designed to determine the feasibility of directly introducing fertilized clam eggs into the lagoon and allowing them to grow and disperse as a natural population. This approach to clam population enhancement allows us to circumvent the expensive and labor-intensive process of growing clams in the laboratory, while still ensuring that large numbers of fertilized eggs will be available in the natural environment. Larval release also allows us to target areas of the lagoon that are suitable for the growth and survival of clams and to rapidly respond to changing conditions. However, to be able to determine the success

TABLE 1.

Planting and sampling dates for the hard clam (*Mercenaria* spp.) spawner transplant study in the Indian River lagoon.

Season	Plant Date	2-wk Sample	3-mo Sample	6-mo Sample	9-mo Sample	12-mo Sample
Fall	10/28/98 (100)	11/9/98 (127)	2/3/99 (107)	4/28/99 (114)	8/10/99 (57)	10/26/99 (17)
Winter	1/27/99 (100)	2/3/99 (59)	4/28/99 (238)	8/10/99 (53)	10/26/99 (18)	N/A (N/A)
Spring	4/20/99 (100)	4/28/99 (180)	8/10/99 (131)	10/26/99 (38)	N/A (N/A)	N/A (N/A)
Summer	8/3/99 (121)	8/10/99 (171)	10/26/99 (6)	N/A (N/A)	N/A (N/A)	N/A (N/A)

Numbers in parentheses indicate sample size of hard clams collected on that date. Note that on each initial seasonal sampling date, clams were randomly sampled from all of those harvested, whereas on later dates sample size reflects the number of clams actually collected from each study plot.

TABLE 2.

Planting and sampling dates for the hard clam (*Mercenaria* spp.) spawner transplant study in the Banana River lagoon.

Season	Plant Date	2-wk Sample	3-mo Sample	6-mo Sample	9-mo Sample	12-mo Sample
Fall	10/28/98 (100)	11/9/98 (73)	2/3/99 (73)	4/28/99 (96)	8/11/99 (97)	10/25/99 (3)
Winter	1/27/99 (100)	2/3/99 (200)	4/28/99 (144)	8/11/99 (91)	10/25/99 (16)	1/25/00 (2)
Spring	4/20/99 (100)	4/28/99 (161)	8/11/99 (87)	10/25/99 (31)	1/25/00 (10)	4/25/00 (1)
Summer	8/3/99 (121)	8/11/99 (229)	10/25/99 (20)	1/25/00 (7)	4/25/00 (9)	8/16/00 (2)

Numbers in parentheses indicate sample size of hard clams collected on that date. Note that on each initial seasonal sampling date, clams were randomly sampled from all of those harvested, whereas on later dates sample size reflects the number of clams actually collected from each study plot.

of this enhancement strategy, it is necessary to be able to track and sample the animals during the planktonic phase of their life.

Spawning

The larval release strategy requires the production of large numbers of viable hard clam embryos that can be successfully transported to and released at the site targeted for enhancement. Adult hard clams were collected from various areas of the Indian River Lagoon on several dates during 1999. The clams were transported to a holding area at Harbor Branch Oceanographic Institution, where they were conditioned in preparation for spawning. Conditioning consisted of holding the clams for several months in a small lagoon on the Harbor Branch campus. On the day before spawning, the clams were transferred from the holding lagoon to a refrigerated storage area and held overnight. On the following day, the clams were removed from the storage area, placed in equally spaced rows on each of three spawning tables, and submerged in approximately 10–15 cm of 28 p.s.u. seawater. During the next six hours, the clams were exposed to cycles of cool and warm water and induced to spawn. As each individual clam initiated spawning, the sex of the animal was identified and the clam was removed from the table and isolated in containers with other clams in small groups of males or females. The clams continued to spawn within the containers, and the resultant eggs were pooled and concentrated on a 35-μm-mesh sieve and exposed to a sperm concentration adequate to ensure fertilization of all eggs. Total egg production and fertilization success were determined microscopically, after which the developing embryos were transferred to 20 L aquarium bags and transported to the study site for immediate release.

TABLE 3.

Gonad staging scheme for female hard clams (*Mercenaria* spp.) collected from the Indian River Lagoon, including female clams transplanted to study sites in the Indian River and Banana River lagoons and sampled during various times of the year, and from their undisturbed conspecifics.

Reproductive Status	Description of Gonadal Tissue	Numerical Stage
No Data	Tissue unreadable	0
Inactive	Gonad tissue undifferentiated	1
Developing	Tissue differentiated, eggs present	2
Ripe	Tissue full of eggs	3
Early spawning	Eggs being shed, but follicles still full in appearance	3.5
Spawning	Many eggs shed, follicles appear partially empty	4
Spent	Follicles nearly empty	5

Larval Tracking

Hard clam embryos were transported from the spawning facilities at Harbor Branch to our study site in the Banana River lagoon and released at 2030 EDT on May 16, 2001, at a site approximately 1.75 m deep. Water temperature at the site was 28°C and salinity was 22 p.s.u., whereas the temperature of the water in which the larvae were transported was 25.8°C and the salinity was 27.7 p.s.u. Before larval release, five subsurface drifters (Davis 1985, Hitchcock & Arnold, unpublished data) were deployed in a box-and-one pattern (one drifter at each corner of a 10 m × 10 m box, with a single drifter in the center of the box), and their initial positions were recorded using a differential Global Positioning System. Then, at a depth of approximately 0.5 m, the clam larvae were gently poured from the bags into the center of the drifter array. Gradual mixing between the transport water and the lagoonal water was allowed in an attempt to minimize osmotic shock.

During daylight hours on May 16, we collected thirteen 200 L water samples from the targeted release area to determine the prerelease concentration of hard clam larvae in the study basin. On May 17 and 23, 2000, post-release water samples were collected to determine the distribution and density of the larval mass. For the May 17 sampling, when the larval mass was predicted to be relatively concentrated, the subsurface drifters were visually located and the position of each drifter recorded. Sample collection locations for hard clam larvae were then selected based upon the location and distribution of the subsurface drifters. On May 23, after diffusive processes were anticipated to have spread the larvae throughout the study basin, samples were collected at each of 23 grid nodes equally distributed throughout the basin.

On each sampling date, samples of hard clam larvae were obtained by using a Jabsco Model 34600-0000 diaphragm pump to collect volumes of water that ranged in size from 100–400 L, depending upon the projected density of larvae. Water was pumped through a 150-μm-mesh sieve to remove large objects, and then captured in a 63-μm-mesh plankton net. Each resultant sample was removed from the cod end of the plankton net and carefully distilled to a volume of approximately 30 mL, then transferred to a 50-mL screw-cap centrifuge tube, labeled, and placed on ice until arrival at the laboratory, where it was frozen at approximately −5°C. Within one month of the completion of the study, all of the frozen water samples were sent to the Skidaway Institute of Oceanography for determination of the presence and abundance of clam larvae. Samples were analyzed for the presence of hard clam larvae using a previously developed genetic probe that is both quantitative and *Mercenaria*-specific (e.g., Frischer et al. 2000).

RESULTS

Spawner Transplants

At both the Indian River and Banana River study sites, the mortality of relayed hard clams was severe, particularly during the summer and early fall of 1999. Also at both sites, considerable loss of clams from the plots was associated with the initial transplant. At each site, on all dates, we transplanted an average of 25 clams m^{-2}, and within 2 wk the densities for all plantings had decreased by more than 50% (Fig. 3). After the initial transplant event and the loss of clams associated with that event, clam densities stabilized throughout the winter, spring, and early summer at both sites (Fig. 3). During late summer and early fall of 1999, the clams experienced substantial mortality, possibly as a result of decreased salinity associated with Hurricane Irene (Fig. 4). As noted previously, that storm destroyed our study plots in the Indian River. It also appears to have had a severe detrimental effect on the clams planted in the Banana River, as clam density decreased substantially in all Banana River plots between the August 11 and October 25, 1999, sample dates (Fig. 3). Hurricane Irene swept through our study area on October 16, 1999, and salinity near our Banana River study site decreased to a study-period minimum of less than 15 p.s.u. at the end of October 1999 (Fig. 4).

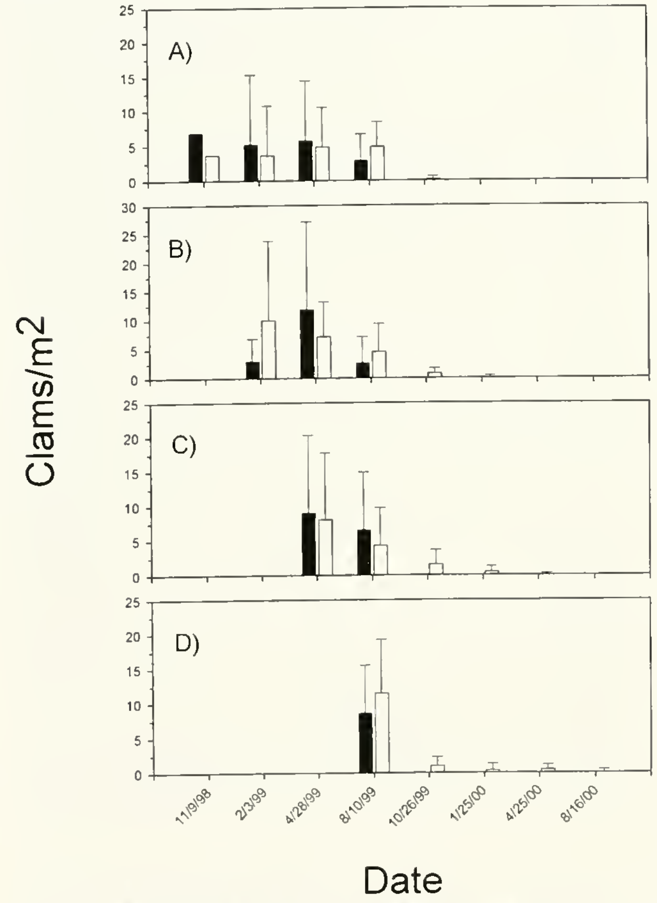

Figure 3. Mean density of hard clams (*Mercenaria* spp.) on various sampling dates after transplantation during (A) fall 1998; (B) winter 1999; (C) spring 1999; and (D) summer 1999 at the Indian River lagoon (filled bars) and Banana River lagoon (open bars) study sites. See Figure 2 for the location of each study site. Note that at the Indian River study site, all four study plots were destroyed by Hurricane Irene during October of 1999 and no further sampling was conducted after that date. Error bars represent one standard deviation. On the November 9, 1998, sampling date, samples were pooled and no standard deviation was calculated.

The high levels of mortality that we observed in our transplant plots may have been influenced by the inability of clams (especially large clams) to reburrow following initial harvest. The Indian River study site was characterized by a soft sand/mud substrate that appeared to provide little resistance to burrowing clams. Upon re-sampling that site two weeks after the fall transplant, 26% of all clams collected remained on the surface, and three months after the fall transplant 20% of all clams collected still remained on the surface. In contrast, the Banana River study site was characterized by a hard sand bottom that appeared to provide considerable resistance to clams attempting to burrow. Two weeks after the Banana River fall transplant, 47% of all clams collected remained on the surface, and three months after the fall transplant 34% of all clams collected remained on the surface. At both sites, failure to burrow was related to clam size. A comparison of the mean SH of buried versus unburied clams at each site two weeks after transplant during both fall and winter, indicated that the clams that failed to burrow were significantly larger than those that successfully reburrowed (*t*-test, see Fig. 5A, B, E, and F for respective *P* values). We detected no significant difference in SH between buried and unburied clams at either site three months after transplant (Fig. 5C and D), although only clams transplanted during the fall were compared. During the spring and subsequent sampling episodes, we discovered few clams at either site that were both alive and unburied.

After the initial episode of transplant mortality, overall mortality of relayed clams did not appear to be size-related. At the Indian River study site, the size distribution of hard clams did not differ significantly among sampling dates (Simultaneous Test Procedure; Sokal & Rohlf 1995) except during the summer transplant study (Fig. 6). During the final sampling episode (October 26, 1999) of the summer transplant study, the size distribution of planted clams differed significantly from the size distribution recorded during the previous two sampling dates and appears to have shifted towards a preponderance of small clams (Fig. 6). For all four of the transplant episodes at the Banana River study site, a significant shift in clam size distribution was detected for the October 25, 1999, sample date, and for all sample dates subsequent to October 25, relative to all sample dates preceding October 25 (Fig. 7). The only exception to this pattern was from the spring transplant study, for which the size shift was not detected until the January 25 sampling episode (Fig. 7).

During each seasonal harvesting event, a subsample of 15 clams from each plot was returned to the laboratory for analysis of reproductive condition and for a comparison with control samples collected from the natural population on the same date. However, the results from only the fall planting date at both study sites are included in the present analysis because that is the only planting date for which adequate sample numbers were available for all sample dates from both sites. The female clams in the control samples had a pattern of reproductive development typical of Indian River hard clams (Hesselman et al. 1989). During fall and winter, when Hesselman et al. (1989) reported that spawning occurred in Indian River hard clams, most clams that we sampled (control and transplant) were either spawning or were spent (Fig. 8). During spring, the season of peak spawning in Indian River

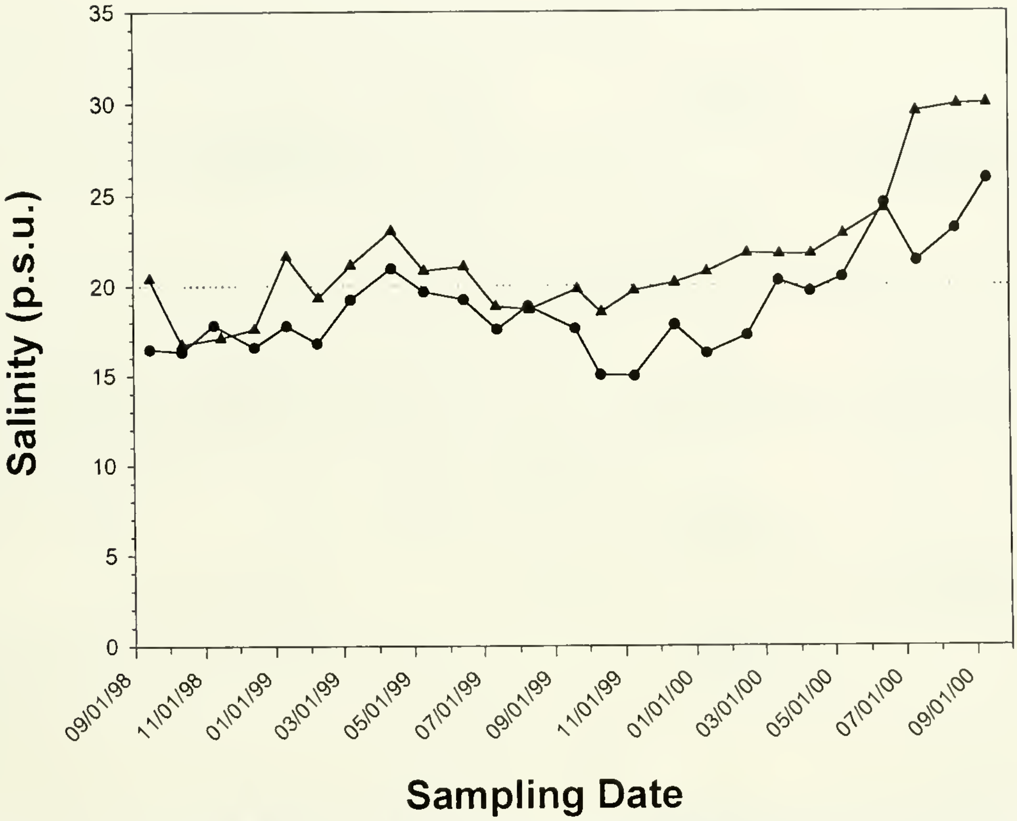

Figure 4. Salinity recorded at the Indian River lagoon (closed triangles) and Banana River lagoon (closed circles) study sites during September 1998 through October 2000. The dotted line provides a 20 p.s.u. reference. Data courtesy of the St. Johns River Water Management District Surface Water Quality Monitoring Program.

hard clams (Hesselman et al. 1989), most of the clams from the control sample were ripe, and lesser proportions were either spawning or spent. In contrast, most animals collected from the Indian River and Banana River transplant plots during spring were in some stage of spawning. Finally, during summer the vast majority of control and transplant clams that we sampled were in the spent condition, which agrees well with the observation of Hesselman et al. (1989) that most Indian River hard clams are reproductively spent during summer.

Gonadal neoplasia is extensive in Indian River hard clams (Hesselman et al. 1988, Bert et al. 1993), and this condition appears to be related to hybridization between the two species of *Mercenaria* (*M. mercenaria* and *M. campechiensis*) that occupy the lagoon (Bert et al. 1993). We recorded neoplasia in 85% of the male clams and almost 92% of the female clams that we collected from the natural clam population of the lagoon. Male clams suffered 93% and 86% neoplasia when harvested after transplant to the Indian River and Banana River, respectively. Female clams suffered 96% and 93% neoplasia when harvested after transplant to the Indian River and Banana River, respectively.

When all samples of clams collected for reproductive analysis during the course of the spawner transplant study were pooled, there were significantly more female than male clams (0.53 males: 1.00 females, $\chi^2 = 42.13$, df = 1, $P < 0.0001$). However, sex ratio was dependent upon clam size-class. For all clams that were ≤ 60 mm SH, we detected no significant difference in sex ratio (0.75 males: 1.00 females, $\chi^2 = 1.43$, df = 1, $P = 0.232$). In contrast, for clams > 60 mm SH, the sex ratio was significantly skewed towards female clams (0.50 males: 1.00 females, $\chi^2 = 42.89$, df = 1, $P < 0.0001$).

Seeding

Mortality of 2 mm hard clams was substantial within 2 wk of planting under all treatment conditions (Table 4). Only under mesh protection did the 2 mm size-class suffer less than 90% mortality, but even with mesh protection the small clams experienced a mean mortality of 85.6%. Survival of clams in the 8 mm size-class was not much better; those clams also experienced >90% mortality in the open plots and at least 50% mortality within 2 wk after being planted in the remaining plots. In contrast, clams in the 16 mm size-class experienced <10% mean mortality in the mesh plots and a mean mortality of 11.3% in the open plots. However, those clams suffered 30.4% mean mortality under the combined protective cover and >70% mean mortality in the shell plots.

Hurricane Irene also severely impacted our seed clam plots. Nevertheless, on November 11, 1999, we attempted to reconstruct the experimental plots and we did conduct suction dredge sampling of the reconstructed plots. We found no live clams in the seed plot 1 y after planting. However, we cannot determine whether that lack of clams was due to the effects of Hurricane Irene or due to factors independent of the hurricane.

Larval Release

Spawning

On May 16, 2000, approximately 550 million hard clam eggs were spawned and collected. The eggs were then exposed to an

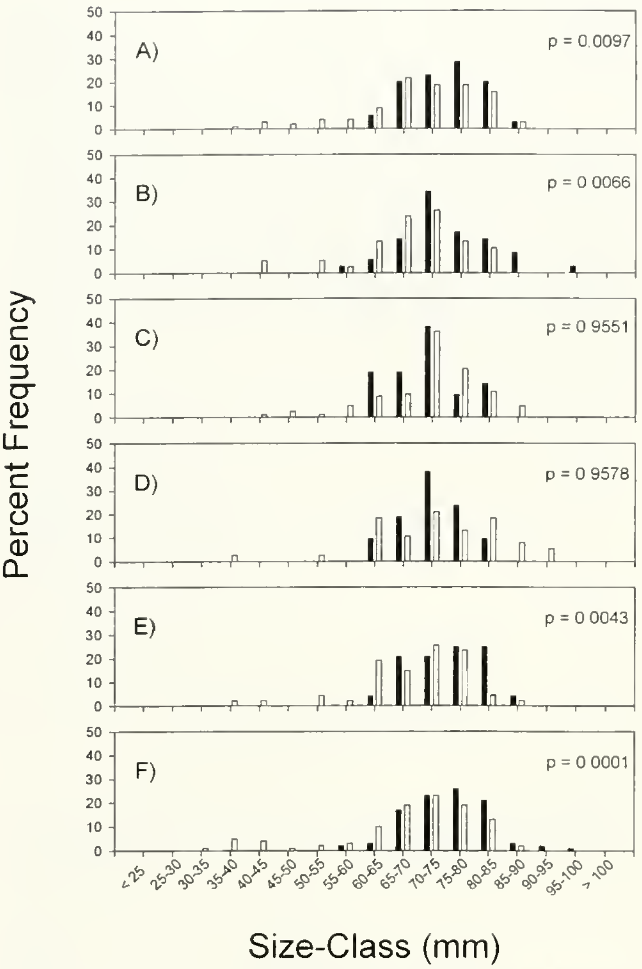

Figure 5. Percent frequency of occurrence of hard clams (*Mercenaria* spp.) at each of the Indian River lagoon and Banana River lagoon study sites during fall and winter sampling events. Filled bars represent the percentage of clams that were recovered on the surface of the plots, and open bars represent the percentage of clams that were buried upon recovery. (A) Indian River fall planting, 2-wk sampling; (B) Banana River fall planting, 2-wk sampling; (C) Indian River fall planting, 3-mo sampling; (D) Banana River fall planting, 3-mo sampling; (E) Indian River winter planting, 2-wk sampling; and (F) Banana River winter planting, 2-wk sampling. The P values included in each plot represent the probability (t-test) that the mean size of clams recovered on the surface was not significantly different than the mean size of clams that were buried upon recovery. A $P \leq 0.05$ indicates a statistically significant difference in the mean size of surficial vs. buried clams.

amount of sperm sufficient to ensure fertilization of all eggs as determined by microscopic examination. Spawning occurred in three "batches" beginning at 1200 EDT and ending at 1600 EDT. Transport from Harbor Branch (1800 EDT departure) to the Banana River release site (2030 EDT release) required approximately 2.5 h, so the clams ranged in age from 4.5 to 8.5 h at the time of release.

Larval Release

Larvae were released at a site in the Banana River lagoon between SR 520 and SR 528 (28°23.320′N latitude, 80°37.951′W longitude) at 2030 EST on May 16, 2000 (Fig. 2). During the next 24 h, the drifters that tracked the water mass within which the larvae were released were transported towards the west until they approached the western shore of the lagoon (Fig. 9). As the drifters approached the shoreline, they gradually swung around to the north, but four of the five drifters contacted the bottom, hung up, and were retrieved. The fifth drifter passed through a small bridge at the western end of the SR 528 causeway and was retrieved to prevent its loss.

Analysis of water samples collected on May 16, before the release of cultured larvae, detected no evidence of naturally occurring hard clam larvae in the study basin. On May 17, after our larval release during the evening of the 16th, we detected larvae at only one sampling station (Fig. 9), probably because the larval mass remained tightly constrained in the area around that station. By May 23, the larval mass had spread throughout the basin (with the exception of the southeast corner), although several peaks of abundance were detected. Two of these peaks were in the northwest corner of the basin, where larval density exceeded 7 veligers L^{-1} (Fig. 10).

DISCUSSION

We tested three strategies for enhancing the abundance of harvestable hard clams in the Indian River Lagoon, Florida. The first strategy involved harvesting adult clams from low-density populations and concentrating them in high-density patches in an effort to increase fertilization success and the production of viable larvae. This strategy does not appear to be cost-effective because most of the clams that we transplanted did not survive the 12-mo monitoring period. Furthermore, the vast majority of the clams that we transplanted were infected with gonadal neoplasia, a disease that progressively reduces fecundity and probably proceeds to a fatal outcome (Yevich & Barry 1969, Hesselman et al. 1988, Eversole & Heffernan 1995). The second strategy involved planting small seed clams at relatively high densities, again with the intention of creating concentrated patches of reproductively active clams. This approach has one anticipated advantage (a longer life span for the spawners) and one unanticipated advantage (avoidance of gonadal neoplasia) when compared with the spawner transplant strategy. However, the first advantage may be offset by the high rates of mortality experienced by most size-classes of the seed clams, and the second advantage may be temporary because the seed clams become increasingly susceptible to gonadal neoplasia, as they grow older (Bert et al. 1993). Although survival rates were relatively high for the largest size-class of clams (16 mm mean SH), the considerable cost of those clams ($0.036 each) reduces the cost-effectiveness of this strategy. The third strategy involved circumventing the entire process of natural fertilization by releasing already fertilized eggs directly into the lagoon. Our results suggest that this strategy may be effective, but more information is needed. It appears that large numbers of clam larvae survived to an age at which settlement can be reasonably expected (8 days to set in culture conditions during May using Indian River water; B. Leeming, personal communication). However, the ultimate fate of those larvae has not been determined, and successful settlement needs to be demonstrated *in vivo* for this approach to have any validity.

The common currency with which to gauge the success of each of these strategies will be an increase in the abundance of hard clams available for harvest by the fishermen. Hard clams in the Indian River Lagoon require approximately 18 months to achieve the legal harvest size of 2.54 cm in shell width (Arnold et al. 1996).

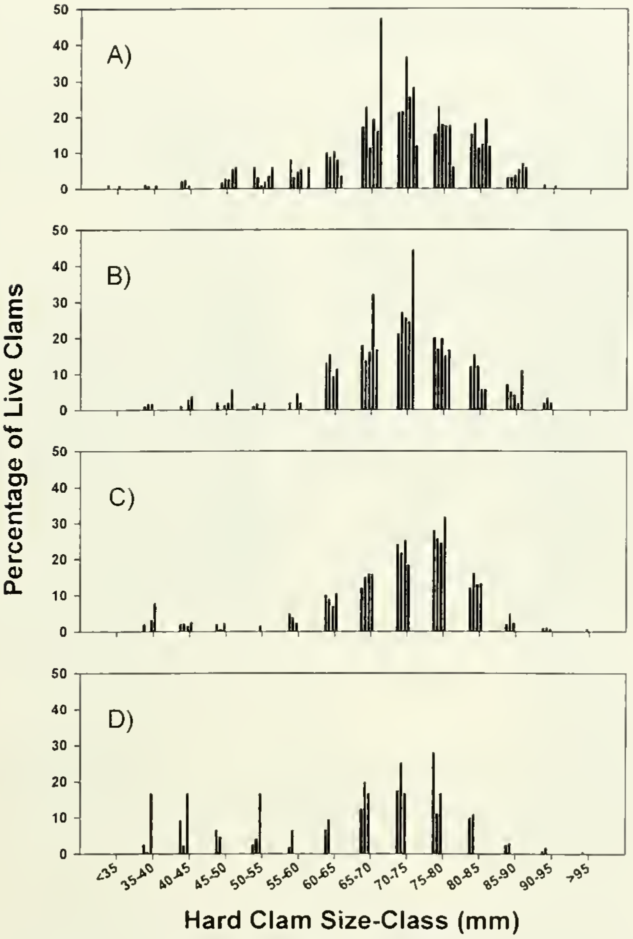

Figure 6. Percentage of live hard clams (*Mercenaria* spp.) recorded within various size-classes during each seasonal sampling event following the (A) fall; (B) winter; (C) spring; and (D) summer transplants in the Indian River lagoon. For each size-class category within each transplant season, sampling dates are plotted from left to right. Thus, the leftmost bar within each size-class represents the sample collected on the original date of harvest, and the rightmost bar represents the sample collected on the final sampling date. See Table 1 for sampling dates and sample size for each date. Note that samples were collected on only five dates for the winter transplant, four dates for the spring transplant, and three dates for the summer transplant because of the impacts of Hurricane Irene, which destroyed the sample plots on October 16, 1999. Otherwise, missing bars indicate that no clams were collected from that size-class on that date.

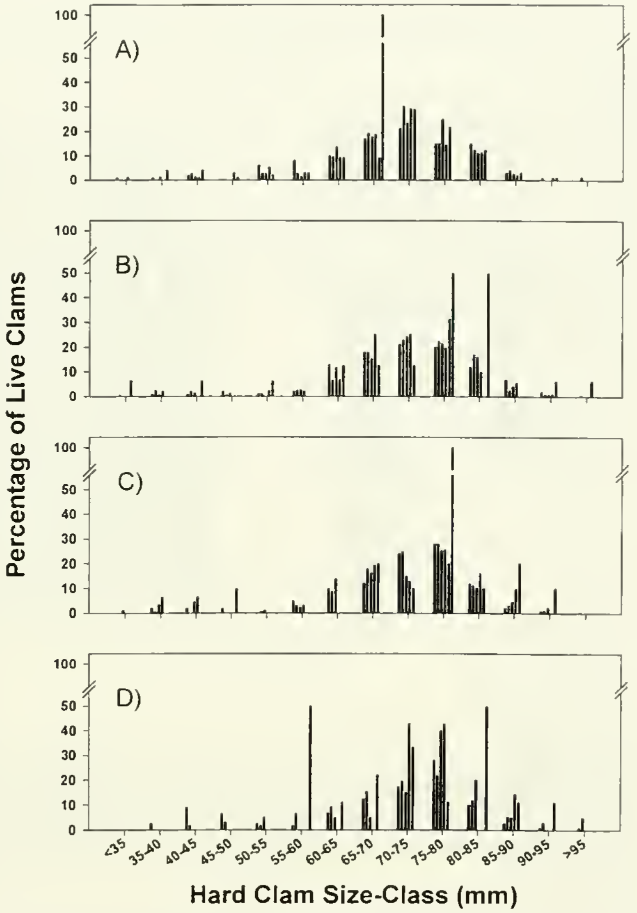

Figure 7. Percentage of live hard clams (*Mercenaria* spp.) recorded within various size-classes during each seasonal sampling event following the (A) fall; (B) winter; (C) spring; and (D) summer transplants in the Banana River lagoon. For each size-class category within each transplant season, sampling dates are plotted from left to right. Thus, the leftmost bar within each size-class represents the sample collected on the original date of harvest, and the rightmost bar represents the sample collected on the final sampling date. See Table 2 for sampling dates and sample size for each date. Missing bars indicate that no clams were collected from that size-class on that date.

For the adult transplants, it would have been possible to detect adult offspring as early as spring 2000, assuming that clams transplanted in fall 1998 spawned very soon after transplant. That is a reasonable assumption considering that a fall spawn has been described for Indian River hard clams (Hesselman et al. 1989) and was similarly indicated by our fall 1999 reproductive data. For the seeding study initiated in fall 1998, we would have expected that maternally derived offspring would be available for harvest no earlier than fall 2000. Hard clams as small as 27 mm SH are reproductively active in the Indian River Lagoon (Hesselman et al. 1989), but clams are generally male during the first year of life (Loosanoff 1937). Thus, we would not have expected egg production from the 16 mm size-class of seed clams for at least 6 months post-planting (i.e., spring 1999). For the larval release study conducted in May 2000, surviving animals would have been expected to achieve harvest size during fall 2001. Information from the fishery and preliminary results from our own sampling efforts provide no evidence of a substantial yield of harvestable hard clams, as might be expected from the above timetables. Of course, our study was conducted on a much smaller scale than would be necessary to realize a significant contribution to future year-classes (McHugh 1981). Our primary objective was to experimentally compare three possible approaches to hard clam population enhancement in the lagoon. From those results, we hoped to be able to choose a single approach that has the greatest likelihood of success and then to apply that approach on a scale appropriate for success.

For the Indian River hard clam fishery, success is a quantifiable parameter. The primary fishing grounds fall within the boundaries of Brevard County, and commercial clam harvest in that county is strictly regulated. Each clammer must be licensed, and only 500 licenses are allocated for the fishery. The goal of our enhancement work is to provide the clam harvesters with a resource base that

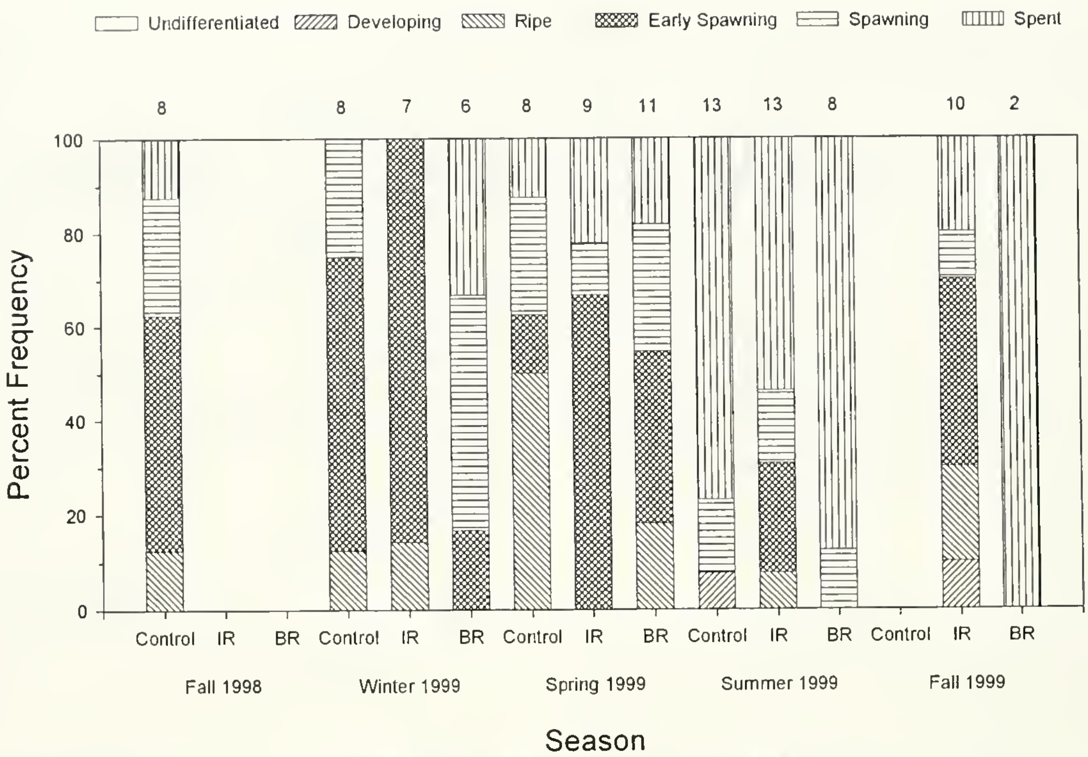

Figure 8. Reproductive stages of female hard clams (*Mercenaria* spp.) collected on various seasonal sampling dates after the fall transplant. Stages are as described in Table 3. Numbers across the top of the plot are sample sizes. Control, clams that were harvested from the natural population during each season; BR, clams that were transplanted to the Banana River lagoon study site during fall and sampled during each season; IR, clams that were transplanted to the Indian River lagoon study site during fall and sampled during each season.

would allow them to survive a nadir in the abundance of naturally occurring clams. If it is assumed that $20,000 is a minimum acceptable annual income for each clam fisherman and that the sale price for each clam is $0.20, then it can be estimated that 50 million harvestable clams must be produced each year to satisfy that harvest goal. Using the larval release strategy as an example, it is apparent that 50 million larvae must be released and must all survive to harvest size to meet that goal. Similarly, if only 1% of the larvae survive to produce clams of legal harvest size, then 5 billion larvae must be released. We have been able to consistently produce approximately 50 million eggs per spawning table per day during subsequent tests of the larval release strategy, and we have used four spawning tables per day during those trials. At that rate, it would require 25 spawning days to produce 5 billion larvae. Carriker (1961) estimated the mean survival rate of hard clam larvae to be 2.6% when rates of flushing were low, but the minimum survival rates that he reported were 0.1% or less. At those minimum larval survival rates it would be necessary to increase daily larval production by a factor of five and to increase the time span of larval release to approximately 50 days to achieve our stated goal. That may be possible, but it would require a broad-scale effort that includes participation by the clam fishermen. Additionally, our best-case larval production estimates require the provision of 800 broodstock per day, because we place approximately 200 clams on each spawning table (equivalent to about 135 female clams with a 2:1 ratio of females to males). Considering the present status of the hard clam fishery in the Indian River Lagoon, the limited availability of broodstock to support egg production may prove to be a serious problem that limits the effectiveness of the larval release strategy. Moreover, broodstock availability may also be adversely affected by the high incidence of gonadal neoplasia in Indian River hard clams. Large clams, which are economically less valuable than small clams and are therefore more available for use in the spawning program, are relatively rare in Indian River waters and produce fewer eggs than would be predicted based upon allometric considerations alone (e.g., Peterson 1983, 1986). The clams that we have successfully used in previous spawning efforts generally fall within the "topneck" commercial

TABLE 4.

Percent mortality of hard clam (*Mercenaria* spp.) seed 2 wk after being planted under various protective conditions at the Indian River study site.

Clam Size-Class	Open Plots	Mesh Plots	Shell Plots	Mesh/Shell Plots
2 mm (rep 1)	96.6	86.9	91.0	92.5
2 mm (rep 2)	98.0	84.2	92.5	98.3
2 mm (mean)	**97.3**	**85.6**	**91.8**	**95.4**
8 mm (rep 1)	91.7	53.8	71.7	84.9
8 mm (rep 2)	97.5	88.9	62.5	90.2
8 mm (mean)	**94.6**	**71.4**	**67.1**	**87.6**
16 mm (rep 1)	20.9	5.2	65.2	48.7
16 mm (rep 2)	1.7	7.6	80.9	12.2
16 mm (mean)	**11.3**	**6.5**	**73.0**	**30.4**

Protective treatments are listed across the top of the table and the results from replicate treatments (and the mean of the paired replicates) are presented within the body of the table.

The boldface rows represent the mean of the two observations.

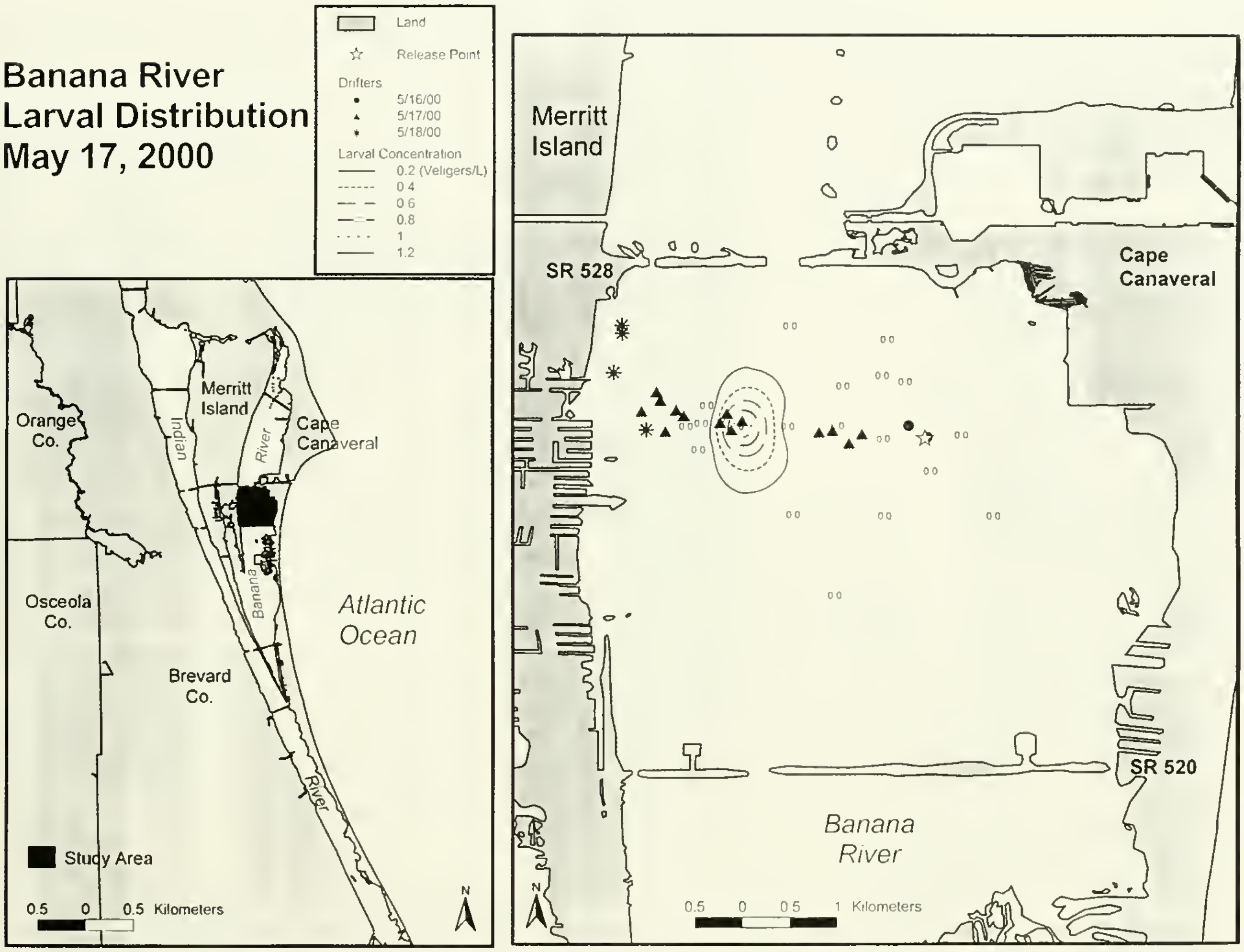

Figure 9. Contour plot of the distribution of hard clam (*Mercenaria* spp.) larvae as estimated by sampling conducted on May 17, 2000, in the Banana River lagoon, Florida. Data are presented as number of clam larvae L^{-1}, and larval concentrations are depicted at the location of each sample station. Also plotted are the locations of the subsurface drifters that were deployed during the evening of May 16, 2000.

classification (average SH = 60 mm). There is a strong market demand for these clams, and seafood processors are reluctant to sell them to us even at a premium price for fear of upsetting previously established buyers. During times when native Indian River broodstock are readily available from seafood processors because of high levels of harvest in the natural fishery, there is little need for an enhancement program, and the contribution that could be realized from enhancement would be swamped by natural production (Kassner & Malouf 1982).

Our estimate of the yield of harvestable clams from larval release does not take into account post-settlement losses, which typically exceed 80% and may approach 100% under some conditions (Gosselin & Qian 1997). However, hard clam populations continue to thrive in Florida and throughout the eastern seaboard of the United States, so conditions suitable for survival must occur. Perhaps it is more appropriate to consider our enhancement efforts within the context of natural clam densities in the lagoon. During the early 1980s in the area near Grant and during the mid-1990s in the area north of Cocoa, clams were extremely abundant and peak densities exceeded 10 clams m^{-2} (Arnold, unpublished data). However, during the time frame of the present study, clams were practically nonexistent in the vicinity of the Banana River study site (Arnold et al. 1997) and were similarly rare in the vicinity of the Indian River study site. As a result, the artificial densities that we initially established in each planting study (spawner transplants and seeding) substantially exceeded the background density at each study site. The 550 million eggs that we contributed during our larval release study equate to an average contribution from the spawn of more than 75 female "cherrystone" size hard clams (Bricelj 1992), assuming 100% fertilization of naturally spawned eggs. Two factors increase the value of the fertilized eggs that we released. First, it is unlikely that 100% fertilization efficiency is realized in the natural environment (Levitan 1995). Second, because of the prevalence of gonadal neoplasia, egg production in Indian River hard clams may be considerably less than that presented by Bricelj (1992) for northern US waters. If we estimate mean production to be 1 million eggs per female, and we estimate a fertilization efficiency of 1% at the low clam densities ($<<1$ m^{-2}) currently found in the lagoon, then the number of larvae that we released is equivalent to the number of larvae produced by approximately 55000 female clams or a bed of >80000 clams assuming a 2:1 female:male sex ratio.

We did not anticipate the rate of loss of transplanted clams that was actually realized during this study. Similar transplants have

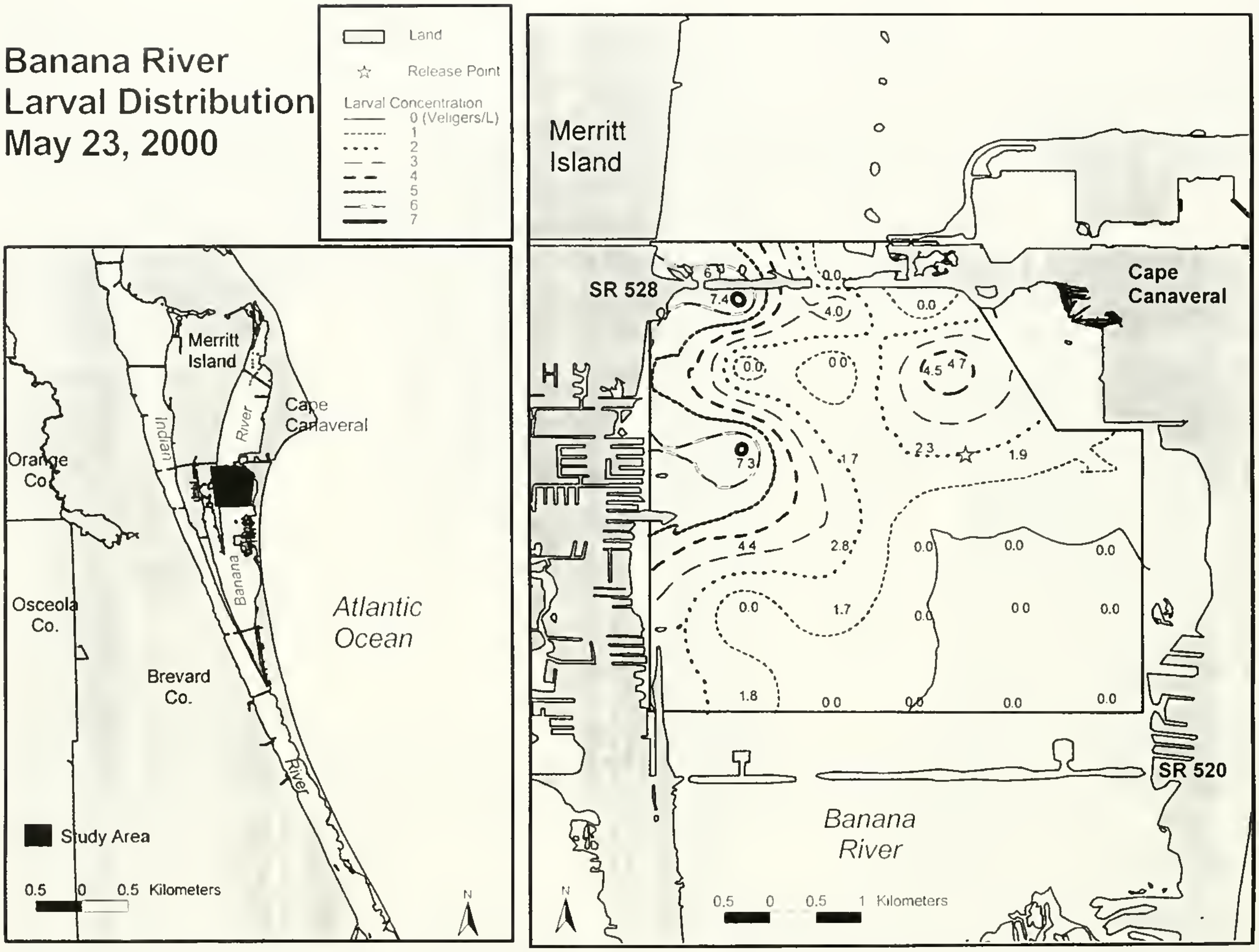

Figure 10. Contour plot of the distribution of hard clam (*Mercenaria* spp.) larvae as estimated by sampling conducted on May 23, 2000, in the Banana River lagoon, Florida. Data are presented as number of clam larvae l^{-1}, and larval concentrations are depicted at the location of each sample station.

been conducted in other areas throughout the range of *Mercenaria*, either for eventual harvest of the relayed clams (e.g., Rice et al. 2000) or to increase or expand larval production (e.g., Kassner & Malouf 1982, Ganz 1991). However, the high frequency of gonadal neoplasia in the clams that we collected for transplant will mitigate against the success of this approach. Gonadal neoplasia is common in Indian River hard clams (Hesselman et al. 1988), particularly in the northern lagoon where we collected clams for transplant (Bert et al. 1993). Although gonadal neoplasia has been reported in hard clams collected from northeastern US coastal waters (e.g., Barry & Yevich 1972), the frequency of occurrence was less than 5% versus greater than 80% in our study. That disease appears to substantially reduce the reproductive potential of hard clams (Hesselman et al. 1988) and probably contributes to the relatively short life span of hard clams in Indian River waters (Jones et al. 1990). Considering that the reproductive potential of *Mercenaria* increases with age (Bricelj & Malouf 1980, Peterson 1983, 1986), the high frequency of gonadal neoplasia in Indian River hard clams appears to render spawner transplants an ineffective strategy for enhancing the abundance of harvestable clam populations in the lagoon.

In all cases, gonadal neoplasia was more prevalent in the transplanted clams than in their undisturbed conspecifics, and the difference in the frequency of neoplasia between the transplants and their undisturbed conspecifics was greater in the Indian River than in the Banana River. This difference was minor and may reflect sampling bias related to differences in the frequency of neoplasia that have been reported for various size-classes and genotypes of hard clams (Bert et al. 1993). Nevertheless, gonadal neoplasia was considerably more prevalent in clams collected during our study than in clam collections reported by either Hesselman et al. (1988) or Bert et al. (1993). Neoplasia is more common in hybrid hard clams (Bert et al. 1993) and hybrid clams are more common in the northern Indian River Lagoon where we collected our transplant animals (Bert & Arnold 1995). Our spawner transplant study would perhaps have been more successful if we had collected clams from more southerly Indian River waters, but we were limited in our choice of harvest sites by the availability of clams.

Seeding as a means of increasing the abundance of hard clams has been attempted in various areas throughout the range of *Mercenaria*, including both coasts of Florida (Menzel & Sims 1962, Menzel et al. 1976, Marelli & Arnold 1996), Georgia (Walker 1985), North Carolina (Peterson et al. 1995), Virginia (Castagna & Kraeuter 1977), New York (Flagg & Malouf 1983), and Rhode Island (Rice et al. 2000). With the exception of the work in North Carolina (Peterson et al. 1995), these efforts have met with limited success due to the high rate of loss of seeded clams even when protective measures are used. In North Carolina, relatively large seed clams (14–22 mm shell length) were planted at relatively low density ($1\ m^{-2}$) in shell hash habitat in late fall, resulting in 35% survival after 14 months (Peterson et al. 1995). However, clam density below $5\ m^{-2}$ is considered to be inadequate for commercial harvest in Florida waters (Arnold et al. 2000), and increasing the density of planted seed clams might result in a loss of economic viability attributed to this approach. Moreover, achieving even the limited enhancement goals that we have set for our project (50 million harvestable clams) would require seeding almost 15,000 hectares of submerged land with almost 1.5 billion clams. Even if the available clam hatcheries could produce that many clams, the cost of the clams alone would be exorbitant. At least for the 500 hard clam fishermen currently licensed to work Brevard County waters, seeding for direct harvest does not appear to be a cost-effective means of ensuring a minimum annual income, although that approach may be feasible for projects of a smaller scale.

We are familiar with only one application of larval release as a means of stock enhancement. Shepherd and colleagues (Preece et al. 1997, Shepherd et al. 2000) released various densities of abalone larvae at several sites in South Australia and monitored their survival. They found that because larval and post-larval survival was density-dependent, the releases of relatively low densities of larvae were more successful than were the high-density releases. The overall conclusion of those authors (Shepherd et al. 2000) was that larval release was not a viable strategy for abalone stock enhancement because of the density-dependent nature of larval mortality. Those results, and results from our own analyses of diffusive processes acting on artificially introduced hard clam larvae (Hitchcock & Arnold, unpublished data), suggest that a point release of the larvae is not the best strategy. Instead, higher survival rates may be obtained by spreading the larvae throughout the target basin, thereby enhancing diffusive processes that will take place anyway (Hitchcock & Arnold, unpublished data). We will test that approach in future experiments.

Efforts to enhance the population abundance of commercially important marine molluscs have been ongoing for decades, and the published record of those efforts indicates that success has been rare if not nonexistent. Clearly, efforts to enhance even moderately dense populations are superfluous, as the reproductive potential of the natural population is sufficient to swamp any directed enhancement efforts. Only when population density is very low, such as it would be in an essentially collapsed population, might such efforts yield success. At that point, we shift from an enhancement effort to a restoration effort, and the goals of the project shift from increasing the abundance of an ecologically functional population to restoring reproductive viability in an ecologically dysfunctional population (Arnold 2001). Nevertheless, user groups and management agencies continue to request that population enhancement efforts be undertaken, and we will continue our efforts to determine if or under what conditions we can meet those requests.

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ABUNDANCE OF OCEAN QUAHOG, *ARCTICA ISLANDICA*, ASSESSED BY UNDERWATER PHOTOGRAPHY AND A HYDRAULIC DREDGE

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ABSTRACT Abundance of ocean quahog (*Arctica islandica*) was estimated with underwater photography and a hydraulic dredge at 15 m depth in Önundarfjördur, NW Iceland. Abundance estimates based on counts of siphons from the underwater photographs were much higher than from analysis of the dredge catches. A total of seven taxa were identified from photographs, of which only ocean quahogs and brittle stars were found in sufficient abundance for further analysis. The large scale spatial distribution of ocean quahogs and brittle stars was contagious, where the data corresponded well with a negative binomial distribution but differed significantly from a Poisson distribution. The Morisita index of dispersion was used to analyze distribution patterns within each photograph. These analyses revealed that even on small spatial scales the distribution of ocean quahog could be contagious.

KEY WORDS: ocean quahog, distribution, abundance, underwater camera, hydraulic dredge, *Arctica islandica*

INTRODUCTION

The ocean quahog, *Arctica islandica*, is an important commercial species in Icelandic (Thórarinsdóttir & Einarsson 1996) and US waters (Kennish & Lutz 1995). Stock assessments of ocean quahog have generally been performed using hydraulic dredges. However, accurate quantitative abundance estimates cannot be provided if the efficiency of the dredge is not known. Besides, efficiency may vary with environmental conditions and between different types of hydraulic dredges (Eleftheriou & Holme 1984, Kennish & Lutz 1995, Anonymous 1998). Abundance of ocean quahog has also been estimated quantitatively from grab samples (Arntz & Weber 1970, de Wilde et al. 1986) but because of the small surface area the grab collects, relative to the low abundance of ocean quahogs in sediments, this laborious method requires a very large sample size to obtain reasonable quantitative estimates.

Many studies have been performed in the last decade using underwater photographic techniques to estimate abundance of epifaunal bivalves, such as scallops (Langton & Robinson 1990, Stokesbury & Himmelman 1993, Goshima & Fujiwara 1994) and deep burrowing crustaceans, based on counts of burrows (Chapman et al. 1975, Hughes & Atkinson 1997). However, to our knowledge only one photographic study has estimated abundance of infaunal bivalves (Wigley & Theroux 1970). Estimation of abundance from photographs is more difficult for infaunal bivalves than for epifaunal bivalves because identification depends solely on siphon characteristics and the protruding parts of the shell. However, siphon characteristics can be very distinctive and infaunal bivalves have been identified to species and genus level by using these features (Siferd & Welch 1992).

The stock size of ocean quahogs off Iceland has been estimated using a hydraulic dredge (Eiríksson 1988, Thórarinsdóttir & Einarsson 1996). The stock size in Önundarfjördur NW Iceland was assessed by this method in 1994 (Thórarinsdóttir & Einarsson 1996). Assessments conducted with dredges provide only information about the larger scale (>10 m^2) distribution patterns of ocean quahogs. However, variation in distribution patterns may occur on much smaller spatial scales (0.1–10 m^2). At these scales, distribution of ocean quahogs could be influenced by small scale variation in sediment grain size, bottom topography, and the presence of other infaunal bioturbators.

Assessments of ocean quahog stocks using underwater photography has the clear advantage that quantitative information on abundance of ocean quahogs can be assessed more rapidly and cheaply compared with surveys conducted with hydraulic dredges. The aim of this study was to assess whether underwater photography can be used to estimate the abundance of ocean quahogs and secondly to assess how reliable these estimates are by comparing them with estimates obtained with hydraulic dredge within the same area. Furthermore, large and small scale distribution patterns of ocean quahogs and brittle stars were investigated.

MATERIALS AND METHODS

The study was performed in July 1999 in Önundarfjördur NW Iceland (66°02′N–23°34′W) at a depth of 15 m. A 35 mm vertically oriented underwater camera (Photosea 1000A) loaded with a roll of 10 m (250 exposures) of 200 ASA film (KODAK) and an obliquely oriented 150 watt strobe (Photosea 1500S) were placed on a steel frame. A videocamera directly connected to a monitor was also attached to the frame. While the vessel was drifting, the frame was repeatedly (at 30-sec intervals) lowered until the trigger weight touched the bottom, activating the camera and the flash to take a photograph (at a speed of 1/100 sec). Because the flash from the strobe could be clearly seen in the video monitor, the camera equipment was hauled up ≈2 m immediately after each photograph was taken. Photographs were taken at a constant distance (0.9 m) from the bottom (the distance from the camera to the trigger weight) and therefore the area of seabed covered in each photograph was always the same (0.46 m^2). The position of the start and the end of each photographic transect was located by a global positioning system.

To analyze the photographs, they were displayed using a slide projector onto a board (144 cm × 100.5 cm), the size of the displayed image matching the size of the board. The board was divided into 24 squares (i.e., sampling units), each of 0.019 m^2 and all animals on the photographs were counted and identified to the lowest taxonomic level possible. To estimate numbers of ocean quahogs per photograph, all siphons were counted. The siphon of the ocean quahog is very short and consists of inhalant and exhalant apertures, which lie close to each other on the inner fold of the mantle. These are the only parts of the bivalve that can be seen on the sediment surface (Fig. 1).

Only two species were found in sufficient abundance for statistical analysis, ocean quahogs (*Arctica islandica*) and brittle stars

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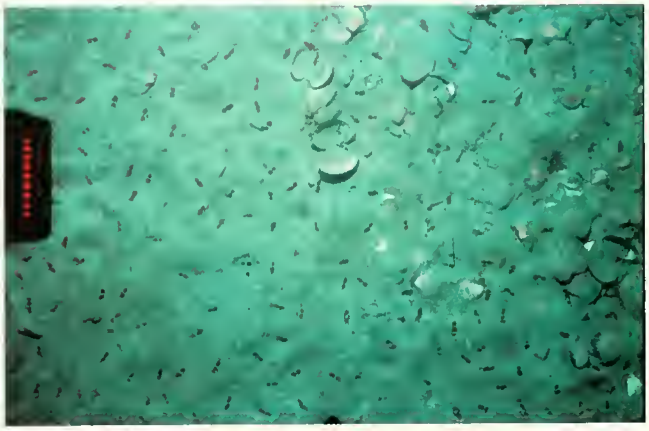

Figure 1. Underwater photograph of the seabed showing siphons of ocean quahogs.

(Ophiuroidea). The large scale distributions of the ocean quahog and brittle stars were compared with Poisson (random) and negative binomial (contagious) distributions using a chi-square test (Elliot 1977). To calculate the goodness-of-fit (chi-square) of the Poisson distribution, only variance and average abundance statistics are required. The negative binomial distribution is based on two parameters, arithmetic mean and the exponent k, which is related to the spatial distribution, and is calculated iteratively using the maximum-likelihood equation (Elliot 1977). In the negative binomial distribution, the variance is greater than the mean, indicating that the distribution of individuals is patchy, whereas if it is the same as the mean, the distribution is random (Poisson). The significance of departures from random and negative binomial distributions were tested using chi-square tests. The extent of contagion in the distribution of ocean quahog was furthermore assessed using the variance to mean ratio (Elliot 1977).

The abundance of ocean quahogs was in general sufficiently high to allow analysis of small-scale distribution patterns within each photograph using the Morisita index of dispersion (Elliot 1977). This index is ideal for this analysis because it is independent of the mean and total number of individuals per photograph and the number of sampling units (i.e., photographic squares) was always the same. The index is greater than one for a contagious distribution, less than one for a regular distribution, and equal to one for a random distribution. Data for analysis were based on abundance of individuals within each of the 24 squares of the photograph. Photographs with less than 20 individuals were not considered suitable for this analysis.

A single cage hydraulic dredge was used to make a comparative investigation of the abundance of ocean quahogs in the same area where the underwater photography took place. The dredge had an overall dimension of 590 × 103 × 230 cm, with a 150 cm wide cutting blade. The bar spacing in the dredge was 34 mm. To determine ocean quahog densities, the distance covered by the dredge was calculated. Ocean quahogs from the catch were weighed, and the total catch weight was divided by the size of the area covered in the tow (5 min duration) to give biomass caught in kg m^{-2}. Individuals in a subsample of 25 kg were counted, measured and weighed. The towing speed was 2.4 nautical miles per hour.

RESULTS

Six invertebrate taxa were identified from a total of 180 seabed photographs. Of these only ocean quahogs (*Arctica islandica*) and brittle stars (Ophiuroidea) were found in high numbers (Table 1). The brittle stars could not be identified down to species level from the photographs. The remaining species, *Asterias rubens* (starfish), *Buccinum undatum* (whelk), *Cucumaria frondosa* (sea cucumber), and *Hyas araneus* (spider crab) were all found with mean abundance less than 0.1 m^{-2}. Ocean quahog was present in 97% of the photographs (max = 148 m^{-2}; Fig. 2a) and brittle stars in 71% of photographs (max = 252 m^{-2}; Fig. 2b). The large scale distribution patterns of ocean quahogs and brittle stars agreed with the negative binomial distribution but differed significantly from the Poisson distribution (Table 1). Furthermore, the variance-to-mean ratio exceeded unity for both ocean quahog and brittle stars (Table 1). The findings from both these analyses suggest that the large scale distribution of ocean quahogs and brittle stars were patchy.

The Morisita index of dispersion ranged greatly between photographs. In 90% of photographs, the Morisita index ranged from 0.68 to 1.62 for ocean quahogs and from 0.46 to 2.58 for brittle stars. These results indicate that dispersion patterns within photographs can be very variable i.e., in some photographs the dispersion patterns of ocean quahogs were highly contagious whereas in others more regular (Fig. 3).

The estimated mean biomass of ocean quahogs in the dredge catch was 1.6 kg m^{-2}, equivalent to 14 indiv. m^{-2} (mean live weight of an individual caught by the dredge was 118 g). Because of the selectivity of the hydraulic dredge, 85% of the clams caught ranged in shell length between 70 to 95 mm, the mean shell length was 82 mm, and no clams <30 mm were observed. Ocean quahog was the only bivalve species found in the dredge samples.

DISCUSSION

This study shows that underwater photography can be used to estimate the abundance of infaunal bivalves, although it may be limited to areas dominated by a single species because identification depends solely on siphon characteristics. Bivalves of size comparable to ocean quahog have not been found in Önundarf-

TABLE 1.

Analysis of the distribution patterns of ocean quahogs and brittle stars.

	Poisson				Negative Binomial		
	Mean	V/M	df (n = 1)	Chi-square	df	k	Chi-square
Ocean quahog	52.8	13.5	177	1343.64*	4	3.68	6.74
Brittle star	43.5	28.9	128	4682.8*	5	1.08	10.37

The goodness of fit of the observed frequency distributions compared with the negative binomial distribution and the Poisson distribution were assessed with the chi-square test.

* $P < 0.005$, mean, mean number of individuals m^{-2}; V/M, variance to mean ratio.

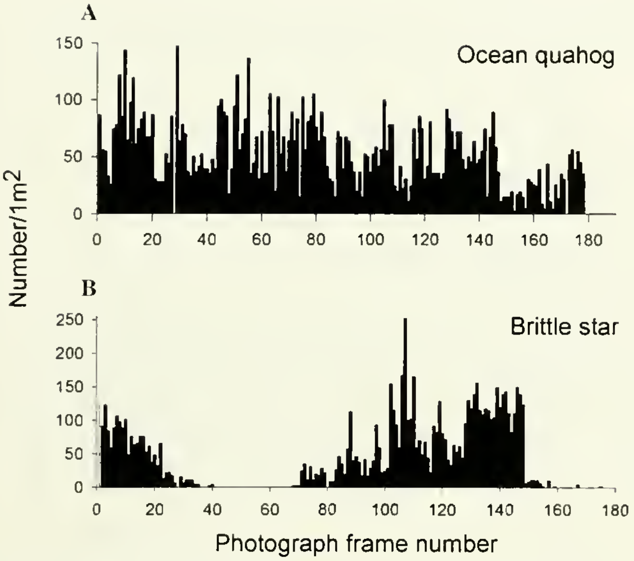

Figure 2. Abundance of ocean quahogs (a) and brittle stars (b), per each successive photographic frame.

jörður in previous surveys (Thórarinsdóttir & Einarsson 1996), suggesting that all siphons seen on the photographs belonged to ocean quahog. Several smaller sized species have been recorded in Önundarfjörður (Gardarsson et al. 1980) but the siphons of these are likely to be too small to be seen on the photographs.

The abundance estimates of ocean quahog based on dredge catch (14 m^{-2}) were about four times lower than based on the photographs (53 m^{-2}). The proportion of individuals (i.e., efficiency) caught by the dredge relative to the total number of individuals in sediments (based on counts from photographs) was therefore 21%. Although this can be regarded as a crude method to calculate efficiency, Thórarinsdóttir and Ragnarsson (2001) obtained a very similar estimate (19%) using the same hydraulic dredge in a different area.

Dredge efficiency may vary with sediment type, current speed, the ratio warp length:water depth, towing speed, hydraulic pressure of the jet, vertical distribution of the clams, and the size of the dredge. This may explain why efficiency estimates can differ so greatly between different studies (Medcof & Caddy 1971, Anonymous 1998).

Ocean quahogs are known to periodically burrow deeper in sediments until the siphons can no longer be seen on the sediment surface (Taylor 1976). Prolonged absence of food (e.g., during winter) has been suggested as one of the factors that triggers them to bury deeper in sediments (Oeschger 1990). Ocean quahogs are able to reduce their metabolic rate to 1% of the aerobic rate, enabling them to remain deeply buried without feeding over long periods of time (Theede et al. 1969, Oeschger 1990). Clearly, if a large proportion of the ocean quahogs are deeply buried, stock assessment with underwater photography or hydraulic dredges will always underestimate abundance.

Recent evidence suggests that the vertical movements of ocean quahogs within sediments are seasonal in Icelandic waters. Siphons were seen on the sediment surface from April to September 2000 but not between the period of November 2000 to February 2001 (Thórarinsdóttir, unpublished data). These findings suggest that ocean quahogs can burrow to greater depths during winter but may emerge to the sediment surface and start feeding when seawater temperature rises and the levels of phytoplankton biomass increases during summer (Thórdardóttir & Eydal 1996). If this proposition remains true, this may explain why catches of ocean quahog per hour towed tend to be much lower in winter than in summer as the catchability of this bivalve is likely to be lower while deeply burrowed in sediments (Thórarinsdóttir unpublished data). It is therefore likely that only a small proportion of individuals were deeply burrowed in sediments when this study was performed.

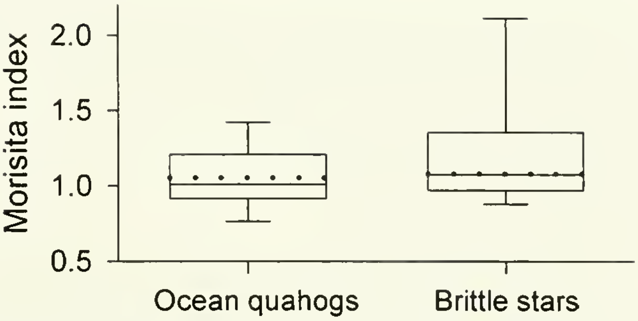

Figure 3. Small-scale distribution patterns (within each photograph) of ocean quahogs and brittle stars assessed with the Morisita index of dispersion. The box plot shows the range in the distribution of the Morisita index in all photographs with more than 20 individuals. The box and whiskers represents 50 and 80% of the data, respectively. Mean (dotted line) and median (solid line) are shown as well.

The present study demonstrates that ocean quahog and the brittle stars were contagiously distributed. Analysis of the distribution patterns within each individual photograph revealed that even on such small scales, the distribution of ocean quahogs and brittle stars can be highly contagious. In some photographs this was caused by the presence of physical features such as stones, which limited their distribution, whereas on other photographs, the underlying reasons for an aggregated distribution were not clear.

Patchy distribution of brittle stars is a common feature widely reported in other studies (e.g., Warner 1971, Piepenburg & Juterzenka 1994). Density-dependent effects (Fujita & Ohta 1990, Summers & Nybakken 2000), predation pressure (Aronson 1989), and local heterogeneity in environmental conditions (Summers & Nybakken 2000) have been suggested to explain contagion of brittle stars.

Stock assessments performed with underwater photographic techniques may provide more accurate quantitative estimates of ocean quahog abundance than using dredges. However, such investigations performed during winter months may greatly underestimate the abundance of ocean quahogs because a large proportion of individuals may be buried deeply in sediments. Furthermore, the small surface area covered by each photograph requires a relatively large sample size to obtain reasonable quantitative estimates. This method is not without limitations and may only be useful in areas where no large infaunal bivalve other than ocean quahog occurs, as identification depends solely on siphon characteristics. Future research should focus on investigating those factors that can influence stock assessment of ocean quahog such as vertical movement in sediments and the efficiency of hydraulic dredges.

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TETRAPLOID INDUCTION BY MEIOSIS INHIBITION WITH CYTOCHALASIN B IN THE DWARF SURFCLAM, *MULINIA LATERALIS* SAY: EFFECTS OF TEMPERATURE

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ABSTRACT Tetraploidy, which is useful for the production of all-triploids, can be induced in marine bivalves by manipulating meiosis. In this study, we tested the effects of temperature on cytochalasin B (CB)-induced meiosis inhibition in a model bivalve, the dwarf surfclam, *Mulinia lateralis* Say. Newly fertilized eggs of the dwarf surfclam were treated with CB (0.75 mg/L) at proper times to block the release of polar body I (PB1) or polar bodies I and II (PB1&2) at 19°C, 24°C, and 28°C. Inhibition of PB1 produced largely triploid and aneuploid-tetraploid (3.7n to 3.8n) larvae, and sometimes small percentages of pentaploids. Inhibition of PB1&2 produced primarily pentaploids and occasionally triploids and aneuploid-tetraploids. Most of the larvae with ploidy levels higher than triploid developed as trochophores only and died a few days after fertilization. At Day 25 post-fertilization, four tetraploids (2%) were detected among 196 juvenile clams in one of the eight PB1&2 groups. No tetraploids were observed in the eight PB1 groups produced. In general, higher (28°C) temperatures did not improve tetraploid production measured as survival to juvenile stage. Low temperature (19°C) made CB treatment less effective, producing all diploid juveniles by Day 17. This work indicates that tetraploidy can be tolerated in *M. lateralis* and scope for further work on this species therefore exists.

KEY WORDS: tetraploidy, triploidy, cytochalasin, temperature, clam, *Mulinia lateralis*

INTRODUCTION

Tetraploid induction in bivalves has been widely investigated because of its potential to provide an ideal approach to triploid production. All-triploid Pacific oysters have been produced by mating tetraploids and diploids (Guo et al. 1996). Triploids are of interest in shellfish aquaculture for their superior growth and improved meat quality. Since the original work on triploidy by Stanley et al. (1981) with the American oyster (*Crassostrea virginica*), triploids have been produced in over 20 bivalves by manipulation of polar body 1 (PB1) and polar body 2 (PB2) as reviewed by Beaumont and Fairbrother (1991) and Guo (1999).

Viable tetraploids have been produced with variable success in fish (reviewed by Pandian & Koteeswaran 1998), and in amphibians (Humphrey & Fanhkauser 1949, Fischberg 1958, Reinschmidt et al. 1979, Nishioka & Ueda 1983). In bivalves, tetraploid larvae have been produced by several methods, including meiotic and mitotic inhibition, meiotic inhibition of gynogenetically activated eggs, and cell fusion (Beaumont & Fairbrother 1991, Guo 1991). However, tetraploid embryos rarely survived beyond metamorphosis in bivalves. In the Pacific oyster *Crassostrea gigas*, tetraploid gynogenesis produced high percentages (95%) of tetraploid embryos, but no viable juveniles were later obtained (Guo et al. 1993). Similar results were obtained by inhibition of mitosis I (Guo et al. 1994), inhibition of PB1 (Stephens 1989, Guo et al. 1992a, 1992b), and blastomere fusion (Guo et al. 1994). There are probably many other failed attempts that have not been reported.

A small number of viable tetraploids have been obtained by manipulating meiosis in blue mussel (Scarpa et al. 1993), Manila clam (Allen et al. 1994), and zhikong scallop (Yang et al. 2000). These sporadic successes have been difficult to reproduce and no breeding populations of tetraploids have been established by manipulating meiosis in eggs from diploids. A different method of tetraploid induction was developed in the Pacific oyster in 1993, when inhibiting PB1 in eggs from triploids produced 2000 viable tetraploids (Guo and Allen 1994). This method produced small numbers of tetraploids in the pearl oyster *Pinctada martensii* (He et al. 2000) and the eastern oyster (Supan et al. 2000). Large numbers of tetraploid spat were subsequently obtained in the Pacific (Eudeline et al. 2000) and eastern (Guo et al. 2002) oysters, demonstrating the reproducibility of the Guo–Allen method. Although the Guo–Allen method is reproducible, it is limited to species in which triploids produce significant numbers of eggs. Therefore, effective methods for direct induction of tetraploids from diploids are needed in some species.

In the search of new candidate species for tetraploid induction and of conditions that might favor its production, we examined the suppression of PB1 alone and both PB1&2 in the dwarf surfclam, *Mulinia lateralis*, by use of cytochalasin B (CB) at different temperatures. Different levels of tetraploids have been produced by inhibiting PB1 at different temperatures in the Pacific oyster (Stephens 1989, Guo 1991). We expect that higher or lower treatment temperatures might affect cellular properties and chromosome segregation patterns, possibly enhancing the effectiveness of the chemical and finally improve survival of tetraploids.

Several characteristic of the dwarf surfclam make it suitable as model for genetic investigations in marine bivalves: a short generation time, a gametogenic activity that occurs over much of the year under cultured conditions, a relatively high reproductive rate, a sex differentiation easily discernible through the shell of sexually ripe specimens, easy conditioning, and small space requirements (Calabrese 1969).

MATERIALS AND METHODS

Broodstock and Gamete Collection

Adult *M. lateralis* were collected from wild populations of Rhode Island. They were conditioned in recirculating seawater at 20°C (salinity 30 ppt) for 2–4 wk before use. During this period, they were fed daily with *Isochrysis galbana* (C-ISO) at densities of 80–100,000 cells/mL. For natural spawning, ripe clams were selected, air-dried for 1 h and induced to spawn in individual beakers containing filtered (1 μm) and UV-sterilized seawater at 24°C. Clams that did not respond to thermal stimulation were dissected for gamete collection. Eggs were passed through a 100-μm-nytex screen to remove any tissue debris, collected, and rinsed on a 20-μm screen. Sperm were passed through a 20-μm screen, and their motility was checked under microscopy before fertilization. The total number of eggs per female was estimated under microscope by counting small aliquots, and the eggs checked for spontaneous development or possible contamination.

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Artificial Fertilization, Chemical, and Thermal Treatments

Sperm were added to egg suspension at about 3–10 sperm/egg. Fertilization and incubation were conducted using filtered seawater with a salinity of 30 ppt at 19–28°C according to the experimental design.

Fertilized eggs were treated with CB, dissolved in dimethylsulfoxide (DMSO) at a final concentration of 0.75 mg/L. All CB treatments started approximately at 5–7 min post-fertilization (PF). The control group was used to gauge the timing of the treatment at all times. For PB1 inhibition, treatments lasted approximately 15–20 min or until about 75% of the untreated eggs released PB1 in the control group. For the retention of both PB1&2, CB treatments were extended for an additional period of 15–20 min, or until the majority of untreated eggs in the control group were ready for the first mitosis (as indicated by the formation of the first polar lobe). After each treatment, the eggs were separated from the chemical by passing them onto a 20-μm screen and gently rinsed with 0.5% DMSO in seawater. Finally, they were suspended in fresh seawater and left undisturbed.

Experimental Design

Experiments were performed using pooled eggs from 4–6 females and pooled sperm from three males. Soon after fertilization the gametes were divided into three groups: in the first group, fertilized eggs were allowed to develop as controls, and in the second and third group the eggs were treated with CB to block PB1 or both PB1&2.

Treatments were tested at normal (24°C), high (28°C), and low (19°C) temperatures. All experiments were replicated three times except for the experiment at low temperature that was conducted twice. Control groups were exposed to DMSO only at the same temperature as the treated groups and for as long as the longest treatment lasted.

Larval Culture and Growth-Out

Larvae were reared in 20-L buckets at 22–24°C and fed daily a diet of *Isochrysis galbana* at densities of 100–120,000 cells/mL starting at 24 h PF. Larvae were cultured at a maximum density of 20 larvae/mL and reached metamorphosis in approximately 8–10 days at a shell length of 180–250 μm. Culture water was completely changed every 48 h, and larvae were collected and washed gently on screens of proper size. D-stage larvae were separated from trochophores by using a 44-μm screen placed on top of a 25-μm screen. At Day 2, the percentages of development (D-larvae and/or trochophores) were calculated from at least 100 individuals per group. Larval survival was determined for all groups at each water change.

After metamorphosis, *M. lateralis* specimens were cultured in upwellers and then in trays contained in a well-aerated recirculating seawater system at a temperature of 19–20°C. Changes of culture water (approximately 1/5 of total volume) were performed every other day.

Determination of Ploidy

Ploidy status of larvae (Day 1–14 PF) and juveniles (Day 17–55 PF) was determined by flow cytometry with DAPI staining according to Allen and Bushek (1992). For larvae, several hundred were sampled after concentrating on a nytex screen and mixed with a DAPI/DMSO stain (Guo et al. 1993). Samples were kept frozen at −80°C and thawed to room temperature before analysis. They were vortexed, passed three times through a 26-gauge needle, and then filtered through a 25-μm-mesh filter. Larvae were analyzed in pooled samples (n = 500–1000), whereas juveniles (n = 100) were analyzed individually. Juvenile samples were prepared by mincing the whole body in a 1.5-mL test tube. Haploid sperm and diploid cells from untreated clams were used as standards at all times.

Statistical Analysis

Fertilization level was determined as percentage of divided eggs at 2–4 h PF. Cumulative survival at Day 2 (D-stage), Day 7, Day 17, and to juvenile was calculated relative to the number of fertilized eggs. Statistical analyses were performed using SYSTAT 10 (SYSTAT Inc.). Percentage data for fertilization and survival were arcsine-transformed and analyzed by ANOVA followed by Tukey's HSD post-hoc comparison. All differences were accepted as significant when $P < 0.05$.

RESULTS

Meiotic and Mitotic Events

At temperatures of 24°C, control eggs started to release PB1 around 12 to 15 min PF, and PB2 at 30–35 min PF. At higher (28°C) or lower (19°C) temperatures, timing was respectively shortened or delayed by 10–15 min. Nevertheless timing of PB1 and PB2 release varied among replicates according to egg quality. In CB-treated groups, no release of PBs was observed during treatments, and meiotic events were restored 3 to 5 min after complete removal of the chemical. Untreated eggs reached mitosis 40–50 min PF except at low temperatures (19°C), where eggs were slightly retarded in development and started cleaving at 55–65 min PF. As expected, mitotic events in CB-treated eggs, particularly in PB1&2 groups, were delayed compared with their control.

Survival and Development

Treatment temperatures did not significantly affect fertilization level within any of the experimental groups, and there was no difference among treated or control groups at any given temperature. Overall, fertilization level ranged between 75% and 99% and varied among replicates (Table 1).

At Day 2, no differences in survival were found between control and treated groups at 24°C or 28°C. At 19°C, the percentage development in the PB1&2 group was 21%, which was significantly lower than in control (53%, $P = 0.009$) and PB1 (51%, $P = 0.01$) groups. At 28°C, eggs in most of the PB1&2 groups survived as trochophores only and did not develop any further, unlike most of the control or PB1 groups where variable proportions of D-larvae (among all larvae) were found (Table 2). At this temperature, survival in the PB1 group was generally low (13–

TABLE 1.

Inhibition of PB1 or PB1&2 in *M. lateralis* under different temperatures: number of eggs used; fertilization level; percent survival of fertilized eggs to Day 2, 7 and 17; and the number of juvenile obtained.

Group	Eggs (×1000)	Fertilization (%)	Day 2 (%)	Day 7 (%)	Day 17 (%)	Juvenile (*n*)
Low temperature (19°C)						
LT1-C	88	90	58	35	6	5,000
LT2-C	103	84	49	25	0.7	600
LT1-PB1	86	86	51	22	1.6	1,200
LT2-PB1	70	89	52	32	0.8	480
LT1-PB1&2	136	90	21	10	0.2	300
LT2-PB1&2	83	93	21	4	0.2	150
Normal temperature (24°C)						
NT1-C	251	97	100	63	56	50,000
NT2-C	278	99	90	20	0	0
NT3-C	158	94	87	66	0.2	350
NT1-PB1	1,045	94	21	8	4	2,000
NT2-PB1	500	99	98	48	20	8,000
NT3-PB1	144	90	40	43	0.6	102
NT1-PB1&2	1,105	94	15	2	0.8	800
NT2-PB1&2	405	99	63	24	0.1	300
NT3-PB1&2	194	89	40	5	0.3	104
High temperature (28°C)						
HT1-C	323	91	5[a]	0	0	0
HT2-C	113	84	29	0.8	0.3	218
HT3-C	265	94	69	53	4	1,000
HT1-PB1	420	89	13	0.7	0.3	200
HT2-PB1	91	84	34	1	0.1	59
HT3-PB1	163	91	15	3	0.3	78
HT1-PB1&2	280	90	2	0	0	0
HT2-PB1&2	156	75	18[a]	0	0	0
HT3-PB1&2	182	93	4[a]	0	0	0

[a] Trochophores only.

TABLE 2.

Inhibition of PB1 or PB1&2 in *M. lateralis* under different temperatures: percentages of D-larvae and trochophores observed in experimental groups at Day 2.

Group	D-larvae (%)	Trochophores (%)
Low temperature (19°C)		
LT1-C	100	0
LT2-C	100	0
LT1-PB1	81	19
LT2-PB1	84	16
LT1-PB1&2	73	27
LT2-PB1&2	75	25
Normal temperature (24°C)		
NT1-C	100	0
NT2-C	100	0
NT3-C	100	0
NT1-PB1	51	49
NT2-PB1	74	26
NT3-PB1	85	15
NT1-PB1&2	26	74
NT2-PB1&2	41	59
NT3-PB1&2	71	27
High temperature (28°C)		
HT1-C	0	100
HT2-C	22	78
HT3-C	92	8
HT1-PB1	25	75
HT2-PB1	23	77
HT3-PB1	35	65
HT1-PB1&2	17	83
HT2-PB1&2	0	100
HT3-PB1&2	0	100

34%) and extremely variable in the control (5–69%). In one control group (HT1-C), all the larvae developed as trochophores only. Overall, percentages of D-larvae at 24°C were significantly higher in control groups (100%) than in PB1 (70%, $P = 0.01$) and PB1&2 (46%, $P = 0.002$) groups, whereas no differences were found between treated groups. At lower temperatures (19°C), again control groups had higher percentages of D-larvae (100%) than PB1 (82%, $P = 0.001$) or PB1&2 (74%, $P < 0.001$) groups. D-larvae percentages were not analyzed for the high-temperature experiment where 100% trochophores were observed in all PB1&2 groups and one control. Trochophores in the CB-treated groups appeared to be deformed and/or swam in a circular motion.

Larvae in all groups metamorphosed between Day 7 and Day 10. At Day 7 and until Day 17, survival of larvae did not differ among treated and control groups at any temperature and varied among replicates (Table 1). Percentage survival to juvenile was generally low within the high-temperature groups, averaging 2.2% and 0.2% in the control and PB1 groups, respectively. An unexpected and complete mortality was encountered after Day 7 in one of the control groups (NT2-C).

In general, the number of surviving juvenile and juvenile clams (Days 16–54) varied greatly among replicates but equally among groups (Table 1).

Ploidy of Larvae and Juveniles

Ploidy of larvae was analyzed on Day 1 and Day 2 to detect possible difference due to differential mortality. No differences in ploidy composition were found between Day 1 and Day 2, and only data from Day 2 are presented.

Ploidy of 2-day-old larvae from treated groups varied greatly among treatments and replicates, ranging from diploidy to pentaploidy (Fig. 1A–D). In particular, PB1 inhibition produced variable proportions of triploid, aneuploid-tetraploid and some pentaploid larvae (Fig. 1A and B), whereas pentaploids were most commonly observed and dominant ploidy in PB1&2 groups. The aneuploid-tetraploid peaks were between 3.6–3.8n, sometimes overlap with 4n. One PB1&2 group had distinctive aneuploid (3.7n) and pentaploid peaks (Fig. 1C), whereas others had clear diploid, triploid, tetraploid and pentaploid peaks (Fig. 1D). After separation of D-stage larvae and trochophores, the flow cytometric analysis of isolated larvae, showed that all or nearly all tetraploid and pentaploid larvae developed as trochophores only (Fig. 2B and D), whereas virtually none or small proportions (Fig. 2C and E) were detected among the D-stage larvae. Control groups from treatments at 19°C and 24°C contained diploid larvae only, unlike controls at 28°C that showed variable proportions of diploid, triploid and pentaploid larvae in two of the three replicates (HT1 and HT2), indicating some influence of temperature on meiotic events. In general, there was no clear pattern that higher or lower temperatures affected the proportions of poly-

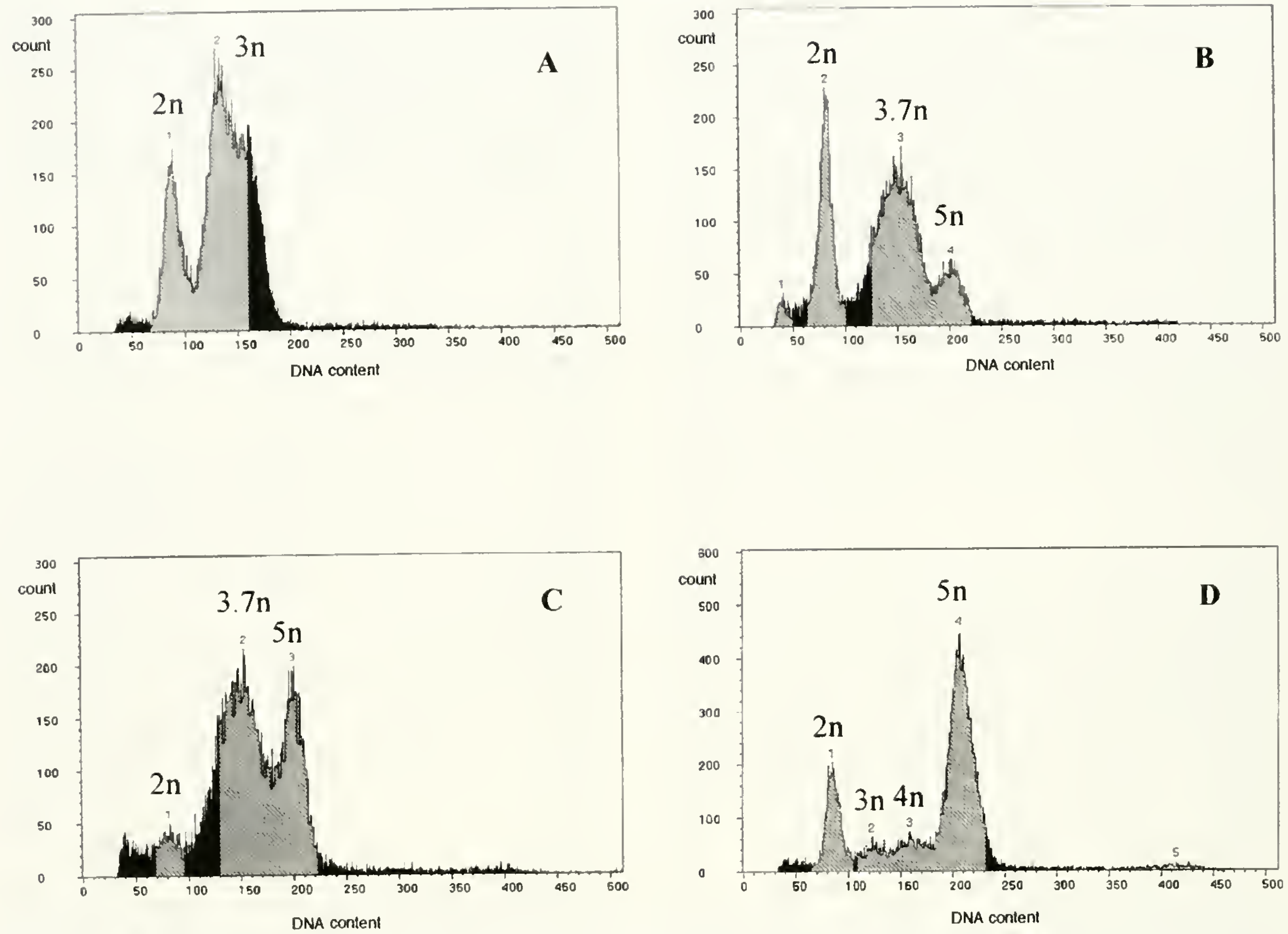

Figure 1. Flow cytometry analysis of 2-day old *Mulinia* larvae from inhibition of PB1 (A, B) and PB1&2 (C, D) showing various ploidy levels induced.

ploids among the treated groups, although precise characterization of ploidy composition was difficult in this study because of the occurrence of aneuploids and tremendous variation among replicates.

The majority of trochophores died within the first 3 days, although some survived to Day 4. Ploidy analysis of larvae was performed several times before metamorphosis and showed virtually no detectable aneuploid, tetraploids, or pentaploids among the surviving larvae after Day 4. Diploids remained the most frequent ploidy in most groups. Distinctive triploid peaks were observed in most PB1 groups at 24°C and 28°C.

After metamorphosis, individual measurements of juvenile and juvenile clams (Days 16–55) showed variable percentages of diploids and triploids in CB-treated groups (Table 3). Of 100 juvenile sampled from one of the PB1&2 groups, two were tetraploid (2%; Table 3 and Fig. 3B). To confirm this finding, another group ($n = 96$) of individuals were analyzed and resulted again in 2% of tetraploids. This group contained juvenile with a measured shell height of 1.5–2.5 mm (Fig. 3A).

At juvenile stages, mean percentages of triploids in PB1 groups did not differ significantly between 24°C and 28°C (Table 3). Eggs treated with CB to inhibit PB1&2 produced viable triploid juvenile in the experiment at 24°C only. No triploids were found among any of the surviving juvenile in the experiment at low temperature (19°C). Three triploids were found among 100 juvenile in one of the control groups (HT2-C) at 28°C, thus confirming the results obtained during early rearing.

DISCUSSION

A low percentage of tetraploid clams (2%) was obtained in one of the eight groups treated to inhibit PB1&2. Although the tetraploid production was low in frequency and inconsistent among replicates, this result provides the first evidence that tetraploid *Mulinia* can survive beyond metamorphosis and reach juvenile stage. This finding provides another example that a small number of viable tetraploids can be produced by manipulating meiosis in normal zygotes. Similarly, small numbers of tetraploids have been obtained in the blue mussel (Scarpa et al. 1993), Manila clam (Allen et al. 1994), and zhikong scallop (Yang et al. 2000).

The production of tetraploids from inhibition of both PBs is unusual. Mature eggs of marine bivalves are arrested at prophase I. Before fertilization, eggs of oysters and clams are actually tetraploids and, theoretically, pentaploids should be produced after fertilization and successful inhibition of PB1&2. The production of pentaploids by blocking both PBs has been demonstrated in the Pacific oyster (Cooper & Guo 1989), in blue mussel (Scarpa et al. 1993), and in this study. Two possible explanations exist why tetraploids were produced in PB1&2 groups. First, it is possible that the CB treatment produced pentaploids, which subsequently reverted to tetraploids through chromosome loss. There is indica-

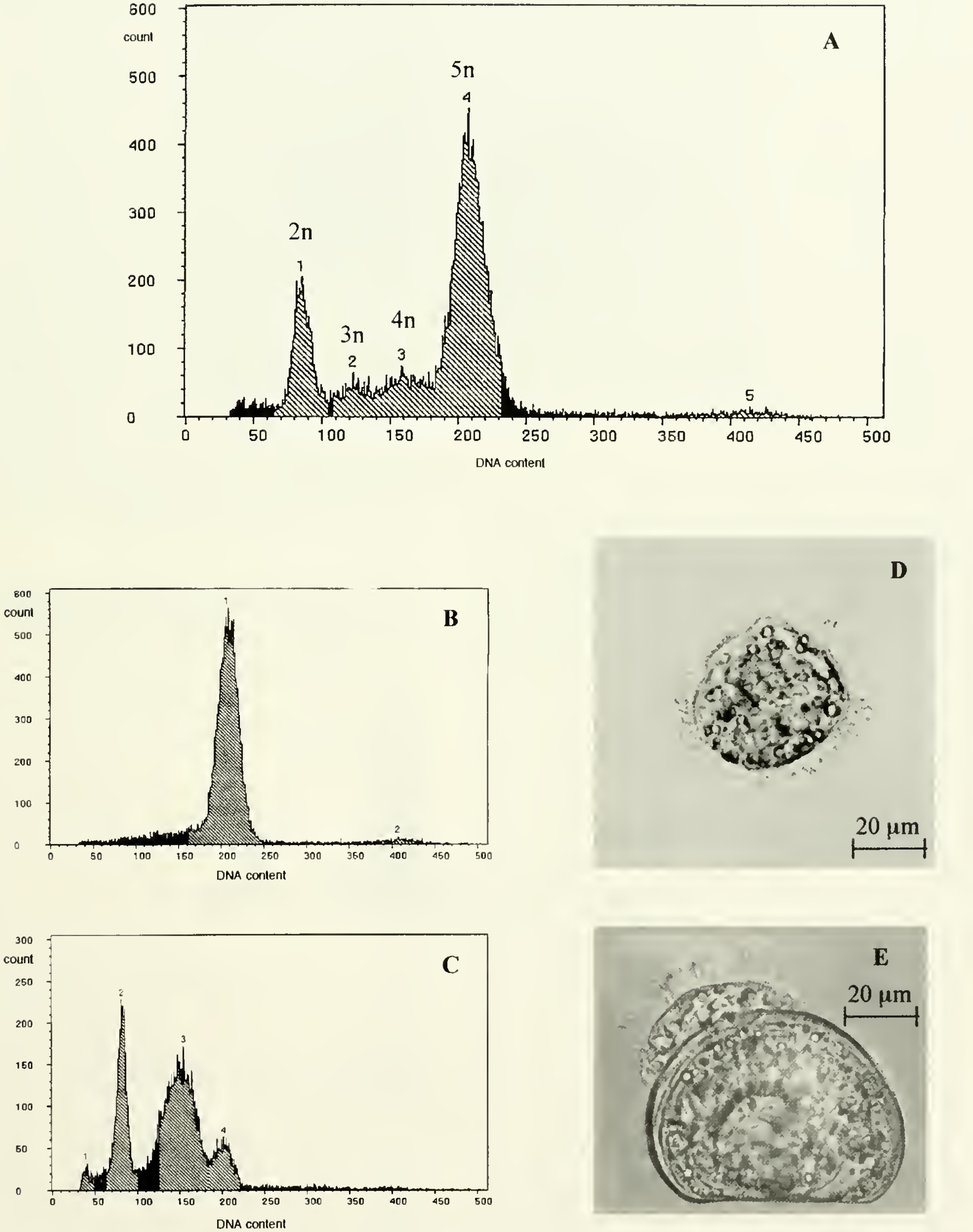

Figure 2. Flow cytometry analysis of 2-day old *Mulinia* larvae from PB1&2 inhibition: A, a sample containing both trochophores and D-larvae; B, trochophores only; C: D-stage larvae only; D: a representative abnormal trochophore; and E: a representative D-stage larva.

tion that tetraploid oysters may revert to triploids or triploid/tetraploid mosaics (Guo et al. 2002). However, reversion is thought to be rare event in triploid and tetraploid oysters (Allen et al. 1997). Secondly, it is possible and even likely that the CB treatment for the inhibition of PB1&2 was not 100% effective and that only PB1 was inhibited in some of the eggs (Guo et al. 1992b, Scarpa et al. 1993). In fact, treatments targeting specific meiotic events are rarely 100% effective because of unsynchronized development of zygotes, which is why triploid induction is rarely 100% effective (Lu 1986, Allen et al. 1989). Therefore, the tetraploids we obtained might be from accidental inhibition of PB1 alone. Interestingly, however, treatments specifically targeting PB1 did not produce viable tetraploids in this study.

Inhibition of PB1 is known to result in a complex segregation pattern that leads to the production of triploids, tetraploids and variable proportions of aneuploids larvae (Guo et al. 1992a, 1992b). These findings were later confirmed in zhikong scallop by Yang et al. (2000). Our study showed that PB1 inhibition had similar effects producing a comparable spectrum of nuclear DNA content in 2-day-old trochophores and D-larvae from CB-treated eggs. Triploids and aneuploids were most commonly observed unlike tetraploids that were rarely detected by flow cytometric analysis. The pentaploid larvae were most likely produced from the incidental inhibition of both PB1 and PB2. Although their ploidy status was not confirmed by chromosome counting, the putative aneuploids (approx. 3.7n) were either hypertriploid or hypotetraploid and possibly produced through tripolar segregation patterns under CB treatment (Guo et al. 1992b). Aneuploids were not found among control groups. Variable proportions of aneuploids were also observed by flow cytometry after PB1 inhibition in zhikong scallop (Yang et al. 2000) and confirmed by chromosome counting. Similar results were obtained on other mollusks by previous workers (Guo et al. 1992a, 1992b, Scarpa et al. 1993, Allen et al. 1994).

Our results show that the higher ploidy levels observed by flow cytometry were mainly represented by populations of trochophores, which declined rapidly over the same period. Clearly, few if any tetraploid embryos survived to D-stage in most groups. The proportion of aneuploids and pentaploids declined abruptly during the first week, and only diploids and triploids were detected among the surviving juvenile. A similar decrease in the proportion of pentaploids and increase in triploids in CB groups was observed in other studies (Scarpa et al. 1993, Guo & Allen 1994). Generally, aneuploidy probably causes imbalance of gene dosage and is often lethal in mollusks with survival depending on specific tolerance to chromosome loss or gain (Guo & Allen 1994, Wang et al. 1999). The failure of tetraploid and pentaploid larvae to survive beyond early developmental stages and metamorphosis has been explained by the unbalanced cytoplasm/nucleus ratios or the cell-number (egg-volume) deficiency hypothesis (Guo 1991, Guo & Allen 1994, Guo et al. 1994). Guo's hypothesis states that the cleavage of eggs of a given size with large tetraploid (or pentaploid) nuclei results in reductions in cytoplasm/nucleus ratio or cell number, both fatal for further development in bivalves. Accordingly, high percentages or large numbers of tetraploids (up to 100%) have been produced using larger eggs from triploids in the Pacific (Guo & Allen 1994, Eudeline et al. 2000) and eastern (Guo et al. 2002) oysters. Supan et al. (2000) also reported the production of tetraploid eastern oysters, but specific numbers and percentages were not available.

Under our experimental conditions, high or low temperature did not improve tetraploid induction as measured by viable tetraploids produced. Effects on ploidy of early larvae were not clear partly because of the tremendous variation within treatments. The presence of large proportions of aneuploids made ploidy determination by flow cytometry difficult. We did not do chromosome counting in this study and had no accurate estimates what chromosome numbers were actually induced under different temperatures. Nevertheless, ploidy of surviving juveniles indicates that high (28°C) and normal (24°C) temperatures produced similar percentages of triploids and that low (19°C) temperature made CB treatment ineffective. Low temperatures did not produce viable polyploids in this study. This result would suggest that a lower temperature might influence the cellular properties and chromosome segregation patterns in this species. In a previous study in the Pacific oyster, high levels of tetraploids were produced under low temperatures (Stephens 1989). High temperatures (28°C) negatively affected the development of larvae deriving from eggs treated to block both PB1&2 and finally provoked complete mortality. It is likely that this result was induced by a combination of long chemical treatment and high temperature. Generally, control groups exposed at the same temperature and for the same duration did produce viable larvae. However, the same temperature was partially effective in blocking PB release and induced 3% triploidy in one control group. Poor egg quality might have favored these

TABLE 3.

Inhibition of PB1 or PB1&2 in *M. lateralis* under different temperatures: age (in days), number, and ploidy composition of *Mulinia* juveniles as determined by flow cytometry.

Group	Age (days)	Juvenile (*n*)	2N (%)	3N (%)	4N (%)
Low temperature (19°C)					
LT1-C	17	100	100	0	0
LT2-C	16	100	100	0	0
LT1-PB1	17	100	100	0	0
LT2-PB1	16	100	100	0	0
LT1-PB1&2	17	100	100	0	0
LT2-PB1&2	16	100	100	0	0
Normal temperature (24°C)					
NT1-C	25	100	100	0	0
NT2-C	na[a]	0	0	0	0
NT3-C	27	100	100	0	0
NT1-PB1	25	100	84	16	0
NT2-PB1	25	100	64	36	0
NT3-PB1	27	102	100	0	0
NT1-PB1&2	25	196	89	9	2
NT2-PB1&2	25	100	83	17	0
NT3-PB1&2	27	104	99	1	0
High temperature (28°C)					
HT1-C	na	0	0	0	0
HT2-C	54	100	97	3	0
HT3-C	51	100	100	0	0
HT1-PB1	55	44	31	69	0
HT2-PB1	54	59	88	12	0
HT3-PB1	51	50	98	2	0
HT1-PB1&2	na	0	0	0	0
HT2-PB1&2	na	0	0	0	0
HT3-PB1&2	na	0	0	0	0

[a] na, not available because of complete mortality.

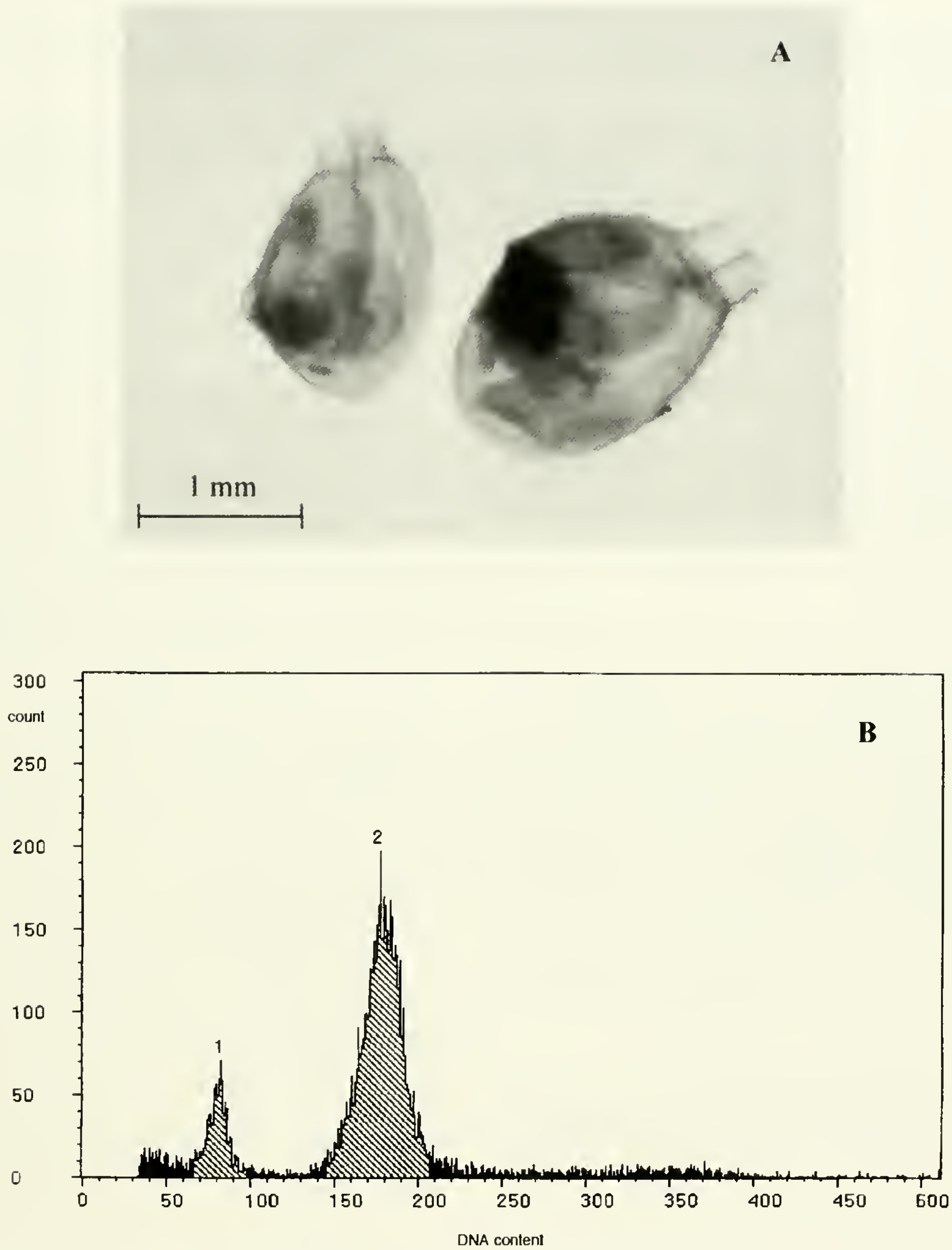

Figure. 3. Flow cytometry analysis of 25-day-old *Mulinia* clam: A, representative juvenile clams analyzed; B: analysis of a diploid (1) and a tetraploid clam (2).

conditions too. Heat shocks in the range of 32–40°C are most commonly required in blocking the release of PBs in other mollusks (Quillet & Panelay 1986, Yamamoto & Sugarawa 1988, Guo et al. 1994).

In summary, this study indicates that temperature has little effects on the final outcome of tetraploid induction in *M. lateralis*. Temperature affects the timing of meiotic segregation and therefore should affect tetraploid induction frequency. We could not detect such effects in this study probably due to random variation in treatment and eggs quality. Considering the low induction efficiency (2%), manipulating meiosis may not be a viable approach to tetraploid production in this species. Nevertheless, this study provides the first evidence that tetraploids are viable in *M. lateralis* and offers encouragement for further research on tetraploidy in this species.

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CYTOGENETIC STUDY OF *OSTREA CONCHAPHILA* (MOLLUSCA: BIVALVIA) AND COMPARATIVE KARYOLOGICAL ANALYSIS WITHIN OSTREINAE

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ABSTRACT Chromosome preparations of the Olympia oyster *Ostrea conchaphila* Carpenter were studied using conventional Giemsa, silver staining, and C-banding techniques. The karyotype consists of six metacentric (1, 2, 4, 6, 8, and 10) and four submetacentric (3, 5, 7, 9) chromosome pairs. The silver-stained nucleolus organizer regions (Ag-NORs) were terminally located on the short arms of the submetacentric pair 5 (56% of cases) and on the long arms of submetacentric pair 7 (6% of cases). Constitutive heterochromatin was observed as telomeric C-bands on the short arm of the NOR-bearing chromosome pair 5 and as centromeric blocks of several chromosome pairs. Comparative analysis of patterns of karyotype, Ag-NORs, and C-bands of this species and of five other flat oysters, *Ostrea angasi, O. chilensis, O. denselamellosa, O. edulis,* and *O. puelchana,* for which data have been previously published, were performed, allowing the inference of cytotaxonomic relationships within Ostreinae.

KEY WORDS: *Ostrea conchaphila*, cytogenetics, cytotaxonomy, Ostreinae

INTRODUCTION

Studies on oyster cytogenetics have been performed so far on 26 species of Ostreacea (see Nakamura 1985, Ieyama 1990, Thiriot-Quiévreux 2002). The first data only concerned chromosome number and gross morphology (Ahmed & Sparks 1967, Menzel 1968). Later, morphometric measurements of chromosomes enabled the comparison among karyotypes at the interspecific and intraspecific level (e.g., Ladron de Guevara et al. 1996, Li & Havenhand 1997). During the last decade, the development of banding techniques has allowed the fine characterization of individual chromosomes (e.g., Leitão et al. 1999a).

According to the morphologically based classification of Harry (1985), which is currently used, the family Ostreidae includes three subfamilies, that is, Lophinae, Ostreinae, and Crassostreinae. These oysters are sequential hermaphrodites and contain both broadcast spawners (Crassostreinae) and brooders (Lophinae and Ostreinae). Recent techniques such as molecular phylogenetic analysis provided novel insights into oyster evolution and systematics (Littlewood 1994, Jozefowicz & Ó Foighil 1998, Ó Foighil & Taylor 2000). Karyological analysis among cupped oysters, the Crassostreinae (Leitão et al. 1999b), has proven complementary to these approaches and has provided additional evolutionary inferences.

Among the flat brooding oyster species, the Ostreinae, five species have been previously karyologically investigated: *Ostrea edulis* (Linné) (Thiriot-Quiévreux 1984), *O. denselamellosa* (Lischke) (Insua & Thiriot-Quiévreux 1991), *O. puelchana* (Orbigny) (Insua & Thiriot-Quiévreux 1993), *O. chilensis* (Philippi) (Ladron de Guevara et al. 1994), and *O. angasi* (Sowerby) (Li & Havenhand 1997).

The Olympiá oyster, *O. conchaphila* (Carpenter 1857), previously known as *O. lurida* (Carpenter 1864), has been studied by Ahmed and Sparks (1967) and Ahmed (1973) using squash techniques and tentative grouping of chromosomes. *Ostrea conchaphila*, native to the western United States and Canada, ranges from the southeast Alaska to Baja California (in tidal channels, estuaries, bays, and sounds). Commercially important in the late 19th century, this species was cultured in the state of Washington until near-collapse of the industry in the 1950s (Baker 1995).

In the present work, the karyotype, nucleolus organizer regions (NORs), and constitutive heterochromatin distribution were studied in *Ostrea conchaphila* (Carpenter 1857) and a comparison with previously published karyological data on the five other flat oyster species mentioned above was performed to analyze cytotaxonomical relationships within Ostreinae.

MATERIALS AND METHODS

Specimens of the Californian Olympia oyster *Ostrea conchaphila* (G0) were imported from the Pacific Institute (Olympia, WA). Oysters were strictly confined to the quarantine facilities of the IFREMER hatchery of La Tremblade, Charente-Maritime, France, according to international recommendations. After reproduction, the progeny (G1) used in this experiment was reared in the same quarantine facilities for at least 5 mo before sampling.

Whole juvenile animals (ca. 2.5 cm length) were incubated for 7–9 h in a 0.005% solution of colchicine in seawater. The gills were then removed by dissection and treated for 30 min in 0.9% sodium citrate in distilled water. The material was fixed in a freshly prepared mixture of absolute alcohol and acetic acid (3:1) with three changes of 20 min each. Fixed pieces of gill from each individual were dissociated in 50% acetic acid with distilled water solution. The suspension was dropped onto heated slides at 44°C and air-dried (Thiriot-Quiévreux & Ayraud 1982).

For conventional karyotypes, gill preparations were stained with Giemsa (4%, pH 6.8) for 10 min. The silver-staining method for NORs was performed on unstained slide preparations according to the procedure of Howell and Black (1980). This method only detects those NORs that were active at the precedent interphase (Miller et al. 1976). Chromosomal Ag-NORs can serve as characters for inferring phylogenetic relationship (e.g., Amemiya

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& Gold, 1990). Constitutive heterochromatin regions (C-bands) were revealed using the method of Sumner (1972) with the counterstain propidium iodide. The evolutionary significance of the heterochromatin has previously been discussed in vertebrates (e.g., Hsu & Arrighi 1971, Saffery et al. 1999, Chaves et al. 2000).

Images of Giemsa-stained metaphases and C-banding were acquired with a CCD camera (Axioplan, ZEISS) coupled to a ZEISS Axioplan microscope. Digital images were processed using Adobe Photoshop 5.0 (Windows) using functions affecting the whole of the image only. Microphotographs of Giemsa stained metaphases and C-banding were taken with a ZEISS Axioplan microscope. Digital images were processed using Adobe photoshop 5.0 (Windows). Microphotographs of suitable NOR-stained metaphases were taken with a ZEISS III photomicroscope.

After karyotyping, chromosome measurements of 10 suitable metaphases were made with a digitizer table (Summa Sketch II) interfaced with a Macintosh. Data analysis was performed with an Excel macro-program. Relative length was expressed as 100 times the absolute chromosome length (in μm) divided by the total length of the haploid complement. Centromeric index was calculated by dividing 100 times the length of the short arm by the total chromosome length. The arm ratio was determined (length of short arm divided by length of long arm). Both centromeric index and arm ratio are given because each expresses centromere position and allows comparison with other karyological studies. Terminology relating to centromere position (m: metacentric, sm: submetacentric) follows that of Levan et al. (1964).

To elucidate similarities between Ostreinae species, a hierarchical agglomerative flexible clustering program was used (Lance & Williams 1966). Both NOR and centromeric index information of *O. conchaphila* and five previously studied Ostreinae species were used to cluster species. The Manhattan metric was used to discriminate and then to associate individual species. Manhattan distance appears appropriate to this kind of combination of quantitative (centromeric index values) and qualitative (NORs positions) data and to measure an association between individual objects (species) (Legendre & Legendre 1998).

RESULTS

Analysis of 60 mitotic metaphase spreads from 15 individuals of *O. conchaphila* confirmed the diploid chromosome number of $2n = 20$, scored by Ahmed and Sparks (1967). For karyotyping, the chromosomes of 21 well-spread metaphases were paired on the basis of chromosome size and centromere position. From these, the 10 best spreads were used for chromosome measurements and classification (Table 1). The karyotype (Fig. 1A) consists of ten chromosome pairs. Pairs 1, 2, 4, 6, 8, and 10 were metacentric. Pairs 3, 5, 7, and 9 were submetacentric.

The Ag-NORs were examined in another 122 metaphases from 10 animals. A variable number of one to three Ag-NOR chromosomes were identified (Fig. 1B). The NOR site was located terminally on the short arms of the submetacentric pair 5 and on the long arms of the submetacentric chromosome pair 7. The most frequent case (56% of observed silver-stained metaphases) was one active silver-stained NOR chromosome in pair 5. The Ag-NORs located on pair 7 occurred in few cases (6%).

Constitutive heterochromatin was observed in 31 karyotypes made from well-spread C-banded metaphases from 13 animals. Telomeric C-bands were always observed on the short arm of the NOR-bearing chromosome pair 5. In addition, centromeric blocks were also found in chromosome pair 2 in 84% of observed metaphases, pairs 1, 4, and 5 in 68%, pairs 6 and 8 in 58% of the C-banded karyotypes and in fewer cases in pairs 3, 7, and 9 (35%), and in pair 10 (26%) (Fig. 1C).

To compare the karyological data from *O. conchaphila* and from the other five flat oyster species previously studied, ideograms (Fig. 2) were constructed from relative length and centromeric index values of *O. conchaphila* (see Table 1), *O. edulis* (after Leitão 2000, French population of La Tremblade hatchery, Charentes Maritimes, France), *O. angasi* (after Li & Havenhand 1997), *O. chilensis* (after Ladron de Guevara et al. 1994), *O. denselamellosa* (after Insua & Thiriot-Quiévreux 1991), and *O. puelchana* (after Insua & Thiriot-Quiévreux 1993). The location of Ag-NORs was also included because chromosomal NOR have been used as characters for inferring hypothesis of cytotaxonomic relationships (e.g., Amemiya & Gold 1990, Leitão et al. 1999 b).

The comparison of the relative length and centromeric index of the 10 chromosomes pairs of the studied species showed that pair 1 was similar among all species, pair 2 was also similar except for *O. puelchana*, pair 3 and 4 were similar except for *O. conchaphila*, but taking into account the close relative length and the standard deviation of pair 3 and 4 of *O. conchaphila*, they may be inverted. Pair 5 was variable among species, pairs 6 and 7 were identical except for *O. denselamellosa*, but in this case, the pairs 6 and 7 cannot be inverted because of their different relative length and the

TABLE 1.

Chromosome measurements and classification in 10 cells of *Ostrea conchaphila*.

Chromosome Pair No.	Relative Length		Arm Ratio		Centromeric Index		Classification
	Mean	SD	Mean	SD	Mean	SD	
1	12.77	0.99	2.39	0.21	42.12	1.78	m
2	11.60	0.55	2.54	0.18	43.87	1.89	m
3	10.64	0.57	1.26	0.20	27.76	2.86	sm
4	10.54	0.50	2.37	0.27	41.94	2.81	m
5	10.47	0.87	1.72	0.16	34.05	2.28	sm
6	9.88	0.78	2.48	0.25	42.81	2.67	m
7	9.46	0.38	1.26	0.16	27.56	2.59	sm
8	9.30	0.58	2.53	0.39	43.59	3.92	m
9	8.79	0.63	1.52	0.25	31.27	3.37	sm
10	6.56	0.65	2.43	0.26	42.18	2.60	m

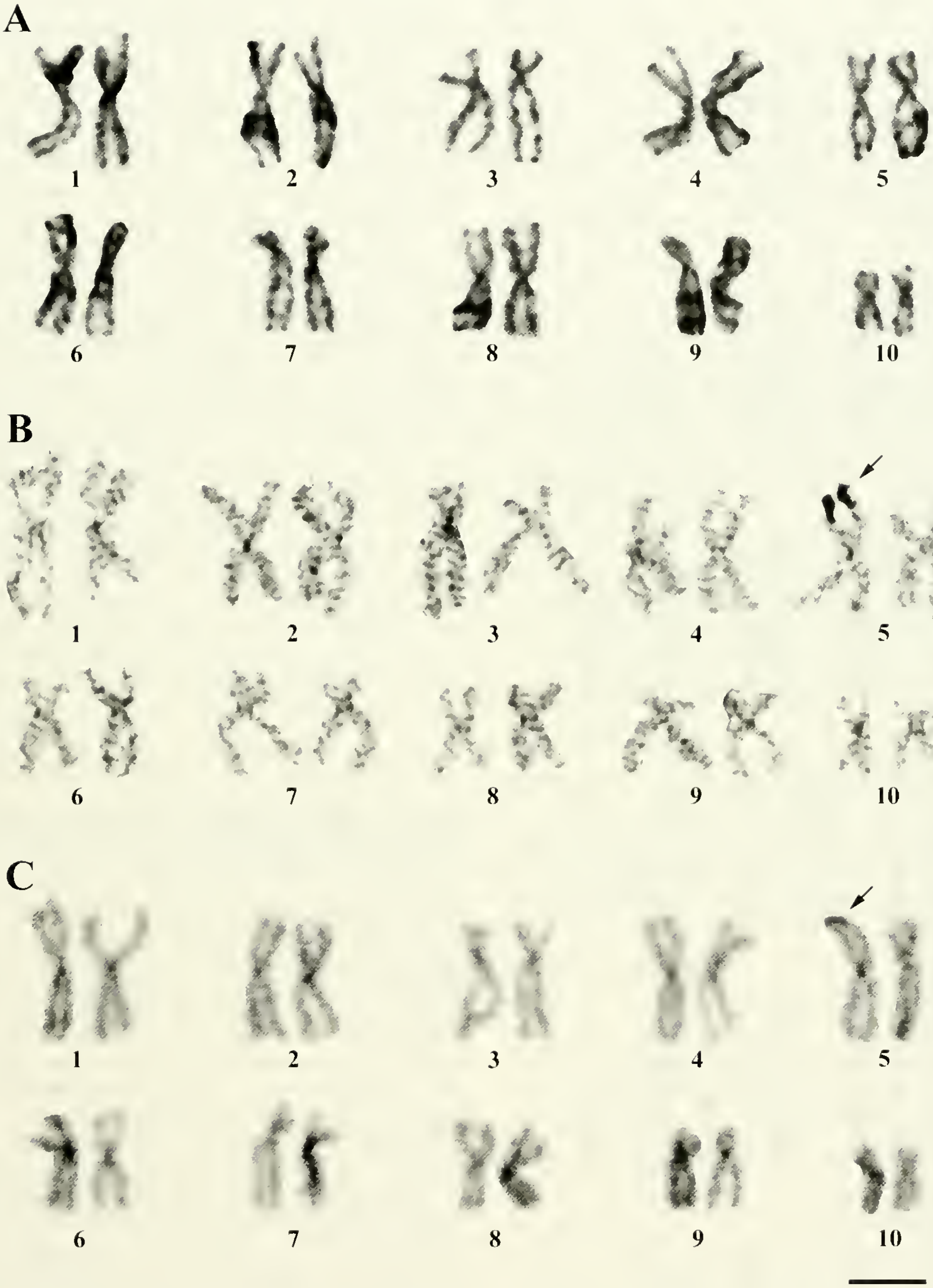

Figure 1. Karyotypes of *Ostrea conchaphila*. A, Conventional Giemsa staining; B, silver-stained nucleolus organizer regions (Ag-NORs); C, C-banding. Note the simultaneous presence of Ag-NOR and C-bands in a telomeric position on the short arms of pair 5 (arrows) and the centromeric heterochromatic blocks on chromosome pairs 1, 2, 4, 5, and 9. Scale bar = 5 μm.

C-banding of pair 6. Pair 8 was variable among species. Pair 9 was identical except for *O. angasi* and pair 10 was variable.

A statistical analysis based on Ci and NORs (Fig. 3) highlighted the clustering of *O. edulis* and *O. angasi* and of *O. denselamellosa* and *O. chilensis* with *O. conchaphila* placed near this cluster. *O. puelchana* is separated from the other species by the highest dissimilarity.

DISCUSSION

This is the first report on karyotype after chromosome measurements and NORs and C-banding patterns of the Olympia oyster. The diploid chromosome number $2n = 20$ observed is characteristic of the genus *Ostrea* and is common throughout the Ostreacea (Nakamura 1985, Thiriot-Quiévreux 2002). The karyotype,

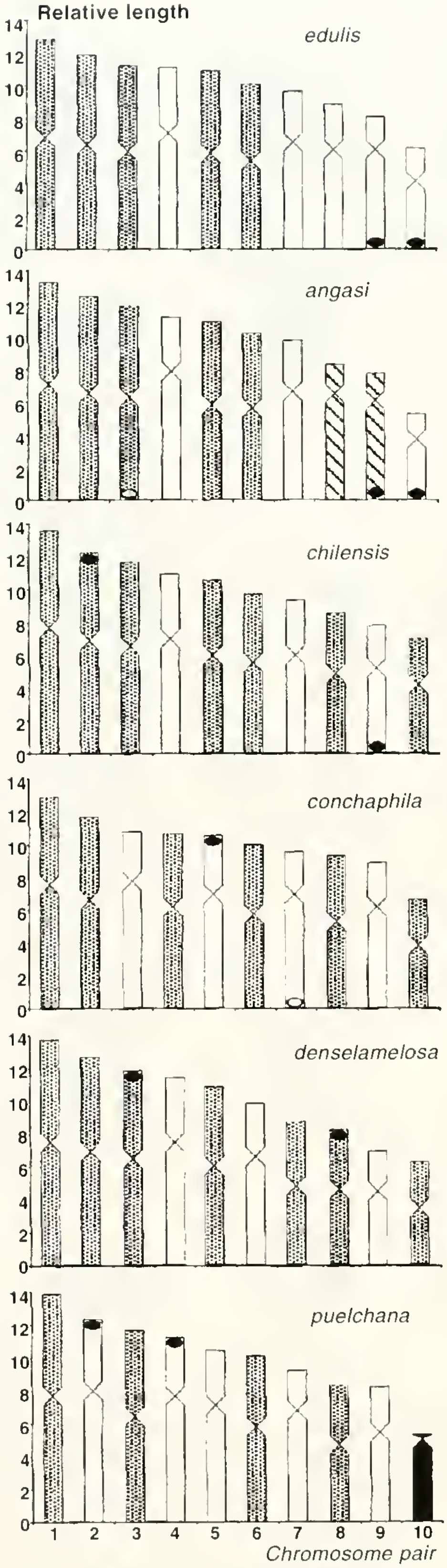

Figure 2. Ideograms of six flat oysters constructed from relative length and centromeric index values. Stippled chromosome: metacentric, white chromosome: submetacentric, striped chromosome: subtelocentric, black chromosome: telocentric. Circles indicate Ag-NORs, dark circles the most frequent case.

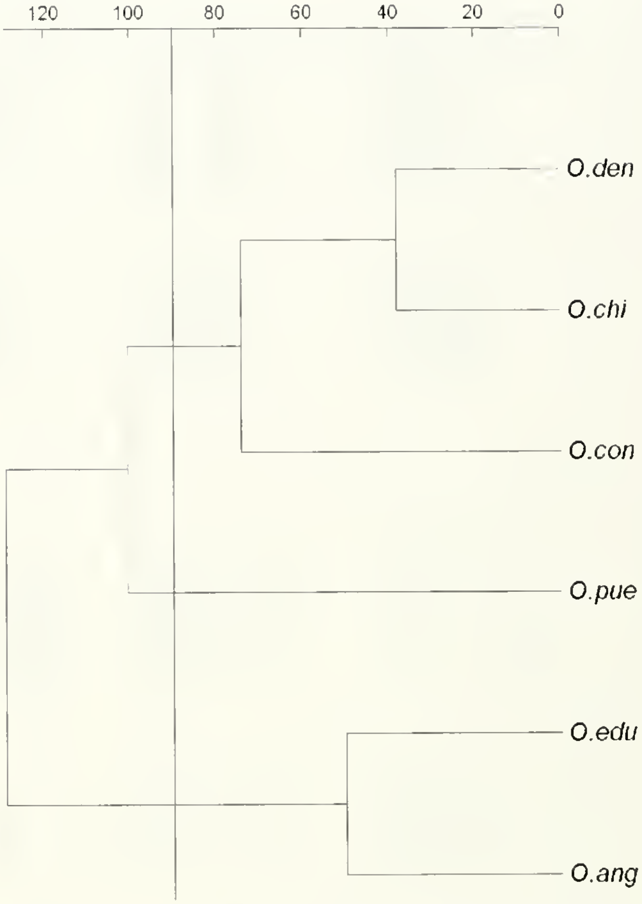

Figure 3. Hierarchical agglomerative flexible clustering of *Ostrea* spp. O. den: *Ostrea denselamellosa;* O. chi: *O. chilensis;* O. con: *O. conchaphila;* O. pue: *O. puelchana;* O. edu: *O. edulis;* O. ang: *O. angasi.*

including six metacentric and four submetacentric chromosome pairs, and the NOR and C-band distribution differ from the other ostreinid species studied. The comparison of the relative length and centromeric index of the 10 chromosome pairs of the studied species shows that, if one postulates that shared chromosome pairs with the same relative length and centromeric index may be considered as primitive, pairs 1, 3, and 4 are primitive and pairs 5, 8, and 10 the most derived. However, these chromosome homologies should be confirmed by other banding techniques.

The comparison of karyotypes and location of Ag-NORs among species highlighted first the chromosome similarity between the European species *O. edulis* and the Australian and New Zealand species *O. angasi*, already pointed out by Li and Havenhand (1997). Their karyotypes differ slightly (5m, 5 sm in *O. edulis* and 5m, 3 sm, 2 st in *O. angasi*), but the phenomenon of variation in the number of submetacentric and subtelocentric chromosomes have been reported in French populations (Thiriot-Quiévreux 1984). More striking is that the most frequent Ag-NOR patterns are similar in both species.

The isolated karyotype of *O. puelchana* is remarkable because of the single telocentric chromosome. The occurrence of telocentric chromosomes has been only seen in one other species of Ostreidae. *Dendrostrea folium* (Lophinae) (Ieyama 1990).

The three other flat oysters bear high karyotype resemblance, that is, seven metacentric and three submetacentric pairs for *O. denselamelosa* and *O. chilensis* and six metacentric and four submetacentric pairs for *O. conchaphila*. Their NOR chromosomal location revealed that there is a higher resemblance between the NOR patterns of *O. chilensis* and *O. conchaphila* than between these two species and *O. denselamellosa*. *O. chilensis* and *O. conchaphila* showed terminally located NORs on the short arms of one chromosome pair and on the long arms of another chromosome pair. On the contrary, in *O. denselamelosa*, Ag-NORs were always terminally located on the short arms of chromosome pairs.

Data on constitutive heterochromatin distribution only concerned three species, *O. denselamellosa* (Insua & Thiriot-Quiévreux 1991), *O. angasi* (Li & Havenhand 1997), and *O. conchaphila* (this study). Centromeric C-bands were observed in chromosome pairs 3, 6, 8, 9, and 10 in *O. angasi* and in pairs 6, 8, 9, and 10 in *O. denselamellosa*. Occasional C-bands were seen on the centromere of pairs 4 and 7 in *O. angasi* and on telomeres of pairs 3, 5, 6, 8, 9, and 10 in *O. denselamellosa*. A substantial proportion of the eukaryote genome consists of constitutive heterochromatin. This genomic fraction includes, among other repetitive sequences, satellite DNA. Sequence analysis of these repeats suggests that the sequences are rapidly evolving, and hence they are valuable as evolutionary markers; consequently, constitutive heterochromatin analysis can give insights about the phylogeny relationships of related species (Saffery et al. 1999, Chaves et al. 2000). The observation in *O. denselamellosa* and *O. conchaphila* of the simultaneous presence of Ag-NORs and C-bands on telomeric position in the same chromosome pair, that is, pairs 3 and 8 in *O. denselamellosa* and pair 5 in *O. conchaphila*, might corroborate the close karyological relationship between these two species noted above.

The cytotaxonomic relationships pointed out here are incongruent with the morphologically based classification of Harry (1985), who stated that *O. chilensis* and *O. angasi* were junior synonymous of *O. puelchana* in the subgenus *Eostrea* of the genus *Ostrea* and that *O. edulis* and *O. denselamellosa* were included in the subgenus *Ostrea* ss. The species *O. lurida* was considered as a junior synonymous of *O. conchaphila* in the genus *Ostreola*. Li and Havenhand (1997) have also previously disagreed with Harry (1985), placing *O. angasi* as a separate species, very close to *O. edulis*.

Our results show greater congruence with molecular phylogenetic analyses of the Ostreinae, based on partial mitochondrial 16S rDNA (Jozefowicz & Ó Foighil 1998) and nuclear 28S rDNA (Ó Foighil & Taylor 2000) datasets. This is most evident for *O. edulis* and *O. angasi*, where a sister species relationship for these European and Australian flat oysters is strongly supported by both karyological and gene tree data. The ostreinid mitochondrial gene trees place the six karyologically-characterized flat oysters into two clades: one containing (among other taxa) *O. puelchana*, *O. conchaphila*, and *O. denselamellosa*, the other composed of *O. edulis*, *O. angasi*, and *O. chilensis*. With the exception of positioning of *O. chilensis*, which in our study is closer to *O. denselamellosa*, these results are in broad agreement with the topology generated by our statistical analysis based on Ci and NORs.

All Ostreinae species are of the brooding type with an extended planktotrophic larval development with the exception of *O. chilensis*, which shows a greatly abbreviated pelagic phase (Walne 1963). This peculiarity is not reflected at the karyological level. However, *O. puelchana* is the only brooding oyster with a distinct dwarf male and it shows a unique phenomenon of settling the larvae on an expansion of the anterior shell margin (Pascual et al. 1989). These unique morphologic features could be related to the karyological isolation of *O. puelchana*.

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THE INHERENT EFFICIENCY OF OYSTER DREDGES IN SURVEY MODE

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ABSTRACT To develop a quantitative stock assessment for the New Jersey oyster (*Crassostrea virginica*) seed beds, oyster dredge efficiency was measured on 10 different oyster beds in the Delaware Bay. Depending on size class and location, mean dredge efficiency for market-size oysters varied from 7.8 to >85%. The sampled beds could be allocated into two groups, one characterized by low dredge efficiency and the other characterized by high dredge efficiency. The low-efficiency group, Group 1, had mean dredge efficiencies for market-size oysters that ranged from 10.9 to 19.5%. The high-efficiency group, Group 2, had mean dredge efficiencies for market-size oysters that always exceeded 45%. A strong tendency existed for market-size oysters to be captured with higher efficiency than smaller oysters. In addition, live oysters tended to be captured with higher efficiency than boxes (articulated valves). Although a conclusion cannot be reached unequivocally, the differential in dredge efficiency observed between Group 1 and Group 2 beds may represent the difference between dredge efficiencies on beds routinely fished and those not routinely fished. An effect of salinity regime cannot be excluded as a possible explanation, however. The differential in dredge efficiency between the two bed groups, about a factor of 4.5 for market-size oysters indicates that variations in bed consolidation may have a large influence on dredge efficiency and may significantly bias estimates of abundance if not taken into account in stock assessments.

KEY WORDS: oyster, dredge efficiency, stock assessment, survey, fishery

INTRODUCTION

Dredges are frequently used survey tools. Knowing the efficiency of the dredge is, therefore, paramount in a quantitative estimate of stock abundance. Dredge efficiencies have been evaluated for a number of bottom-dwelling commercial species, including scallops, *Placopecten magellanicus* and *Zygochlamys patagonica* (Giguère and Brulotte 1994, Lasta and Iribarne 1997), surf clams, *Spisula solidissima* (NEFSC 2000a), ocean quahogs, *Arctica islandica* (NEFSC 2000b), and blue crabs, *Callinectes sapidus* (Vølstad et al. 2000).

The stock assessment for the New Jersey oyster seed beds uses a standard 1.27-m oyster dredge (Fegley et al. 1994). Quantification of this survey depends upon knowing the efficiency of the dredge under survey conditions. Oyster dredges are not among the most efficient of sampling gear. Estimates of dredge efficiency range between 2 and 32% in survey mode (Chai et al. 1992). As used by the industry in the normal routine of fishing, the dredge efficiency consistently falls into the lower portion of this range (Banta et al. in press).

To develop a quantitative stock assessment for the New Jersey oyster seed beds, we performed a series of measurements of dredge efficiency for a standard oyster dredge (Fig. 1). Because previous estimates had varied over a wide range, we conducted these measurements on a number of oyster beds covering a range of salinities and degrees of fishery impact to evaluate whether changes in bed environment and fishing history affect dredge efficiency.

METHODS

Field Program

Dredge efficiency measurements were conducted in summer 1999 and summer 2000 on 10 different oyster beds in the Delaware Bay. Eight of these were in New Jersey waters and two were in Delaware waters (Fig. 2).

Normally, three separate experiments were conducted on each bed. Time constraints limited the number to less than three in several cases. Each experiment was conducted in an identical manner in the following way.

The oyster boat F/V Howard W. Sockwell carried out a 1-min dredge tow using a standard 24-tooth 1.27-m dredge (Fig. 1). Tooth length was approximately 44 mm, and the mouth opening was 1.27 m × 51 cm. The bag consisted of 17 rows of 50.8 mm rings. During the dredge tow, a data logger recorded DGPS position and time at 5-s intervals. A second boat, the R/V Zephyrus, ran immediately parallel but about 5 m off the oyster boat. A buoy was dropped from the R/V Zephyrus at the point immediately opposite dredge deployment and another immediately opposite dredge retrieval as the tow progressed.

The dredge haul was brought on board and a one-bushel sample taken for analysis. The remainder of the haul was measured volumetrically and discarded overboard. A full oyster dredge holds about 12 bushels of material. Presumably, dredge efficiency declines as this volume is approached. In this study, dredge volumes exceed 8.5 bushels at only two sites, Bennies and Arnolds, where dredge volumes routinely fell between 9 and 10 bushels. However, efficiency estimates for these sites did not materially diverge from efficiency estimates for other sites, suggesting that tow distances were short enough so that dredge capacity did not influence measured efficiency.

A buoyed 23-m transect line was dropped from the R/V Zephyrus near the first buoy and the line payed out towards the second buoy. Limitations in visibility prevented divers from routinely sampling within the tow path. As a consequence of the need to maintain a consistent sampling protocol, the diver line was positioned about 5 m from the dredge path so that all diver samples were taken near to and in parallel with the tow path. Twelve collection sacks were affixed at equal intervals along this line. Divers were then deployed to sample along the transect line using an 0.5- × 0.5-m quadrat (0.25 m^2). A random 0.25-m^2 sample was taken at the location where each collection sack was affixed. Divers attempted to retrieve all of the loose bottom material, including all live oysters and boxes (dead, articulated valves). Collection was facilitated by the use of small hand-held scratch rakes. The divers were instructed to take only the loosely consolidated

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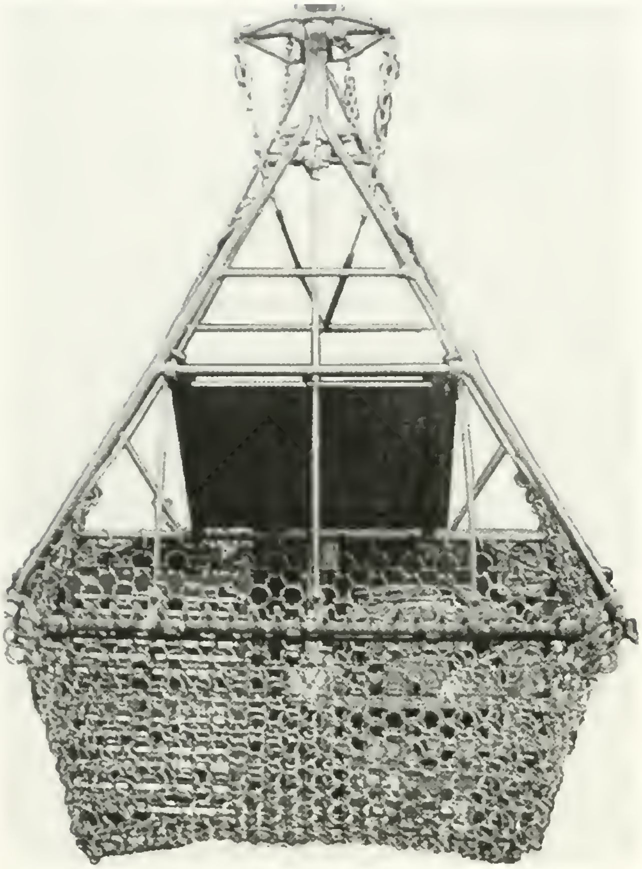

Figure 1. A standard 1.27-m New Jersey oyster dredge.

material on the surface that would normally be taken by the dredge. In most cases, quantitative retrieval was simple because the consolidated portion of the bed was near the surface. In a few cases, unconsolidated shell extended downwards for some distance. In these cases, the diver took the upper portion of the shell until the collection sack was filled.

Laboratory Analyses

Each bushel sample and each diver sample was sorted into live oysters, boxes, and shell and other debris and the respective volumes measured. The longest dimension of each oyster and box >20 mm was measured. Swept area was calculated for each dredge tow from the 5-s position logs and the dredge width.

Population abundance (N) is normally related to survey abundance (n_s) by a catchability coefficient: $N = qn_s$. The conversion requires four parameters: A, the area of the survey; d, the distance traveled per tow; w the cross-section sampled by the dredge; and e, the efficiency of capture by the dredge (NEFSC unpubl.). Thus, $q = A/dw\ 1/e$. Because, in this study, the dredge boat and diver samples were individually normalized to a 1 m^2 area ($A/dw = 1$), catchability q becomes the reciprocal of efficiency e, and was calculated as follows:

$$q = \frac{1}{e} = \frac{\left(\dfrac{\sum_{i=1}^{12} \textit{number of oysters or boxes (diver sample)}^{-1}}{\textit{number of diver samples} * 0.25\ m^2}\right)}{\left(\dfrac{\textit{number of oysters or boxes (bushel)}^{-1} * \#\ \textit{bushels}}{m^2\ \textit{dredge swept area}}\right)}$$

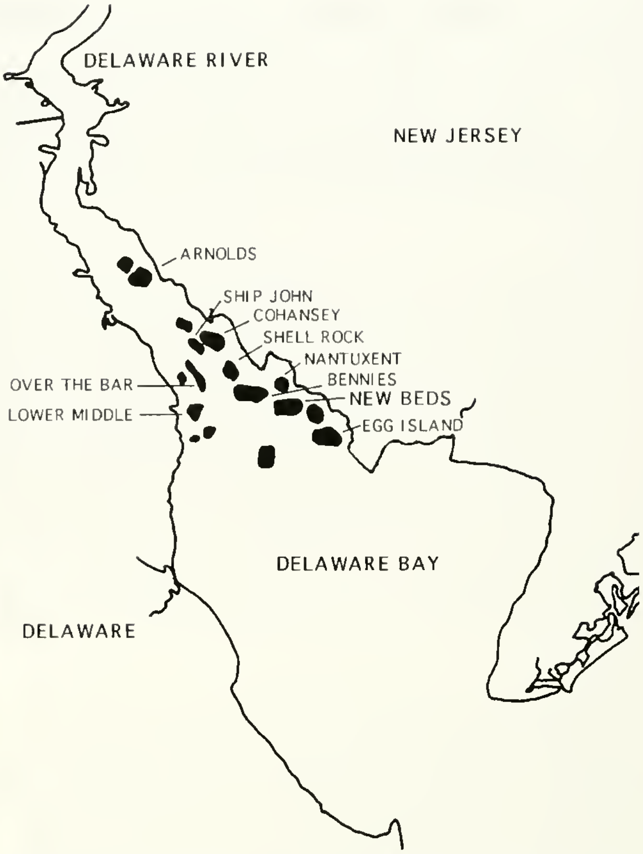

Figure 2. Location of the 10 oyster beds where dredge efficiency measurements were conducted in survey mode. Banta et al. (in press) obtained estimates of dredge efficiency under commercial use from New Beds.

Statistical Analysis

For statistical analyses, we used Spearman's rank correlation and ANOVA on ranked data. When appropriate, differences within the ANOVA were resolved with comparisons that used the least squares means. For some statistical analyses, live oysters and boxes were split into three size classes: juvenile (20–63.5 mm), submarket (63.5–76.2 mm), and market (≥76.2 mm).

RESULTS

Mean values of q for each seed bed are provided in Table 1. Values of q for live oysters ranged from 1.54 (an efficiency of 64.9%) to 11.27 (an efficiency of 8.9%). With one exception, all the size classes of oysters and boxes were correlated with one another (Table 2). The efficiency of collection of shell debris (disarticulated shell and shell fragments), however, was much more rarely correlated with the efficiency of collection of live oysters or boxes and the correlation coefficients were consistently lower. Divers had difficulty determining when to stop digging out shell and shell fragments on some beds, and this uncertainty in diver collection resulted in variation in the computed catchability for debris among diver samples.

Visual observation of Table 1 suggests that the sampled beds can be divided into two groups, those with relatively high values of q (low dredge efficiency) and those with relatively low values of q (high dredge efficiency). This observation was confirmed by ANOVA analysis comparing the efficiency of collection of mar-

TABLE 1.

Mean values of q (the reciprocal of dredge efficiency) for each of the size classes of live oysters and boxes, total live oysters, total boxes, and debris.

	Live Oysters				Boxes				
Oyster Bed	**Juveniles**	**Submarkets**	**Markets**	**Total Live**	**Juveniles**	**Submarkets**	**Markets**	**Total Box**	**Debris**
Group 1									
Arnolds	10.30	2.22	6.46	9.26	10.05	8.81	10.04	9.74	7.47
Cohansey	12.80	7.96	7.81	11.27	17.62	50.54	11.40	18.83	37.23
Over the Bar	9.63	5.88	5.14	7.61	6.81	12.58	5.58	7.33	6.93
Lower Middle	9.33	7.69	6.23	8.80	8.09	7.92	13.40	8.31	15.26
Ship John	10.16	12.06	9.15	10.40	12.79	9.91	13.17	11.88	50.12
Group 2									
Shell Rock	4.16	2.84	1.99	3.70	5.37	4.55	2.44	4.71	12.45
Nantuxent Pt.	3.86	1.98	–	3.30	2.32	2.48	–	1.98	6.06
Bennies	2.57	2.31	1.17	2.32	6.70	4.01	5.48	5.58	5.20
New Beds	2.04	3.12	0.64	2.13	2.88	2.08	–	2.71	9.65
Egg Island	–	–	2.22	1.54	15.41	6.00	20.31	16.99	14.47

Debris includes disarticulated shells and other shell debris. Dash indicates situations where diver sampling did not provide an adequate catch of that variable to permit an estimate of dredge efficiency.

ket-size oysters between oyster beds (Table 3). Note that, in general, the beds Arnolds, Cohansey, Over the Bar, Lower Middle, and Ship John, hereafter termed Group 1, are usually significantly different from the beds Shell Rock, Bennies, New Beds, and Egg Island, hereafter termed Group 2, in this analysis (Table 3). Market-size live oysters were not collected at Nantuxent Point, but perusal of the remaining data in Table 1 suggests that, had they been, Nantuxent Point would have fallen within the latter group of beds.

The average values of q for Groups 1 and 2 are shown in Table 4. With the exception of market-size boxes and debris, the efficiency of collection of live oysters and boxes on Group 1 beds is significantly lower (a higher q) than the efficiency of collection of live oysters and boxes on Group 2 beds.

The efficiency of capture of market-size oysters was higher (lower q) than for submarket-size ($P = 0.04$) and juvenile oysters $P = 0.003$). The latter two were not significantly different. The averages recorded in Table 4 also suggest that the efficiency of capture of live oysters is somewhat higher than boxes. In fact, values of q were significantly lower for all live oysters relative to all boxes ($P = 0.002$), live submarket oysters relative to submarket boxes ($P = 0.04$), and juvenile live oysters relative to juvenile boxes ($P = 0.006$). Market-size live oysters and boxes did not differ significantly, although the mean of the former falls well below the mean of the latter, especially for Group 2 beds. Accordingly, boxes were collected with a lower efficiency than live oysters overall.

The two groups of beds differ in average salinity. Group 1 beds are upbay of Group 2 beds. However, the intensity of fishing also follows the salinity gradient. Visual inspection of samples showed that oysters were much more clumped in samples from Group 1 beds as a consequence of the much lower frequency of dredging that has historically occurred on these beds. Clumping and reef consolidation might decrease dredge efficiency. If so, a correlation might exist between the amount of dredging on the bed during the preceding year and our measurement of dredge efficiency. We evaluated the significance of dredging using Spearman's rank correlations between the number of bushels taken per bed in 1999 and 2000 versus the measured value of q. The number of bushels taken is a reasonable surrogate for the total swept area of dredging (Banta et al., in press). All correlations were negative in accordance with the hypothesis that a higher value of q (lower dredge efficiency) should coincide with lower harvest rates. However, only the correlation with market-size live oysters was significant.

DISCUSSION

Oyster dredge efficiency varied over a wide range among the oyster beds sampled in the Delaware Bay. The range measured

TABLE 2.

P-values from Spearman's rank correlations between the efficiency of capture of the various groupings of live oysters, boxes, and debris.

	Submarket Live Oysters	**Market Live Oysters**	**Total Live Oysters**	**Juvenile Boxes**	**Submarket Boxes**	**Market Boxes**	**Total Boxes**	**Debris**
Juvenile live oysters	0.0013	0.0001	0.0001	0.0001	0.0001	0.0048	0.0001	0.1289
Submarket live oysters		0.0022	0.0005	0.0007	0.0008	0.0073	0.0002	0.0003
Market live oysters			0.0001	0.0001	0.0001	0.0019	0.0001	0.0167
Total live oysters				0.0001	0.0001	0.0047	0.0001	0.1128
Juvenile boxes					0.0001	0.0003	0.0001	0.0231
Submarket boxes						0.0030	0.0001	0.0136
Market boxes							0.0001	0.3216
Total boxes								0.0174

TABLE 3.

P-values from comparisons of least squares means for the efficiency of capture of market-size live oysters among the sampled beds.

	Cohansey	Over the Bar	Lower Middle	Ship John	Shell Rock	Bennies	New Beds	Egg Island
Arnolds	0.5529	0.5360	0.6912	0.2775	0.0310	0.0057	0.0207	0.1967
Cohansey		0.2597	0.3290	0.5651	0.0100	0.0019	0.0093	0.0972
Over the Bar			0.7895	0.1312	0.1478	0.0356	0.0645	0.4315
Lower Middle				0.1590	0.0645	0.0121	0.0351	0.3017
Ship John					0.0061	0.0014	0.0057	0.0539
Shell Rock						0.3778	0.3680	0.7253
Bennies							0.7784	0.3337
New Beds								0.3111

No market-size oysters were collected at Nantuxent Point, hence this bed is not included in the table. Boxed area delineates the comparisons between Group 1 and Group 2 beds discussed in the text.

encompasses dredge efficiencies higher than those recorded by Chai et al. (1992) in the Chesapeake Bay. In that study, dredge efficiencies varied from 2 to 32%. In this study, depending on size class and location, mean dredge efficiency for market-size oysters varied from 7.8 to >85%. Such high efficiencies are achieved when the dredge is used in survey mode, with short one minute tows that do not result in the complete filling of the dredge. The oyster fishery, as it routinely fishes, rarely achieves a dredge efficiency above 5% (Banta et al., in press) because the tows are longer and the dredge is routinely full when retrieved.

The range of efficiencies measured is large. Extreme values, whether high or low, probably are due to patchiness in the sampled area. Diver samples were not taken from the dredge tow path, but rather along a transect run parallel and close to the dredge tow path. Nevertheless, the sampled beds could readily be allocated into two groups, one characterized by low dredge efficiency and the other characterized by high dredge efficiency. The low-efficiency group, Group 1, had mean dredge efficiencies for market-size oysters that ranged from 10.9 to 19.5%. The high-efficiency group had mean dredge efficiencies for market-size oysters that always exceeded 45%.

A strong tendency existed for market-size oysters to be captured with higher efficiency than smaller oysters. Presumably, a greater tendency exists for the smaller oysters to pass between the dredge teeth or through the rings of the collection bag and, thus, not be collected. Dredge efficiencies were particularly low for juveniles, many of which may be attached to smaller pieces of shell that are poorly sampled. Very likely, dredge samples routinely result in a significant bias against juveniles. In this study, market-size oysters were captured with about twice the efficiency of juveniles.

In addition, live oysters tended to be captured with higher efficiency than boxes. The difference was highly significant, particularly in Group 2 beds where boxes tended to be captured with an efficiency of about one-third the efficiency of living oysters. Two possible reasons exist for the lower capture efficiency of boxes: 1) collection by dredge may result in disarticulation. This possibility is not supported by experiments designed to evaluate this source of disarticulation, however (Powell et al. 2001); 2) some boxes taken by divers may be deeper in the reef than the dredge normally samples. Efficiency of collection of debris was significantly correlated with efficiency of collection of total boxes in accordance with this hypothesis (Table 2); however, the efficiency of collection of debris was also correlated with some live oyster variables. Thus, a conclusive explanation for the variation in efficiency of capture between boxes and live oysters is not provided by the present analyses.

Box counts are routinely used as a method to estimate mortality rates in shellfish populations (Merrill and Posgay 1964, Fegley et al. 1994, Christmas et al. 1997). The differential in dredge efficiency measured in this study could result in a significant bias in the live:dead ratio and a significant underestimation of mortality rate from box counts if diver collections are unbiased. On the other hand, Christmas et al. (1997) observed that disarticulation often took more than one year, whereas mortality estimates from box counts usually assume that boxes were produced in the current year. This bias would tend to counterweigh the potential bias imposed by dredge efficiency. However, Powell et al. (2001) ob-

TABLE 4.

Mean values of *q* (the reciprocal of dredge efficiency) for each of the size classes of live oysters and boxes, total live oysters, total boxes, and debris for two groups of beds.

	Live Oysters				Boxes				
Oyster Bed	Juveniles	Submarkets	Markets	Total Live	Juveniles	Submarkets	Markets	Total Box	Debris
Group 1	10.46	6.89	6.93	9.40	11.26	18.98	11.00	11.47	21.49
Group 2	3.33	2.57	1.54	2.83	6.78	4.03	8.85	6.50	9.55
P value	0.0009	0.04	0.0001	0.0002	0.04	0.0008	NS	0.02	NS

Group 1 contains Arnolds, Cohansey, Over the Bar, Lower Middle, and Ship John. Group 2 contains Bennies, Shell Rock, Nantuxent Point, Egg Island, and New Beds. Debris includes cultch and other debris. *P* values record the results of ANOVA analysis comparing the two groups with respect to the variable listed as the column heading. NS, not significant ($\alpha = 0.05$).

served that disarticulation rates might be higher in the Delaware Bay than observed in Chesapeake Bay by Christmas et al. (1997). Consequently, the potentially offsetting biases in the interpretation of box counts cannot, as yet, be fully evaluated.

Why Group 1 beds yielded such low dredge efficiencies in comparison to Group 2 beds cannot unequivocally be identified. Group 1 beds are all upbay of Group 2 and, thus, exist at lower average salinities. Unfortunately, Group 1 beds also have been impacted less over the long term by dredging because effort in the Delaware Bay oyster industry also follows the salinity gradient, with lower effort on the lowest salinity beds (HSRL 2000, Fegley et al. 1994, Banta et al., in press). We attempted to assess the influence of dredging using catch data for 1999 and 2000, without much success. However, whether the catch data for the year before sampling is the correct estimator of the effect of dredging is questionable. Visual observation, for example, shows that oyster clumps are larger and contain more living oysters on these upbay beds. These clumps, very likely, are more firmly attached to the underlying bed than are the oysters on Group 2 beds. Greater bed consolidation on the upbay beds (Group 1) would reduce the effectiveness of the teeth in scraping shell material up into the dredge and, consequently, reduce dredge efficiency.

Oyster catchers normally report that catch rates are lower on beds that have not been fished for a time and that catch rates improve after repeated dredging over a few days. Very likely, this repeated dredging breaks the bottom up and results in a substantial increase in the efficiency of capture. Although the conclusion cannot be reached unequivocally, it seems most likely that the differential observed between Group 1 and Group 2 beds represents the difference between dredge efficiencies on beds routinely fished and those not routinely fished. The differential is large, about a factor of 4.5 for market-size oysters, indicating that variations in bed consolidation may have a large influence on dredge efficiency and may significantly bias estimates of abundance if not taken into account in stock assessments.

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NOVEL REPEAT ELEMENTS IN THE NUCLEAR RIBOSOMAL RNA OPERON OF THE FLAT OYSTERS *OSTREA EDULIS* C. LINNAEUS, 1758 AND *O. ANGASI* SOWERBY, 1871

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ABSTRACT The nucleotide sequence of the ribosomal RNA operon, from the 18S rRNA gene through ITS-1, 5.8S rRNA gene, and ITS-2, was determined for single clones of the flat oysters *Ostrea edulis* from Europe and *O. angasi* from Australia. The genic regions, the 18S and 5.8S rDNAs, were identical between the two species and displayed a high degree of similarity with available sequence from *Crassostrea gigas*, *C. nippona*, *Saccostrea commercialis*, and *S. cucullata*. In ITS-1, the *Ostrea* species had a characteristic repeat region, with subrepeats of 54 base-pairs (bp) (*a*) flanking a 27-bp fragment (*b*), where *b* remained constant while *a* changed by 1 bp through successive iterations (*a'*, *a''*). In *O. edulis*, the pattern was *a*, *b*, *a'*, *b*, *a''*, whereas in *O. angasi* it was *a*, *b*, *a''*. Apart from the difference in repeats, the ITS-1 of the two *Ostrea* species was identical except for a single substitution and two 2-bp insertions/deletions. ITS-2 was identical between the two *Ostrea* species except for a single substitution and a 3-bp insertion/deletion, and contained a nearly complete repeat of *a* near the 5' end. These ITS sequences were markedly dissimilar to available sequence from the other oyster species examined, and the repeat region was unique to *Ostrea*, with possible functionality in transcription. On the grounds of sequence similarity, it is suggested that *O. edulis* and *O. angasi* are conspecific.

KEY WORDS: rDNA sequence, ITS, *Ostrea*

INTRODUCTION

The taxonomy and systematics of oysters is far from being resolved (Carriker & Gaffney 1996), and confusion over the recognition of species persists, although the recent application of genetic techniques has been very successful in providing valuable characters to address phylogenetic relationships of the major species groups and families (Buroker et al. 1979a, Buroker et al. 1979b, Brock 1990, Banks et al. 1993, Anderson & Adlard 1994, Littlewood 1994, Boudry et al. 1998, Jozefowicz & Ó Foighil 1998, Ó Foighil et al. 1999, Ó Foighil & Taylor 2000). Among the remaining contentious issues is the question of the phylogenetic relationship between *Ostrea edulis* Linné, 1758, the European flat oyster, and *O. angasi* Sowerby, 1871, the Australian flat oyster.

In the most recent comprehensive revision of the living oysters, Harry (1985) reduced *O. angasi* to a synonym of the Southern Hemisphere species *O. puelchana* D'Orbigny, 1841, placing it in a different subgenus from *O. edulis* based on larval morphological and developmental characters. However, this classification has not been universally accepted (e.g., Jozefowicz & Ó Foighil 1998), and further data have since become available to suggest a closer relationship between *O. angasi* and *O. edulis* than had been supposed. Comparison of karyotypes, nucleolus organizer regions, and constitutive heterochromatin from chromosome preparations of *O. angasi* and *O. edulis* shows a very high degree of similarity, which is not seen between *O. angasi* and its supposed Southern Hemisphere conspecifics *O. puelchana* and *O. chilensis* Philippi, 1845, or between *O. angasi* and the Indo-West Pacific *O. denselamellosa* Lischke, 1869 (Li & Havenhand 1997). Partial mitochondrial 16S rRNA gene sequences position *O. edulis* and *O. angasi* in a well-supported clade with a low degree of DNA sequence divergence relative to the other conspecifics (Jozefowicz & Ó Foighil 1998). These results are also supported by partial 28S rRNA gene sequences (Ó Foighil & Taylor 2000). Furthermore, the concept of *O. puelchana* (previously thought to be native to Argentina) as a single circumglobal species in the Southern Hemisphere has been convincingly challenged by partial mitochondrial 16S rRNA (Jozefowicz & Ó Foighil 1998) and 28S rRNA gene sequences (Ó Foighil & Taylor 2000). These data support resurrection of the regional taxa in the southern hemisphere, namely *O. chilensis* in Chile and New Zealand, *O. angasi* in Australia and *O. algoensis* Sowerby, 1871 in South Africa.

To date, genetic information on the living oysters has focused on the rRNA gene (rDNA) array. In the Bivalvia, and in many other taxa, the rRNA genes have been useful in inferring phylogenetic and taxonomic relationships (e.g., Field et al. 1988, Ghiselin 1988, Nakamura 1989, Rice et al. 1993, Kenchington et al. 1994, Kenchington et al. 1995, Steiner & Müller 1996, Campbell et al. 1998, Frischer et al. 1998). These genes are co-transcribed, producing a single transcript consisting of the 18S, 5.8S, and 28S rRNA coding regions (or their homologues), separated by two internal transcribed spacers (ITS-1 and ITS-2) with an external transcribed spacer (ETS) located upstream of the 18S rRNA gene (Fig. 1). The transcribed precursor rRNAs are separated by intergenic spacers (IGS or NTS) consisting of transcribed and nontranscribed spacer sequence. This rRNA cistron is tandemly repeated head to tail at one or more chromosomal sites termed nucleolus organizer regions (NORs) (Hadjiolov 1985). The primary transcript is cleaved via a series of processing reactions into the 18S, 5.8S and 28S rDNAs, which along with approximately 85 ribosomal proteins and 5S rDNA, form the 40S and 60S preribosomal subunits (Hadjiolov 1985).

Regions of the rRNA cistron are under varying degrees of functional constraint and therefore provide a wide range of phylogenetic resolution. The IGS, a noncoding region, is under the least selective constraint and is therefore expected to show the greatest sequence divergence, rendering it useful for intraspecific comparisons. The ITS regions are also rapidly evolving, but to a lesser degree than the IGS. In contrast, the coding region of the rRNA repeat unit, that is, the 18S, 5.8S, and 28S genes, is highly conserved within and among species both in sequence and in secondary structure, with the 18S being the most conservative over a

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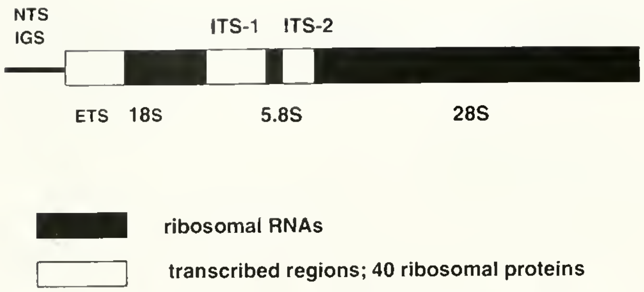

Figure 1. A schematic of the ribosomal RNA (rRNA) cistron indicating the genic regions in black and the noncoding regions in white.

broad taxonomic range (Appels & Honeycutt 1986). However, within the 18S rRNA gene, there are both conserved and variable regions associated with its secondary structure. Although some regions of this gene are conserved across all eukaryotes, other regions (loops) can be highly variable. Therefore, it has been suggested that this gene is valuable for phylogenetic investigations at a variety of taxonomic levels (Sogin et al. 1986), particularly when the time since divergence of the taxa is not known. A similar argument has been made for the much larger 28S rRNA gene (Hillis & Dixon 1991).

Here, we examine the nuclear rRNA cistron for sequence differences in the 18S, ITS-1, 5.8S, and ITS-2 regions to provide additional support for the level of differentiation between *O. angasi* and *O. edulis*. In the course of our studies, novel repeat elements were identified in the ITS which are described and discussed in context of their possible evolution.

MATERIALS AND METHODS

Sample Location

Specimens of *O. angasi* were collected from Cloudy Bay Lagoon, south end of Bruny Island, Tasmania, Australia on June 23, 1993 by Dr. John Thomson and shipped alive to our laboratory. Specimens of *O. edulis* were obtained from Department of Fisheries and Oceans research stock, originally imported from Europe via the United States to Canada during the late 1970s and used as broodstock at a hatchery in Port Medway, Nova Scotia. Animals were dissected and voucher specimens of the shells were archived at the Bedford Institute of Oceanography, Dartmouth, Nova Scotia.

DNA Extraction and Purification

High-molecular-weight DNA was extracted from the adductor muscle of a single animal of each species according to the protocol of Rice and Bird (1990). Briefly, tissue was ground in liquid nitrogen, proteins were removed by mixing with an equal volume of phenol:chloroform:isoamyl alcohol, followed by a final wash with an equal volume of chloroform:isoamyl alcohol to remove the phenol residues, and DNA was precipitated with sodium acetate in cold ethanol. The DNA was further purified on a cesium chloride gradient by high-speed centrifugation.

DNA Amplification

The 18S rRNA gene, ITS-1, 5.8S rDNA, and ITS-2 regions of the rRNA cistron were amplified as two fragments, one approximately 1825 base pairs (bp) and corresponding to the 18S rRNA gene, and the second approximately 1220 bp and containing the ITS spacers and 5.8S sequences. Synthetic oligonucleotides corresponding to conserved regions of the 18S rRNA gene and appended to restriction endonuclease sites were used as amplification primers for the first fragment (Bird et al. 1992). Deoxy-uracil monophosphate (dUMP) primers, with four trinucleotide repeat at the 5′ ends and complementary to the coding strand of the 18S (dUMP 18SF: 5′-CAUCAUCAUCAUGGTTTCCGTAGGTGAACCTGC-3′) and the 28S rDNAs (dUMP 28SR: 5′-CUACUACUACUAATATGCTTAAGTTCAGCGGGT-3′), were used to amplify the second fragment. The change in protocol to the dUMP primers was purely a matter of convenience because these primers were available in our laboratory at the time for another project (i.e., Patwary et al. 1998). Amplification was performed in a Perkin-Elmer (Irvine, CA) DNA Thermal Cycler programmed for an initial cycle of 5 min. at 94°C followed by 30 cycles of 2 min at 37°C, 3 min at 72°C, and 1 min at 94°C, plus a final cycle of annealing for 2 min at 37°C and one of extension for 5 min at 72°C. The same amplification conditions were used for each of the primer pairs and a negative control (no DNA template) was included in each run.

Cloning Polymerase Chain Reaction Products

Amplified DNA containing the 18S rRNA gene was ligated directly into the plasmid sequencing vector pCR 1000 using the TA Cloning™ kit, following manufacturer's instructions (Invitrogen Corp., Carlsbad, CA). Recombinant plasmids were maintained in the OneShot™ *Escherichia coli* INVαF′ cells provided in the kit. Transformants were grown on YT agar plates supplemented with kanamycin (100 μg mL^{-1}) and were detected by blue/white selection in the presence of X-gal and IPTG (Gold Biotechnology, St. Louis, MO).

Amplification products for the second fragment containing the ITS spacers and 5.8S gene generated with the dUMP primers were purified using the Geneclean II™ elution kit (BIO/CAN Scientific, Mississauga, ON). The purified DNA was directionally cloned into Epicurean competent cells (Stratagene, La Jolla, CA) using the CLONEAMP™ System (Life Technologies, Rockville, MD) for rapid cloning of amplification products.

The size of the inserts was screened by protoplasting (Sekar 1987), followed by digestion with *Not*I (New England Biosystems, Mississauga, ON) and *Kpn*I (Pharmacia, Peapack, NJ). A single colony was inoculated into 2xYT medium, following the protocols for a large-scale plasmid preparation (Maniatis et al. 1982).

DNA Sequencing and Alignment

Single clones of purified plasmid DNA containing the 18S rRNA gene were subjected to dideoxy sequencing according to the USB Sequenase version 2.0 protocol (USB Corporation, Cleveland, OH). Amplification primers plus the oligonucleotide primers described in Bird et al. (1992) were used to sequence the complete 18S rRNA gene in both directions. dITP was substituted for dGTP in the sequencing protocol to resolve compressions caused by secondary structure.

Purified DNA containing the ITS spacers and 5.8S rRNA gene was sequenced on an ABI 373 Automated DNA Sequencer, using the PRISM™ Dye Terminator Cycle Sequencing Kit (PE/ABI; Applied Biosystems, Foster City, CA) according to the manufacturer's instructions. M13 universal forward (5′-GTAAAACGACGGCCAGT-3′) and reverse (5′-TTCACACAGGAAACAG-3′) primers (USB) were used for initial sequencing. The DNA was sequenced completely in both directions by using a set of internal

TABLE 1.

Forward (F) and Reverse (R) Primers used to sequence the ITS-1, 5.8S, and ITS-2 regions of the rRNA cistron.

18S (end)	F1:	5′-GGTTTCCGTAGGTGAACC-3′
ITS1	F2:	5′-CTCCGTCGCCTTAAATGC-3′
	F3:	5′-GATTCCGAGCTCACGGAC-3′
	F4:	5′-CGTTTCACGTACATCAATAGT-3′
5.8S	F5:	5′-TCTAAGTGGTGGATCACTCG-3′
	F6:	5′-CATTGAACATCGACATCTTG-3′
ITS2	F7:	5′-CTCCGTCGCCTTAAATGC-3′ (in repeat *a*)
	F8:	5′-CAAGTACGGACGGCGACG-3′
ITS1	R1:	5′-CGCCGTCGGGAAGACGG-3′
	R2:	5′-GCTCGGAATCACCGAAAGT-3′
	R3:	5′-ACTATTGATGTACGTGAAACG-3′
5.8S	R4:	5′-CGAGTGATCCACCACTTAGA-3′
	R5:	5′-ACGCACCTGGCTGCGCTC-3′
ITS2	R6:	5′-GATTAGTGTTGTACGAGGCG-3′
	R7:	5′-GCCATCCGTCCGAGCAGACG-3′

primers that overlapped one another, providing at least 4 and up to 8 sequence streams from separate reactions for most regions of the fragment (Table 1). A consensus sequence from these reactions was produced via the software program Sequencher™ (Gene Codes Inc., Ann Arbor, MI).

Sequences were aligned with CLUSTALW (Thompson et al. 1994) and then checked manually. Repeat regions were identified by visual inspection, and a BLAST search (Altschul et al. 1997) was performed on these elements. Our data were compared with sequences downloaded from GenBank: *Ostrea edulis* 18S rDNA (Accession No. U88709); *Saccostrea commercialis* Iredale & Roughley, 1933, ITS-1, 5.8S rDNA (L28702); *Crassostrea gigas* Thunberg, 1793, ITS-1, 5.8S rDNA, ITS-2 (AF280609, AF280610); *C. nippona* Seki, 1934, ITS-1, 5.8S rDNA (AB041760); *C. cucullata* G.P. Deshayes, 1836, 18S (AJ389634).

RESULTS

Ostrea edulis Versus *O. angasi*

Nucleotide sequences of the rRNA operon, from the 18S rDNA through ITS-2, were similar in *Ostrea edulis* and *O. angasi*. Both the 18S (1823 bp, not shown) and 5.8S (159 bp, Fig. 2) rRNA genes were identical in their entirety. The ITS sequences were also very similar except for a large (81 bp) insertion/deletion in ITS-1 (Fig. 3), so that this sequence was 546 bp in *O. edulis* and 465 bp in *O. angasi*. Otherwise, ITS-1 differed between the species by a single substitution and two short (2-bp) insertions/deletions, while ITS-2 differed by only a single substitution and a 3-bp insertion/deletion, for a length of 476 bp in *O. edulis* and 479 bp in *O. angasi* (Fig. 4).

Further inspection of the ITS-1 sequence revealed the presence of three distinct regions (Fig. 3): (1) a short 5′ end of approximately 110 bp, which contained the single substitution and a 2-bp insertion/deletion; (2) a tract of repeated elements which differed in their number and arrangement between the species but were otherwise nearly identical in sequence; and (3) a post-repeat fragment of approximately 225 bp which had the second 2-bp insertion/deletion. The repeated elements were composed of two subrepeats ($a = 54$ nucleotides and $b = 27$ nucleotides) that occurred in tandem, with *b* always flanked on both sides by *a* (Fig. 4). Whereas the *b* sequences were conserved within and between repeats and between species, the *a* sequences of *O. edulis* showed a point mutation between *a* and *a*′, and a second mutation between *a*′ and *a*″. In *O. angasi*, *a*′ and the second *b* were missing, and the single *b* sequence was flanked at its 3′ end by *a*″ (Fig. 3). In *O. edulis*, the repeat sequence formed the pattern *a*, *b*, *a*′, *b*, *a*″, whereas in *O. angasi* it took the form of *a*, *b*, *a*″.

A 49-nucleotide portion of *a* also appeared in the ITS-2 sequences near the 5′ end (Fig. 4). This element was missing 3 bp at the 5′ terminus and 2 bp at the 3′ terminus, but otherwise differed from *a* in only 2 bp, plus a third bp in *O. angasi* which represented the only point mutation in ITS-2 between the species.

Comparison with Other Oyster Species

Both the 18S and 5.8S rRNA genes displayed considerable similarity with those of other oysters of the genera *Crassostrea* and *Saccostrea*. Our *O. edulis/angasi* 18S rDNA sequence differed from *Crassostrea gigas* (GenBank AB064942) at 43 sites over 1824 bps for 97.6% similarity, and from *Saccostrea cucullata* (GenBank AJ389634) by 49 sites over 1755 bp for 96.5% similarity. However, another sequence of *O. edulis* 18S rDNA from

```
O.edulis/angasi  CAACTCTAAGTGGTGGATCACTCGGCTCGGGGGTCGATGAAGAGCGCAGCCAGCTGCGTG 60
C.gigas          CAACTCTAAGTGGTGGATCACTCGGCTCGGGGGTCGATGAAGAACGCAGCCAGCTGCGTG 60
C.nippona        CAACTCTAAGTGGTGGATCACTCGGCTCGGGGGTCGATGAAGAGCGCAGCCAGCTGCGTG 60
Saccostrea       CAACTCTAAGTGGTGGATCACTCGGCTCGGGGGTCGATGAAGAGCGCAGCCAGCTGCGTG 60
                 ******************************************* ****************

O.edulis/angasi  AATTAATGTGAATTGCAGGACACATTGAACATCGACATCTTGAACGCACATGGCGGCCTT 120
C.gigas          AATTAATGTGAATTGCAGGACACATTGAACATCGACATCTTGAACGCACATGGCGGCCT- 119
C.nippona        AATTAATGTGAATTGCAGGACACATTGAACATCGACACCTT--- 101
Saccostrea       AATTAATGTGAATTGCAGGACACATTGAA--------------- 89
                 ************************************* *********************

O.edulis/angasi  CGGGTAACTCCCGAGGCTCACGTCTGTCTGAGGGTCGGC 159
C.gigas          CGGGTAACTCCCGAGGC-CACGTCTGTCTGAGGGTCGGT 157
C.nippona        ---------------------------------------
Saccostrea       ---------------------------------------
                 ***************** ********************
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Figure 2. Nucleotide sequence of the 5.8S rRNA gene from the oysters *Ostrea edulis*, *O. angasi*, *Crassostrea gigas* (GenBank Accession Numbers Af280609, AF280610), *C. nippona* (GenBank Ab041760), and *Saccostrea* (GenBank L28702). Asterisks identify points of identical sequence among the available data. Hyphens indicate points of nucleotide deletion/insertion and incomplete sequence at the 3′ end in *C. nippona* and *Saccostrea*. Numbers refer to the number of nucleotides from the 5′ end.

```
O.edulis    ATTAACAAAACAAGGAAACAACCAAGGTCGTTGAGACGTGT--CTCTCTCGTCATAGCCC  58
O.angasi    ATTAACAAAACAAGGAAACCACCAAGGTCGTTGAGACGTGTGTCTCTCTCGTCATAGCCC  60
            ******************* *********************  *****************

O.edulis    GCGACCAGGTCGTGTGGAACGGGCGAGAGAAGGCCAATCGCCGTCCCCGCGGCGCCTTGG  118
O.angasi    GCGACCAGGTCGTGTGGAACGGGCGAGAGAAGGCCAATCGCCGTCCCCGCGGCGCCTTGG  120
            ************************************************************

                           a                                   b
O.edulis    GCCGTCGTAGCCGAACAGGCTCCGTCGCCTTAAATACAGACGAATGGGAAACTCGTCGCC  178
O.angasi    GCCGTCGTAGCCGAACAGGCTCCGTCGCCTTAAATACAGACGAATGGGAAACTCGTCGCC  180
            ************************************************************

                                              a'
O.edulis    GTCTTCCCGACGGCGCCTTGGGCAGTCGTAGCCGAACAGGCTCCGTCGCCTTAAATACAG  238
O.angasi    GTCTTCCCGA--------------------------------------------------  190
            **********

                           b                                   a"
O.edulis    ACGAATGGGAAACTCGTCGCCGTCTTCCCGACGGCGCCTTGGGCAGTCGTAGCCGAACAG  298
O.angasi    -------------------------------CGGCGCCTTGGGCAGTCGTAGCCGAACAG  219
                                           *****************************

O.edulis    GCTCCGTCGCCTCAAATACAGACGAGCTCGACTTTCGGTGATTCCGAGCTCACGGACACG  358
O.angasi    GCTCCGTCGCCTCAAATACAGACGAGCTCGACTTTCGGTGATTCCGAGCTCACGGACACG  279
            ************************************************************

O.edulis    GCATTTGGTCACCGAATATGACGCCAGGCATCGGCGGTCGTGGGCCGACGTTATAAGCA  418
O.angasi    GCATTTGGTCACCGAATATGACGCCAGGCATCGGCGGTCGTGGGCCGACGTTATAAGCA  339
            ***********************************************************

O.edulis    CCCTGGTATGAACGTTTCACGTACATCAATAGTTTTGATCTTGGCGCGGGGGGAAAAACA  478
O.angasi    CCCTGGTATGAACGTTTCACGTACATCAATAGTTTTGATCTTGGCGCGGGGGGAAA--CA  397
            ********************************************************  **

O.edulis    CTCCTCGCGGTCGCGTCCGGCGTTCTTGTTGCGCGCCGAGACCGGCCGAATTTGGCATCT  538
O.angasi    CTCCTCGCGGTCGCGTCCGGCGTTCTTGTTGCGCGCCGAGACCGGCCGAATTTGGCATCT  457
            ************************************************************

O.edulis    CTTTTTGA  546
O.angasi    CTTTTTGA  465
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Figure 3. Nucleotide sequence of the ITS-1 region from the oysters *Ostrea edulis* and *O. angasi*. Asterisks identify points of identical sequence and hyphens indicate points of sequence deletion. Numbers refer to the number of nucelotides from the 5′ end. The 54-bp repetitive element (*a*) is indicated in the *O. edulis* sequence by shading, and nucleotide changes in its second (*a′*) and third (*a″*) iterations are underline; the 24-bp subrepeat (*b*) is outlined.

GenBank (U88709), differed from our *O. edulis/angasi* sequence in 10 sites and was identical with the other oysters at 8 of those sites.

Similarly, the 159-bp 5.8S rRNA genes of *O. edulis/angasi* showed a high degree of similarity with the *Crassostrea gigas* sequence, which differed at only four positions (Fig. 2; 97.5% similarity). Partial sequence from *C. nippona* and *Saccostrea commercialis* was also nearly identical with that of *O. edulis/angasi.*

By contrast, considerable variability was evident among the spacer sequences of the oyster genera (Figs. 4 and 5). None of the other ITS-1 sequences showed repeat elements, and similarity was low in the region corresponding to the *a* and *b* elements. However, the sequences were more conservative in an area coincident with the *a″* repetitive element in *O. edulis* and *O. angasi*. Lacking repeats, the sequences from *Crassostrea* and *Saccostrea* were shorter (445–520 bp) than in *O. edulis* (546 bp), although the length of the *C. nippona* sequence approached that of *O. edulis* because of numerous insertions downstream from the repeat region (Fig. 5). For ITS-2, both *Ostrea* sequences were shorter than that of *Crassostrea gigas*, which was 611 bp (Fig. 4). Overall sequence similarity of the ITS-2 region between the *Ostrea* species and *C. gigas* was on the order of 82%; conserved regions at the 5′ and 3′ ends were identified. In *C. gigas*, the area corresponding to *a* contained six differences from *a* apart from the absent terminal nucleotides, including only one of the two point mutations in the *O. edulis* sequence. A BLAST search (Altschul et al. 1997) on the repeat elements could not identify similar sequence in other organisms.

DISCUSSION

Nuclear ribosomal sequences are often assumed to be homogenized within individuals and populations of a species through concerted evolution processes (Hillis & Davis 1988). Homogeni-

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O.edulis    GAAACTATCCATCGGACA------CCTGGGTTTGTCCGCCTTGGGCCGTCGTAGCCGAAA   54
O.angasi    GAAACTATCCATCGGACA------CCTGGGTTTGTCCGCCTTGGGGCGTCGTAGCCGAAA   54
C.gigas     GAAACTATCAATCGACTAAAATTTCATTATTCTAGTCGCCTTGGGCCGTCGAAGCCTCTC   60
            ********* ****   *      * *   * *   ********* ***** ****

O.edulis    A-GGCTCCGTCGCCTTAAATGCAGACCGACGCCC---------GCTCGA-----------   93
O.angasi    A-GGCTCCGTCGCCTTAAATGCAGACCGACGCCC---------GCTCGA-----------   93
C.gigas     ACGGCTCCGTCGCCTTAAATGCAGACCGATGACCAAAGAAACGGCTCTATTGGTGACTCG  120
            * *************************** * **         **** *

O.edulis    GAGCGTCGCCGG-TCTCACCGCCTATGGGTTTCC---CCCACGGTAGAAGGCTT--CTCG  147
O.angasi    GAGCGTCGCCGG-TCTCACCGCCTATGGGTTTCC---CCCACGGTAGAAGGCTT--CTCG  147
C.gigas     GTGCCTCGGCGGGACGAAACACGTCTCGACTTCCACTCTTTTCGCTAAACTCTTATCCCG  180
            * ** *** ***  *  * * * * * *  ****   *     *   **  ***  * **

O.edulis    AACGTCTGCTCGGACGGATGGCGA--GAGGAT-CGGGG----AGGGCGTTGA------CG  194
O.angasi    AACGTCTGCTCGGACGGATGGCGA--GAGGAT-CGGGG----AGGGCGTTGA------CG  194
C.gigas     AACGGCTGCTCGGTCGGACGGCGAAGGAGGACGCGGGGTGCGAGGGCGCAGAAAAAGACG  240
            **** ******** **** *****  *****  *****    ******  **      **

O.edulis    GCAACGCGGTCTGGTCT--------------------TGACGACCGA--------ACCGA  226
O.angasi    GCAACGCGGTCTGGTCT--------------------TGACGACCGA--------ACCGA  226
C.gigas     GCAACGCGGTCTGGCCACATACGCGAAGCTCCGGCTATAGCGGCGGGTCTCGACCATCAA  300
            ************** *                     *  ** * *         * * *

O.edulis    GAGCGTGCCCGAAGCCC-------CTTACACCAAACT---CGAACCTCGCCATTCGCTTT  276
O.angasi    GAGCGTGCCCGAAGCCC-------CTTACACCAAACT---CGAACCTCGCCATTCGCTTT  276
C.gigas     AAGCGTGCCCGAAAGTGATGCAAACTCGCAACAAACTTCCCCGGACTCTCCAT-CGCTTT  359
             ************           **  ** ******   *    *** **** ******

O.edulis    GTCCTTGC--GGTGCAAGTACGGACGGCGACG-----TACCGAGG---GGGGAGCCGGGG  326
O.angasi    GTCCTTGC--GGTGCAAGTACGGACGGCGACG-----TACCGAGGAGGGGGGAGCCGGGG  329
C.gigas     GGCCTTGCCTGGCCTTAGTACGGATAACAAAGGCGGTTAAAGAAGGAGATCGTTTCGCTG  419
            * ******  **    ********   * * *     **  ** *      *   **  *

O.edulis    CGGA----------TCGAGAACGGC-------GCAAGTTGGAGC----AGGTGTCGCGAG  365
O.angasi    CGGA----------TCGAGAACGGC-------GCAAGTTGGAGC----AGGTGTCGCGAG  368
C.gigas     CAGAGTCCTGCCATTCAAAAAGGGGCTGAAGAGTCAACAGGGGCTCAAGGGCATCGCGAG  479
            * **          ** * ** **        *  *   ** **     **  *******

O.edulis    GAGGGCTCGCCG-------------GCCGCCTCGTACAACACTAATCATGC-----CTCA  407
O.angasi    GAGGGCTCGCCG-------------GCCGCCTCGTACAACACTAATCATGC-----CTCA  410
C.gigas     GGATCCGCGTTTTAAGAAAAAAAGCGCGGACCTCGATAAAACCAAAAACGGGACAACCAA  539
            * ** * **                ** * *    * ** ** **  * *      *  *

O.edulis    TGATTCCTCAA---CTCCGACCTCAGATCAGGCGAGACTACCCGCTGAACTTAAGCATAT  464
O.angasi    TGATTCCTCAA---CTCCGACCTCAGATCAGGCGAGACTACCCGCTGAACTTAAGCATAT  467
C.gigas     TTTTTTTTCAATCACTCCGACCTCAGATCAGGCGAGACTACGCCCTGAACTTAAGCATAT  599
            *  **  ****   *************************** * ****************

O.edulis    TAGTAGTAGTAG  476
O.angasi    TAGTAGTAGTAG  479
C.gigas     CACTAAGGGCAG  611
             * **   * **
```

Figure 4. Nucleotide sequence of the ITS-2 region from the oysters *Ostrea edulis*, *O. angasi*, and *Crassostrea gigas* (GenBank Accession Number F280610). Asterisks identify points of identical sequence and hyphens indicate points of sequence deletion/insertion. Numbers are the number of nucleotides from the 5′ end. The segment that corresponds to the repetitive element (*a*) from the ITS-1 region of *Ostrea* is indicated by shading of the *O. edulis* sequence, and the two substitutions within it are underline, as is a third substitution of *O. angasi*.

zation is most likely to occur if the rRNA genes occur in a single tandem array on one chromosome. In both *O. edulis* and *O. angasi*, two NORs are located terminally on chromosome pairs 9 and 10 (Thiriot-Quiévreux & Insua 1992, Li & Havenhand 1997). Additionally, intraspecific variation in the number of NORs per cell and the size (copy number) has also been identified in *O. edulis* (Thiriot-Quiévreux & Insua 1992). From this, one would predict that sequence variation would exceed homogenization. However, sequence divergence in the rRNA genes is very low between *O. angasi* and *O. edulis*.

The sequences for the coding regions of the 18S and 5.8S rDNAs were identical between the *Ostrea* species. This similarity

```
O.edulis         ATTAACAAAACAAGGAAAC--AACCAAGGTCGTTGAGACGTGT--CTCTCTCGTCATAGC  56
O.angasi         ATTAACAAAACAAGGAAAC--CACCAAGGTCGTTGAGACGTGTGTCTCTCTCGTCATAGC  58
C.gigas          ATTAACAAAACAAAATCGG-GGCTCGTTGGCAAGAGCGACCGAGAGGCTAACGCAA--AA  57
C.nippona        ATTAACAAAAAAAGCATCG-GGCTCGTCGGCA-GAGCACCGAGGACGGCAACGAAACGAA  58
Saccostrea       ATTAACAAACAAAATGGGGCAAACCATTGGCAAACCATCTCATGGTCGAGATGTAACGGT  60
                 *********  **           *   * *                     *  *

O.edulis         CCGCGACCAGGTCGTGTGGAACGGGCGAGAGAAGGCCAATCGCCGTCCCCGCGGCGCCTT  116
O.angasi         CCGCGACCAGGTCGTGTGGAACGGGCGAGAGAAGGCCAATCGCCGTCCCCGCGGCGCCTT  118
C.gigas          ACACAGCCGAGG-G-TTGTTGTT--------AAAGTCG--GATCGACCTCG-AACGCCCA  104
C.nippona        ACACAGCCGAGG-GGTTGCTGCTGTTTGGCAAGTGTCG--TGTCGACCTCT-AACGCCGA  114
Saccostrea       CCAAACCCCGGA-GCCCGTGACC---------AGGTCG--CGCGGA--TGG-GGTGCGGA  105
                  *    **  *  *   *                * *       *          **

                              a                                          b
O.edulis         GGGCCGTCGTAGCCGAACAGGCTCCGTCGCCTTAAATACAGACGAATGGGAAACTCGTCG  176
O.angasi         GGGCCGTCGTAGCCGAACAGGCTCCGTCGCCTTAAATACAGACGAATGGGAAACTCGTCG  178
C.gigas          GCGACCAGGTCGCTAGGCCGAGGG-ATCCAGGCGAACTCGCTGGGGGGGTTTA-------  156
C.nippona        GCGACCAGGTCGCAAGGCCGAGGG-ACAC-GGCGA-CTCGCTTGGGGGGGTCGGTTCAAA  171
Saccostrea       GAGAAAACGCTATCTCTTTCGGTC-GTAC-------CTCGTTGGCAGAGGCG--------  149
                 * *     *                             *    *    *

                                                   a′
O.edulis         CCGTCTTCCCGACGGCGCCTTGGGCAGTCGTAGCCGAACAGGCTCCGTCGCCTTAAATAC  236
O.angasi         CCGTCTTCCCGA------------------------------------------------  190
C.gigas          ----TTTCCTCT------------------------------------------------  164
C.nippona        GCCTTTCCCCCC------------------------------------------------  183
Saccostrea       -----CAACCGA------------------------------------------------  156
                         *

                                      b                                a″
O.edulis         AGACGAATGGGAAACTCGTCGCCGTCTTCCCGACGGCGCCTTGGGCAGTCGTAGCCGAAC  296
O.angasi         ---------------------------------CGGCGCCTTGGGCAGTCGTAGCCGAAC  217
C.gigas          ---------------------------------CAGCGCCTTGGGCCGTCGAAGCCTTCC  191
C.nippona        ---------------------------------CAGCGCCTTGGGCCGTCGAAGCCTTTT  210
Saccostrea       ---------------------------------GAGAGCCTTGGGCAGTCGCACCCGAAC  183
                                                    * ********* **** * **

O.edulis         A---------------GGCTCCGTCGCCTCAAATACAGACGAGCTCGACTTTCGGTGATT  341
O.angasi         A---------------GGCTCCGTCGCCTCAAATACAGACGAGCTCGACTTTCGGTGATT  262
C.gigas          T----------------GCTCCGTCGCCTTAAGTACAGACGAGCTCGACC----GCGACC  231
C.nippona        TTTCTTCGGAACAAAGGGCTCCGTCGCCTTAAGTACAGACGAGCTCGACC----GCGACC  266
Saccostrea       A---------------GGCTCCGTCGCCTTAAATTCAGACGAGCTCGACC----GCGACC  224
                                  ************ ** * **************     * **

O.edulis         CCGAGCTCACGGACACGGCATTTGGTCACCGAATAT----GACGCCAGGCATCGGCGG--  395
O.angasi         CCGAGCTCACGGACACGGCATTTGGTCACCGAATAT----GACGCCAGGCATCGGCGG--  316
C.gigas          CCGGGCTTCCGGAACGGTGGGTCGCTAAAACAAAACAAAAAGCACTCGGCATCGTCGGGG  291
C.nippona        CCGGGCTTCCGGAACGGTGGGTCGC--AAACAAAACTCATAGCACTCGGCATCGTCGGGA  324
Saccostrea       C-GGGCTTT-GACGGGGAAGGTCGC------AAAAC----ACCGCTCGGCATCGTCGG--  270
                 * * ***   *     *    * *       ** *       * *  ******* ***

O.edulis         TCGTGGGGCCGACGTTATAAGCACCCTGGTATGAACGTT-TCACGTACATCAATAGTTTT  454
O.angasi         TCGTGGGGCCGACGTTATAAGCACCCTGGTATGAACGTT-TCACGTACATCAATAGTTTT  375
C.gigas          TGGAAACCTCGACGTTATAAGCACCCGAGTATGTACTTTCTCACGTATACCAACAGATTT  351
C.nippona        GGTTGGCCTCGACGTTATAAGCACCCGAGTATGTACTTTCTCACGTATACCGATAGCTTT  384
Saccostrea       TCATTGTTCCGATGTTGTAA-CGCCCGAGTATGTACATTCTCACGTATACGAAATCCTTA  329
                          *** *** *** * ***  ***** ** ** ******* *        **

O.edulis         ----------GATCTTGGCGCGGGG----GGAAAAA-----------------------C  477
O.angasi         ----------GATCTTGGCGCGGGG----GGAAA-------------------------C  396
C.gigas          CT-TACACAAAACCCGGGAAGGCGGAGTCGGCAAG------------------GCTCTTG  392
C.nippona        TTACACACAANAACCCGGGGGGCGGAGTCGGGAGGCAGGCAATTAAGTCCTCAACTCTGC  444
Saccostrea       TTTTA-ACGAGGA--AGGGGTCGGGAGCAGGCAAAG---------------TCTCTCGGC  371
                                 **     **    ** *

O.edulis         ACTCCTCGC---GGTCG-CGTCCGGCGT-TCTTGTTGCGCGCCGAGACCGGCCGAATTT-  531
O.angasi         ACTCCTCGC---GGTCG-CGTCCGGCGT-TCTTGTTGCGCGCCGAGACCGGCCGAATTT-  450
C.gigas          CCTTTTTTC---GGGGGTCGGCCGGCGTACCTCGTTGCGCGCCGCGACCGACCGAATT--  447
C.nippona        TTTTTTTTCTCGGGGGGTCGGCCGGCGTACCTCGTTGCGCGCCGAGACCGACCGAATT--  502
Saccostrea       TTTTCTTTCCTCGGTCG-CGTCAGGCGTACTTCGTTGCGCGCCTCGACCGGCCGAATCAC  430
                   *  *  *   **  * ** * *****   * **********  ***** ******

O.edulis         ---GGCATC-T-CT-TTTTGA  546
O.angasi         ---GGCATC-T-CT-TTTTGA  465
C.gigas          ---GGCATCATACTATTTTGA  465
C.nippona        ---GGCATCTTATTTTTTTGA  520
Saccostrea       TTTGGCATC----TAT--TGA  445
                    ******    * *  ***
```

Figure 5. Nucleotide sequence of the ITS-2 region from the oysters *Ostrea edulis*, *O. angasi*, and *Crassostrea gigas* (GenBank Accession Number F280609). Asterisks identify points of identical sequence and hyphens indicate points of sequence deletion/insertion. Numbers refer to the number of nucleotides from the 5′ end. The 54-bp nucleotide repetitive (*a*) elements in *O. edulis* are indicated by shading of the *O. edulis* sequence. The 27-bp nucleotide repeat (*b*) is outlined on the same sequence.

```
O.angasi   CTTTCGCTCTCGTCCTTGCGTGCACGTCTCGACCTGTGGAGACTGCTCTCTGGGCAGTGG  720
O.edulis   CTTTCGCTCTCGTCCTTGCGTGCACGTCTCGACCTGTGGATACTGCTCTCTGGGCAGTGG  720
O.edulis   (Littlewood 1994)                       G

O.angasi   CCGCTAACTGCGAGTGCGCACGGGGTGGGAGGCGTGGCGGTGCCCGATGGTCAGTGGCGA  780
O.edulis   CCGCTAACTGCGAGTGCGCACGGGGTGGGATGCGTGGCGGTGCCCGATGGTCAGTGGCGA  780
O.edulis   (Littlewood 1994)             G

O.angasi   ATCGGTCGGGCGTCCACCCGACCCGTCTTGAAACACGGACCAAGGAGTCTAACATGTGCG  840
O.edulis   ATCGGTCGGGCGTCCACCCGACCCTTCTTGAAACACGGACCAAGGAGTCTAACATGTGCG  840
O.edulis   (Littlewood 1994)       G
```

Figure 6. Partial nucleotide sequence of the 28S rRNA gene showing variable positioning between the oysters *Ostrea angasi* (GenBank Accession Number AF137046) and *O. edulis* (GenBank AF137047) as used by Ó Foighil and Taylor (2000) and as determined by Littlewood (1994).

prompted a re-examination of the partial 28S rRNA gene sequences produced for *O. edulis* by Littlewood (1994) and those cited by Ó Foighil and Taylor (2000) for *O. edulis* and *O. angasi* (deposited in GenBank). The sequences by Ó Foighil and Taylor (2000) show three variable positions between the taxa over 911 nucleotides. However, the Littlewood (1994) sequence for *O. edulis* does not differ from *O. angasi* at those positions (Fig. 6). Therefore, it would appear that *O. angasi* and *O. edulis* may have identical nucleotide sequences for the coding regions of the rRNA cistron given that the partial data from the 28S rRNA gene were taken from the variable domains (D1, D2, D3). The 10-bp difference between the *O. edulis* 18S rDNA sequence of this study and U88709 from GenBank is not considered further as the latter is not published and the methodology used to infer it is not known. The 18S rRNA gene was expected to be the most conserved of the coding regions and therefore is unlikely to differ so greatly within a species.

Variation was detected in the non-coding internal spacer regions. The ITS-1 and ITS-2 both show sequence divergence between *O. edulis* and *O. angasi* irrespective of the repeat elements in ITS-1. However, the differences between *O. edulis* and *O. angasi* in ITS-1 are small compared with those observed between congenerics *Crassostrea gigas* and *C. nippona* (Fig. 5). The major difference between the *Ostrea* species is in the repeat unit of the ITS-1.

Repeat elements in the ITS-1 spacer have been identified previously (Long & Dawid 1980), including those with the same subrepeat pattern (*a, b, a*), (van Herwerden et al. 1999). However, it is not known if the repeating elements in these oysters have a function. Those found in the IGS region of both *Drosophila melanogaster* (Kohorn & Rae 1982) and *Xenopus laevis* (Moss 1983) have an influence on the level of transcription (Busby & Reeder 1983, Grimaldi & Di Nocera 1988). The ITS-1 region has an ability to form ribonucleoprotein complexes with cellular proteins and this domain is critical to the efficient maturation of the precursor RNA (Lalev et al. 2000). It is likely that the repeat element in *Ostrea* will have some effect on transcription. The secondary structure formed in *O. edulis* is more thermally stable than that of *O. angasi*. Further, the conserved nature of the base repeat and the repetition of the *a* element in the ITS-2 strengthens the case for functionality of these elements.

In other parts of the genome, repeat regions commonly arise through a preferential gain of a few repeat units at one end of the tandem repeat array, generating a minisatellite sequence (Jeffreys et al. 1994). Slip-stranded mispairing (Levinson & Gutman 1987) is commonly proposed as a model for VNTR repeat formation, expansion and contraction. Chance mutations are necessary for repeat expansion and these produce a few tandem repeats that facilitate the first strand slippage event (Messler 1996, Taylor & Breden 2000). However, different mechanisms may be responsible for the formation of repeats with motifs longer than 5 bp (Jeffreys et al. 1994). Taylor and Breden (2000) have described a general model for minisatellite birth that results in the formation of a locus with long repeats flanked by one unit of the original noncontiguous repeat. The appearance of differently sized repeats in the ITS-1 of *O. edulis* and *O. angasi* can be most expeditiously explained by unequal or misaligned crossing over. Depending on the ancestral configuration, the *a′*, *b* pair has been either lost or gained in one of the two taxa since their geographic separation.

The implication of these data is that *O. edulis* and *O. angasi* are conspecific as suggested by Jozefowicz and Ó Foighil (1998). These findings parallel the example of *Saccostrea commercialis* and *S. glomerata* (Anderson & Adlard 1994), in which the taxa are morphologically similar, yet geographically separated, with identical flanking 18S, ITS-1, and 5.8S regions. Although *O. angasi* and *O. edulis* have identical coding regions, some variation was detected in the ITS-1 and in the ITS-2, primarily through the deletion of repeat elements in *O. angasi*. However, this level of spacer variability is minor compared with that observed among the *Crassostrea* species.

As with the *Saccostrea* species studied by Anderson and Adlard (1994), the species in this study are also geographically separated. *Ostrea angasi* is found in Australia, from Western Australia along the southern coast to Tasmania and north along the east coast to the Clarence River in New South Wales (Thomson 1954). *Ostrea edulis* is mainly European, occurring from Norway to Morocco, including the Mediterranean Sea and the Black Sea (Carriker & Gaffney 1996). As put forward by Jozefowicz and Ó Foighil (1998), this incongruity can be explained by undocumented anthropogenic transport. Although *O. angasi* is widespread in Australia, it is not common throughout its range (Thomson 1954) and its distribution is centered around the area first colonized by Europeans. Such a scenario would suggest that the ITS-1 of *O. angasi* underwent a deletion of the *a′*, *b* region in the approximately 200–300 y after the transfer of *O. edulis* to Australian waters. However, as only one individual of each *Ostrea* species was sequenced, we do not know whether the number of repeats in the ITS-1 varies intraspecifically. Further investigation into intraspecific variation in the ITS-1 may identify a European locale from which *O. angasi* originated and evolved. Closer examination of late Pleistocene deposits at the Largs site in the Lower Hunter Valley, New South Wales, which reportedly contain specimens of *O. angasi*, may also shed some light on this question. In the interim, we submit that *O. edulis* and *O. angasi* are conspecific.

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ULTRASTRUCTURAL AND HISTOCHEMICAL CRITERIA FOR DETERMINING NORMALITY IN MATURE OOCYTES OF THE PACIFIC OYSTER *CRASSOSTREA GIGAS*

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ABSTRACT In hatcheries of bivalve molluscs, female gametes are assessed according to the morphologic stages of oocytic development. Three stages of oocytes immature, mature, and overripe were studied. We identified each category, based on ultrastructural features of the vitelline coat, cytoplasmic membrane, and organelles. Vitelline inclusions in the ooplasm were identified by ultrastructural and histochemical methods. Cytologic alterations involved in the typical degenerative process of mollusc oocytes were observed, including initial damage in the cytoplasmic membrane, rupture and degeneration of the vitelline coat, and damage in ooplasm and vitelline globules. All of these characteristics seem to be associated with the natural process of oocytic degeneration, but they can also be the consequence of handling during reproduction by artificial means.

KEY WORDS: oocyte, ultrastructure, histochemistry, *Crassostrea gigas*, reproduction

INTRODUCTION

Poor quality of gametes is believed to be one of the main causes of irregularities of embryonic and larval development in bivalve mollusc hatcheries (Raven 1966, Galtsoff 1964, Dohmen 1983, Wilson et al. 1996, Gérard et al. 1997, Le Pennec et al. 1998). In aquaculture, the quality of the gametes is defined by its capacity to be fertilized and consequent development of a viable larva (Kjorsvik et al. 1990). Morphologic criteria are commonly used for identification of female gametes of good quality. Three types of oocytes can be identified at spawning: mature oocytes with a round form and healthy appearance, immature oocytes with a pear shape and heterogeneous appearance, and overripe or atretic oocytes (Dorange & Le Pennec 1989, Paulet et al. 1992). This is a simple, quick, and inexpensive method. Nevertheless, these criteria are not sufficient to assess the viability of spawnings of *Crassostrea gigas* (Valdez-Ramirez et al. 1999). Results obtained from hatchery production reveal great variations that do not correlate with the amount of mature or normal oocytes. Germ cells maturation can be disturbed by multiple endogenous and exogenous factors in bivalve molluscs (Pipe 1987, Dorange & Le Pennec 1989, Paulet et al. 1992). A visual inspection does not confirm that mature oocytes will develop after fertilization.

Daniels et al. (1973) described the normal and abnormal characteristics of female gametes of *Crassostrea virginica* in the cytoplasm components, in particular, yolk granule distribution. This study suggests that knowledge of the gamete cytology is significant to evaluate the reproductive capability of the species. For *Pecten maximus*, Dorange and Le Pennec (1989) described, by means of transmission electron microscopy (TEM), the ultrastructural features in the stages of oocyte development and the natural process of oocytic degeneration. These authors showed that, at the ultrastructural level, cytologic alteration could be observed in the plasmic membrane, nuclear envelope, and mitochondria that were not detectable by gross morphologic observations.

The main purpose of this study is to address some questions related to the quality of the oocytes of *C. gigas*. Specifically, we tried to identify and describe the cytologic and cytochemical anomalies that most commonly affect the viability and quality of female gametes.

MATERIALS AND METHODS

Samples of *Crassostrea gigas* (Thunberg 1793) were obtained from the Bay of Brest, Brittany, France during its natural spawning period, June and July. Oocytes were obtained by draining of gonads. The oocytes were kept in seawater for one hour to recover their shape, as they are compressed in the gonads. Some gametes were fertilized to obtain embryos and larvae. Fragments of gonads of different specimens were also used in this study.

Sample Preparation for Transmission Electron Microscopy

Samples (oocytes, embryos or fragments of gonads) were fixed for one hour in 2.5% glutaraldehyde buffered with 0.2 M sodium cacodylate adjusted to 1100 mOsm and pH 7.2 (Cross & Mercer 1993). Postfixation was completed in 1% OsO_4 using the same buffer for 60 min at 4°C. The samples were rinsed, followed by progressive dehydration in ethanol baths (70%, 95%, and 100%). They were embedded in resin (Spurr 1969). Resin polymerization was completed at 60°C for 48 h.

Semi-thin (1 μm) and ultrathin (60–70 nm) sections were cut with glass and diamond ultramicrotome knives (Ultracut-S Leica) according to the method of Cross and Mercer (1993). The semi-thin sections for study under light microscopy were stained with 0.5% toluidine blue. The ultrathin sections were contrasted with uranyl acetate and lead citrate stains (Reynolds 1963). Observations of ultrathin sections were made with a Transmission Electron Microscopy (TEM) (JEOL 100 Cx). In addition, a few semi-thin sections were also treated using techniques for the identification of vitelline globules.

Detection of Lipids

Before postfixation, some samples of oocytes were treated in a solution of methanol-chloroform (1:1) at 60°C for 12 h to extract the lipids (E samples). They were postfixed using the same procedure described above. The identification of the lipid globules was made by comparing sections from the same batch of oocytes from which lipids were not extracted (NE samples).

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Detection of Proteins

The study of basic proteins was made on semi-thin sections treated with the 1.5% periodic acid at 40°C for one hour. They were stained by Ponceau 2r at 40°C for 3 h (Gori 1977). The protein globules revealed by staining were compared with the inclusions observed in ultrathin sections of samples from the same batch, by size, form, and their location in the cytoplasm of the oocyte.

Detection of Corbohydrates

The characterization of carbohydrate reserves in the oocytes was obtained by a PAS reaction (Periodic acid-Schiff) (Gabe 1968). Negative "glycogen" controls were prepared with amylase at 37°C for 3–6 h. After several rinses, these sections were treated at the same time as the positive glycogen controls.

RESULTS

Mature Oocyte

Mature or normal oocytes, measuring from 60 to 65 μm, contain a bulky nucleus (Fig. 1: 1). The germinal vesicle, in the prophase of first meiotic division, shows regular contours, approximately 25 μm in diameter. The nucleolus, when visible, is generally eccentrically located. Nuclear pores are sometimes observed. The cytoplasmic membrane bear numerous regular microvilli embedded in a vitelline coat (oolemma) (Fig. 1: 2). The fibrillar structure of the vitelline coat appeared highly electrondense at the periphery (Fig. 1: 2). This was observed in all the samples of unfertilized (Fig. 1: 2) oocytes and in those still in place in the gonad (Fig. 1: 1). Observations made of the vitelline coat ultrastructure in oocytes before fertilization (Fig. 1: 2), at the moment of fertilization (Fig. 1: 3), and 3 h after fertilization (Fig. 1: 4) showed no real differences in electron-density. The perivitelline space is sometimes visible between the vitelline coat and the plasmic membrane (Fig. 1: 5).

In the cytoplasm, mitochondria, endoplasmic reticulum, and many vitelline inclusions are visible (Fig. 1: 5). Mitochondria are very common and are distributed homogeneously. Their size is variable, the longest measuring approximately 0.5 μm. The lamellae of the endoplasmic reticulum are not abundant in the cytoplasm. Cisternae of endoplasmic reticulum are sometimes visible in the vicinity of the mitochondria. Some dictyosomes are also present.

Cytoplasmic inclusions accumulated during vitellogenesis are numerous and are varied in types. They occupy the larger part of the cytoplasm, differing in size, form, and density. By coupling ultrastructure study with cytochemical observations, the chemical nature of the inclusion was identified. Three types of inclusions were detected (Fig. 2: 1 to 8) and are described in the following three sections.

Type I, Lipid Inclusions

On semi-thin sections of the NE samples, the lipid inclusions (stained with Sudan black) occupy the larger part of the cytoplasm, and their distribution is homogeneous (Fig. 2: 1). At the ultrastructure level, comparison of the samples with or without lipid extraction (Fig. 2: 2 and 2: 3), shows that these compounds correspond to the largest inclusions, reaching a diameter of approximately 1 μm. Their contour is regular and, in most cases, they are enclosed by the granular endoplasmic reticulum. A higher electron density in the center than at the periphery characterizes these inclusions (Fig. 2: 2).

In several NE samples, the lipid globules were partially or completely absent (Fig. 2: 4 and 5).

Type II, Glycoprotein and Carbohydrate Inclusions

Round organelles, from 0.2 to 1 μm in diameter, are surrounded by a membrane. The homogeneous contents are visible in ultrathin sections. They may be dispersed in the cytoplasm, but they are mainly present at the periphery (Fig. 2: 5). The distribution and location of these reserves in ultrathin sections correspond to positive PAS reaction (Fig. 2: 6) and to the globules stained pink by Ponceau 2r (Fig. 2: 8) on the semi-thin sections. This suggests that type II inclusions are glycoproteinaceus in nature.

Glycogen detection by comparison between PAS staining in semi-thin sections (Fig. 2: 6) and the negative control (Fig. 2: 7) does not allow clear observation of this compound in the mature oocyte. However, we note a slightly more intense staining on oocytes untreated with amylase, which suggests the presence of glycogen.

Type III, Lysosome Inclusions

Distribution and occurrence of the lysosome inclusions detected by Ponceau 2R (Fig. 2: 8), are comparable way as type II inclusions. Certain differences were observed in MET. Type III inclusions are an irregular structure, and their location is unusual. They are provided with a single external membrane. They are heterogeneous in size, form, and electron density (Fig. 1: 2 and 1: 5) and always abundant, with a maximum size comparable to the lipid inclusions. Their heterogeneity contrasts with other inclusions of relatively homogeneous forms.

Ultrastructure Anomalies of the Oocytic Degeneration

Different degenerative alterations are observed (Fig. 3: 1 to 5). Degeneration in the female gamete can present a large increase of the perivitelline space (Fig. 3: 1 to 3) and a dilatation of the base of the microvilli. In some cases, the vitelline coat is detached and no microvilli are seen at the periphery (Fig. 3: 4). In the cytoplasm, mitochondria seem to be the first oocytic organelles involved in the degenerative processes (Fig. 3: 3). Other vitelline inclusions can also deteriorate, but lysosome bodies stay intact (Fig. 3: 4 and 5). The most severe damage observed in the cytoplasm include large vacuoles enclosing the vitelline bodies and cytoplasmic components, which are degraded to a lesser or greater level and forming dense globular masses. At this stage, intact lysosomal bodies are common (Fig. 3: 5).

Other Cytologic Alterotions

In some cases, mainly in free oocytes, the vitelline coat can be partially or completely detached from the plasmic membrane (Fig. 4: 1 and 2). In addition, the rupture of the plasmic membrane is sometimes seen (Fig. 4: 3), but the contents of the mature oocyte are not degraded.

DISCUSSION

Mature oocytes of *C. gigas* are comparable to those in other bivalve mollusc species, such as *Spisula solidissima* (Longo & Anderson 1970a, Longo & Anderson 1970b), *C. virginica* (Daniels et al. 1973), and *Pecten maximus* (Dorange & Le Pennec 1989, Devauchelle et al. 1997). In oocytes of *C. gigas*, the vitelline coat

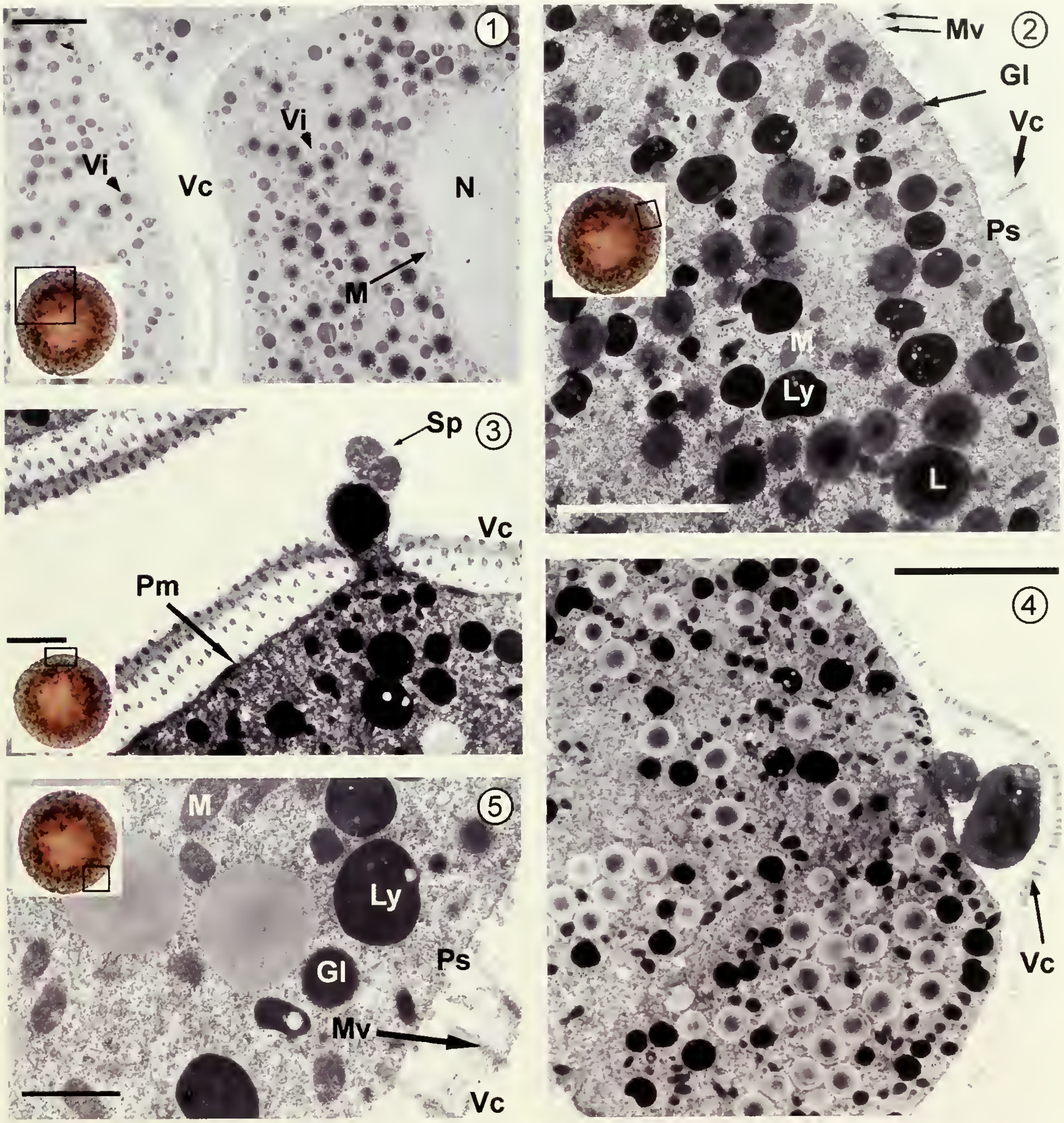

Figure. 1. Ultrastructural characteristics of the normal oocytes (1) Mature *Crassostrea gigas* oocyte prior to breakdown of nuclear envelope (prophase I stage) in the gonad. Scale: 5 µm. (2) Mature unfertilized *C. gigas* oocyte. Fibrillary structure of the vitelline coat: dense zone on the surface. Mitochondria and vitelline inclusions: lipids (type I), glycoproteinaceus (type II), and lysosome (type III). Scale: 5 µm. (3) Vitelline coat of the oocyte at the moment of fertilization. Cytoplasmic and vitelline coat density (→). Scale: 1 µm. (4) Vitelline coat density (→) in fertilized oocyte (3-h-old embryo). Scale: 5 µm. (5) Organelles and vitelline inclusions in ooplasm; lipids (type I), glycoproteinaceus (type II), and inclusions of the lysosomal type (type III). Scale: 1 µm. Legend: Vc, Vitelline coat; Pm, Plasmic membrane; Vi, Vitelline inclusions; M, Mitochondria; N, Nucleus; Ps, Perivitelline space; Mv, Microvilli; Sp, Spermatozoa; L, Lipid inclusions; Ly, Lysusome type inclusions; Gl, Glycoprotein inclusions.

structure seems different because of the tightness of the fibrillar net at the periphery. In this study, we did not detect modifications of the vitelline coat after fertilization, as observed in other bivalve molluscs: *Pecten maximus* (Dorange et al. 1989, Casse, 1995), *Mytilus edulis* (Humphreys 1962), other invertebrates, (Dohmen 1983, Epel et al. 1984, Pashchenko & Drozdov 1998), or in fish (Yemel'yanova 1980, Kjorsvik et al. 1990). In these species, cortical granules take part in the formation of the fertilization membrane. It is recognized that this membrane is a barrier that prevents polyspermy, but most molluscs apparently do not form a fertiliza-

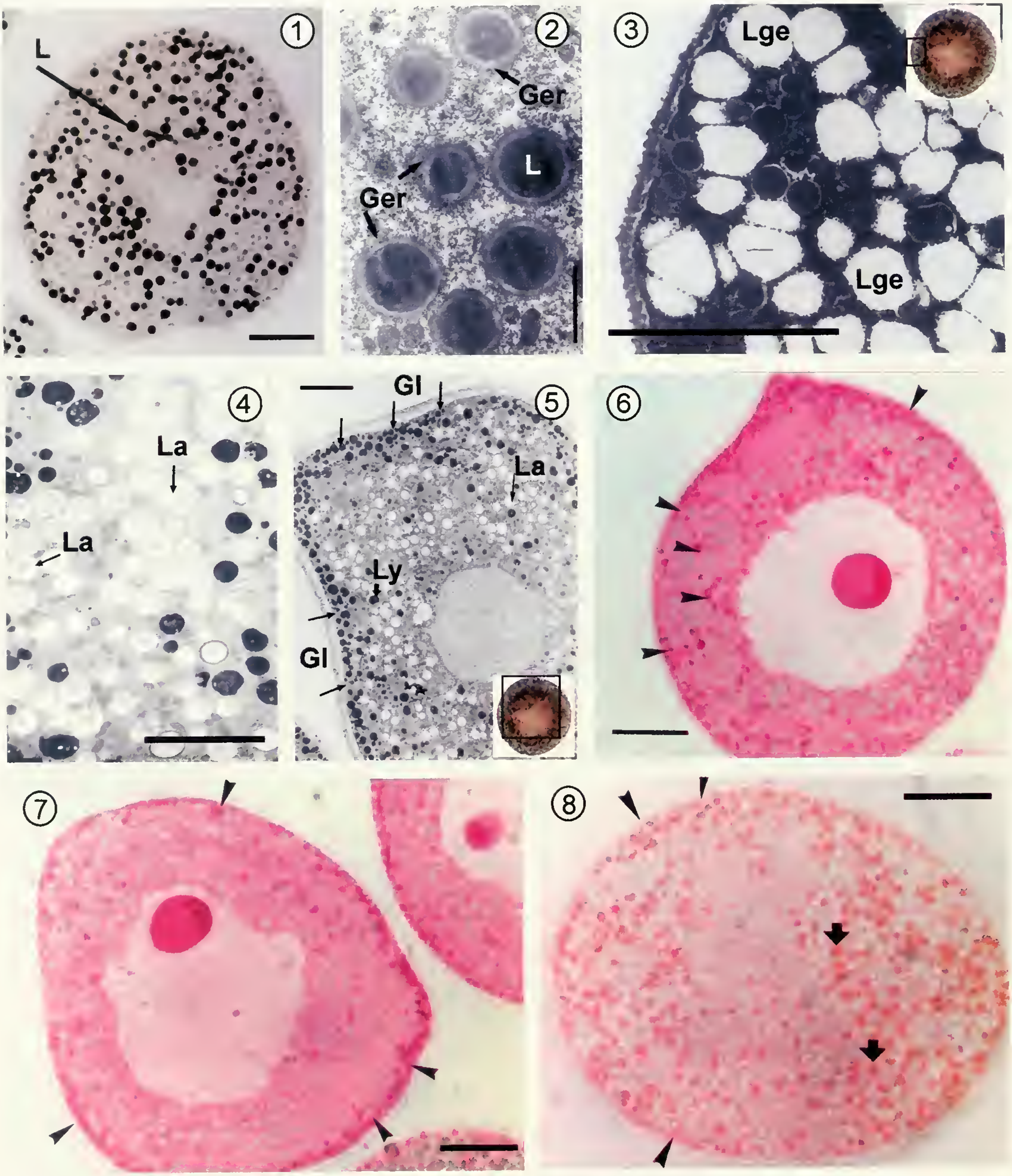

Figure 2. The vitelline inclusions, identification and fine structure (1) Lipid inclusions stained by Soudan black in semi-thin section. Scale: 10 µm. (2) Lipid inclusions in ultrathin section are enclosed by the granular endoplasmic reticulum. They present a higher electron density in the center than at the periphery. Scale: 1 µm. (3) Ultrathin section after extraction of the lipids. Scale: 5 µm. (4) Ultrathin section showing lipid inclusions partially or completely absent in NE preparations. Scale: 5 µm. (5) Distribution of the different types of vitelline inclusions in ultrathin sections. Scale: 5 µm. (6) Carbohydrate granules (type II) in semi-thin sections (➤) after staining with the PAS. Scale: 10 µm. (7) Negative control in glycogen detection by reaction with amylase for 3–5 h at 37°C. The less dense bottom suggests the presence of glycogen. Scale: 10 µm. (8) Glycoprotein inclusions, type II, (➤) and probably type III (→) on semi-thin sections stained by Ponceau 2r for 3 h at 40°C. Scale: 10 µm. Legend: L, Lipid inclusions; Gl, Glycoprotein inclusions; Ger, Granular endoplasmic reticulum; Lge, Lipid inclusions after extraction; La, Lipid absents.

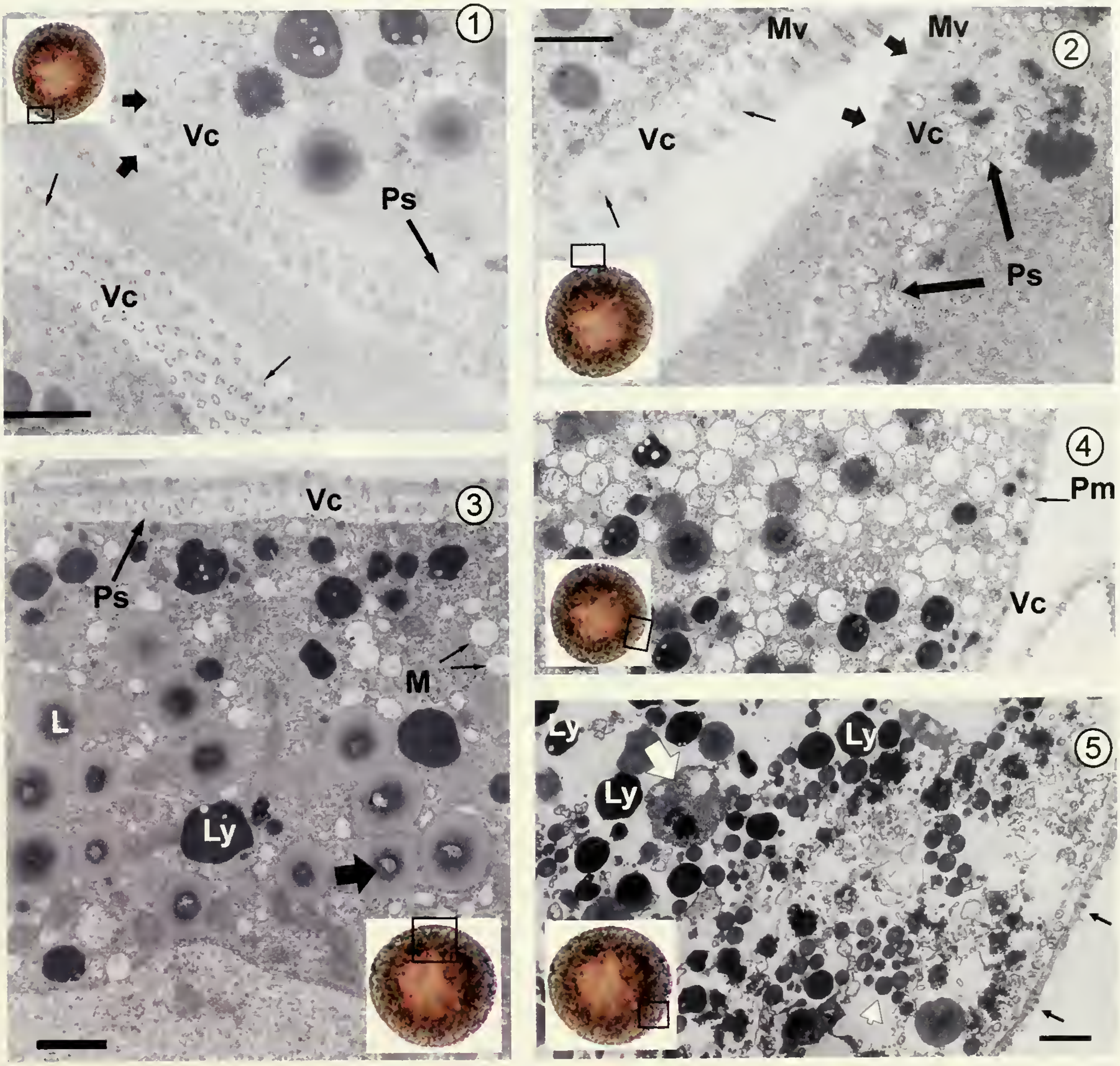

Figure 3. Ultrastructural anomalies of the oocyte; oocytic degeneration (1) Vitelline coat deterioration (→), normal vitelline coat in another oocyte (→). Scale: 1 μm. (2) Vitelline coat, advanced stage of degeneration (→). Perivitelline space increases. Ooplasm and organelles may be modified at the periphery of the oocyte. Normal structure of vitelline coat (→). Scale: 1 μm. (3) Degeneration in the ooplasm. Only mitochondria are damaged (→); their shape is modified, the cristae disappear, and their content becomes clearer. Scale: 1 μm. (4) Advanced degeneration in the ooplasm. The altered organelles are more abundant in the cytoplasm. Deteriorated vitelline coat is observed separated at some places from the plasmic membrane (→). Scale: 1 μm. (5) Advanced oocytic degeneration. Plasmic membrane is not visible. Vitelline coat surrounds the contents of the oocyte (→). In the cytoplasm, organelles are often grouped in vacuoles (➤). Degeneration of the organelles is important (→). Intact type III inclusions are abundant. Scale: 1 μm. Legend: Vc, Vitelline coat; Pm, Plasmic membrane; Vi, Vitelline inclusions; M, Mitochondria; Ps, Perivitelline space; Mv, Microvilli; L, Lipid inclusions; Ly, Lysosome type inclusions.

tion membrane. The precise mechanism for preventing polyspermy is not clearly understood (Dohmen 1983). The formation of this membrane in oocytes of *C. gigas* could be explained as a specific reaction (Thierry & Rambourg 1974).

Three types of vitelline inclusions were identified in the mature oocytes of *C. gigas*. Lipid inclusions, type I are abundant and easily locatable globules in the cytoplasm because of their homogeneous form. We observed a partial or total vacuolization of the contents of these globules in some oocytes. Vacuolization is not the result of extraction from an extended period of dehydration in ethanol because, in the same batch of oocytes, one finds normal oocytes. If a technical problem is excluded, the assumption is that this is a variable state in the maturity of oocytes. Steele (1998) shows comparable modifications of these globules that coincide with other deteriorations in the cytoplasm. Dorange (1989) shows that the density of lipid inclusions can be correlated to the stage of

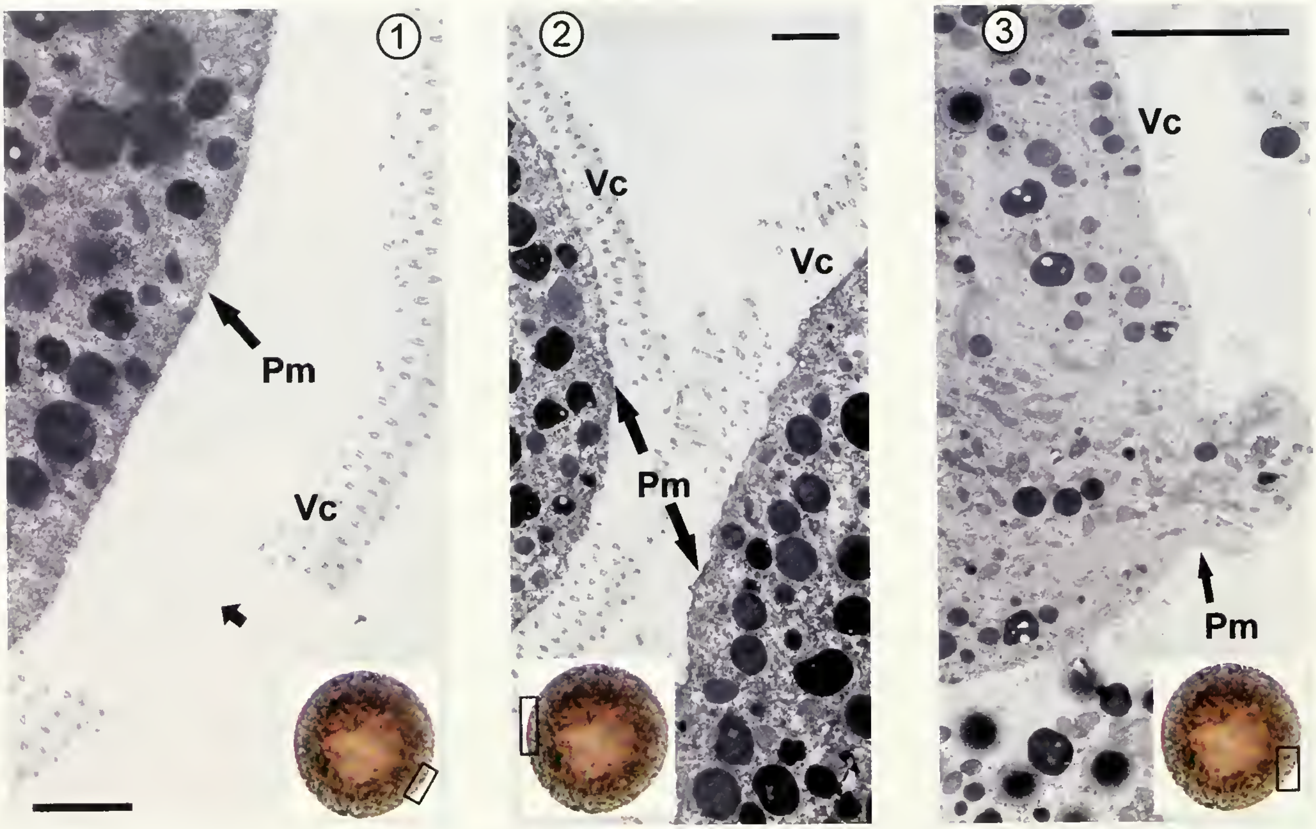

Figure 4. Other ultrastructural anomalies of the oocytes (1) Rupture of vitelline coat in mature oocytes. Plasmic membrane is not broken. Cytoplasm and vitelline inclusions are not modified its normal aspect. Scale: 1 µm. (2) Another example of the rupture of vitelline coat in mature *C. gigas* oocytes. Scale: 1 µm. (3) Rupture of the vitelline coat and plasmic membrane in mature *C. gigas* oocytes can take place at the gonad. In the cytoplasm, the organelles are intact. Cellular remains are observed between the oocytes. Scale: 5 µm. Legend: Vc, Vitelline coat; Pm, Plasmic membrane.

oocytic maturity in *P. maximus*. This also seems to be the case for *C. gigas*.

Lipid inclusions with a granular contour have been described in several species of bivalves such as *C. virginica* (Daniels et al. 1973), *C. gigas* (Steele 1998), and gastropods such as, *Ilyanassa* spp. (Gérin 1976) and *Acmaea* spp. (Kessel 1982). Daniels et al. (1973) and Steele (1998) suggest that it could be lipoprotein granules associated with the endoplasmic reticulum.

Glycoprotein was detected in type II inclusions that were stained by the protein and carbohydrate specific reaction in semi-thin sections. This type of inclusion has been noted in *C. virginica* (Daniels et al. 1973) and in the mussel *Mytilus edulis* (Albertini 1985). Positive staining by PAS in semi-thin sections seems to confirm the presence of glycogen. However, this must be checked at the ultrastructural level (reaction, Thierry & Rambourg 1974, for example). According to Tazawa et al. (1985) and Tazawa et al. (1986), these reserves represent the first source of energy during the first modifications in embryonic development of *C. gigas*.

Type III vitelline inclusions suggest lysosomal bodies. The presence of hydrolases (lysosomal enzymes) has been demonstrated in cytoplasmic globules oocytes of bivalves: *P. maximus* (Lubet et al. 1987, Dorange & Le Pennec 1989, Casse 1995), *Rangia cuneata* (Marsh et al. 1981), *Mytilus edulis* (Pipe & Moore 1985), and the gastropod *Littorina littorea* (Moore et al. 1982). Based on multiple observations, lysosomal bodies that are present during the degenerative stage of *P. maximus* oocytes were clearly identified by Lubet et al. (1987). Since its enzymatic activity is demonstrated, its presence in the oocytes of *C. gigas* must reveal different functions because it is always identifiable in the mature and degenerative stages.

The main causes of the poor viability of the gametes detected in the present study are related to the oocytic degenerative process. This is a phenomenon usually described in studies of the reproduction of fish, invertebrates in general, and the bivalve molluscs in particular (Lubet et al. 1987, Dorange et al. 1989, Paulet 1990, Ntiba et al. 1992, Sarojini et al. 1994, Widowati 1994, Steele 1998). Some serious alterations can be identified by direct examination of the samples using optical microscopy, but initial damage cannot be detected by means of morphologic observation. Ultrastructural techniques allow observation of different stages of degeneration. The first modifications involving the vitelline coat and plasmic membrane do not modify the form of the oocyte, which suggest that these gametes are not excluded during a morphologic examination. When degradation reaches the cytoplasm, oocyte shape is not modified in all cases. Therefore, damage may not be detected by simple microscopic observation.

It is important to mention that, according to our observations, this last stage does not seem to correspond to the same process of degeneration as the preceding one, but this cannot be confirmed now. First, damages may indiscriminately affect the periphery of gametes. Second, damage orients itself only to the content of the organelles, not to the periphery. These vacuolization already have been described in the typical oocytic degeneration process for bivalve molluscs *P. maximus* (Lubet et al. 1987, Dorange & Le

Pennec 1989), *Mytilus edulis* (Pipe 1987), and *Pinna nobilis* (De Gaulejac et al. 1995). In these species, modification coincide with degeneration of the vitelline membrane. This is not the general case observed. A study following oogenesis and oocytic lysis by ultrastructure techniques is required to explain this difference. The only morphologically identifiable degenerative stage corresponds to a severe degree of damage. In this stage, vitelline inclusions and organelles are grouped without order and they are degraded, forming masses of different density in cytoplasm. This causes important modification of the mass, density, and gamete form.

Another cytologic damage that obstructs larval development has been demonstrated in this study. It is the breaking of the vitelline membranes, without apparent alterations in cytoplasm. These damages are probably caused during the collection and handling of the gametes, but it is not possible to be certain that these damages are revealed in the oocyte morphology.

In conclusion, this study demonstrates that oocytes with normal external appearance can, in fact, display biochemical deficiencies or irreversible and lethal cytologic damages. These anomalies can explain the poor development of embryos and larvae. Although observation of these defects is difficult, its detection and quantification in light microscopy is possible by means of the Trypan blue exclusion test, as studied by Valdez–Ramirez et al. (1999) for oocytes of this species.

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AN INTRINSIC MEMBRANE PROTEIN IN OYSTER SPERM STIMULATES SPAWNING BEHAVIORS IN *CRASSOSTREA VIRGINICA*: IMPLICATIONS FOR AQUACULTURE

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ABSTRACT Pheromones are thought to play a critical role in triggering spawning in oysters, but none have been identified to date. Male and female oysters are stimulated to spawn by oyster sperm. Male oysters are also stimulated to spawn by oyster eggs, but females are not. Earlier studies suggest that the spawning activity associated with sperm may be membrane-bound. As a first step toward isolating and characterizing a spawning pheromone in sperm of the oyster *Crassostrea virginica*, we used: (1) an extraction and purification procedure for preparing oyster sperm membranes; (2) a method to selectively remove extrinsic sperm membrane-associated proteins; and (3) a bioassay to monitor the robust, repetitive adductor muscle contractions that occur concurrently with oyster spawning. This report presents evidence that the candidate oyster sperm pheromone is a heat- and trypsin-sensitive intrinsic membrane protein. A synthetic or recombinant fragment of the pheromone could be used in the aquaculture industry to induce spawning in oysters.

KEY WORDS: bivalve, *Crassostrea virginica*, mollusk, oyster, pheromone, sperm

INTRODUCTION

Spawning in the oyster, *Crassostrea virginica* Gmelin, has been known to be a group reaction for more than a century and involves the release of sperm and eggs by a number of oysters. Although spawning in *C. virginica* can be influenced by a seasonal rise in water temperature, male and female oysters are more readily stimulated to spawn by oyster sperm; male oysters are also rapidly stimulated to spawn by oyster eggs (Galtsoff 1938b). Sperm can induce spawning in oysters that do not respond to increased temperature stimulation. For example, approximately 55% of oysters that failed to react to increased temperature spawned immediately upon addition of sperm (Galtsoff 1938b). A minimum concentration of ~100–150 sperm per milliliter of seawater will induce an all-or-none spawning reaction that cannot be inhibited or stopped once it has begun. In physiologically ripe females, the presence of oyster sperm normally initiates ovulation and rhythmic contractions of the adductor muscle, and the latent period to spawning is relatively independent of the concentration of sperm (Galtsoff 1930, Galtsoff 1938b). In males, the latent period to spawning is usually shorter compared with females (Galtsoff 1940).

The active factor(s) that stimulates spawning in *C. virginica* is associated with the sperm and is not a soluble factor released with sperm (Galtsoff 1938b). As a first step toward characterizing the active factor in oyster sperm responsible for stimulating male and female oysters to spawn, we employed a simple extraction and purification procedure for preparing oyster sperm membranes, a method to selectively remove extrinsic sperm membrane-associated proteins, and a bioassay to monitor adductor muscle contractions that normally occur concurrently with oyster spawning.

A major obstacle to purifying and characterizing the factor(s) responsible for spawning in *C. virginica* is that spawning is restricted to a relatively brief reproductive season, and the year-round bioassay of sperm membrane fractions has not been possible. Therefore, a bioassay was used based on the observation that oysters initiate a series of strong, rhythmic contractions of the adductor muscle in response to sperm entering the mantle cavity via the incurrent siphon (Galtsoff 1938a, Galtsoff 1938b). In ripe females, this vigorous and repetitive clamping of the shells normally results in the ejection of a cloud of eggs to a distance of 30 cm or more. This activity is thought to result in a relatively wide and uniform distribution of eggs, improving their chances of fertilization by sperm. In this report, we present evidence that the oyster sperm factor(s) that stimulates the vigorous and repetitive adductor muscle contractions observed concurrently with spawning is a heat- and trypsin-sensitive intrinsic membrane protein.

MATERIALS AND METHODS

Animals

For tissue extractions, oysters (*Crassostrea virginica*) were collected from April to June 2000 and 2001 from Galveston Bay, Texas.

For bioassays, animals were collected from Galveston Bay year-round and temporarily kept on ice; the shell height ranged from approximately 38 to 52 mm. To avoid rapid increases in temperature that could induce spawning, oysters were first acclimated to room temperature (20–22°C) out of water and cleaned to remove external biofouling. Approximately 60 oysters were subsequently transferred to individually numbered clear glass, screw top beakers with lids (Qorpak; Fisher Scientific) containing 500 mL of artificial seawater (ASW; Instant Ocean®, Aquarium Systems, Mentor, OH) at 25 ppt (20–22°C), and acclimated for at least 12 h prior to bioassays. Containers were continuously aerated by an in-house air system using a pipette projecting through a hole in the beaker lid. Oysters that were used in bioassays in late summer and early fall were potentially ripe but may have spawned out, whereas those collected at later times were probably completely spawned out. The majority of bioassay animals used in these studies were presumed to be males since the largest oysters, which are typically females, do not fit into the beakers.

Tissue Extraction and Purification of Oyster Gonad Membranes

The entire visceral mass was removed from sexually mature *C. virginica*, and most of the associated mantle, gill, and palp tissue were excised; the sex was determined by microscopic observation of gametes, and male gonads were stored at −70°C until they were used. A flow diagram showing the extraction and purification

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scheme is shown in Figure 1. The amount of visceral mass tissue used (range: 3.0–9.5 g) was sufficient to: (1) purify an excess amount of sperm membranes; and (2) obtain maximal responses in the bioassay. Visceral mass tissue was homogenized in 100 mL of ice-cold Buffer A (20 mM Tris-HCl, 2 mM ethylenediaminetetraacetic acid [EDTA], 1 mM phenylmethylsulfonyl fluoride, pH 7.4) using a Polytron homogenizer (Brinkmann), and centrifuged for 10 min at 5,000 × *g* (4°C). The resulting low-speed P1 pellets were resuspended by brief homogenization in 7.5 mL of Buffer A and centrifuged for 10 min at 5,000 × *g* (4°C). The combined low-speed S1 supernatants were subsequently centrifuged for 60 min at 100,000 × *g* (4°C) to generate high-speed P2 pellets (membrane fraction) and S2 supernatants.

Extraction of Extrinsic Membrane Proteins Using Sodium Carbonate

The standard procedure used to selectively remove extrinsic proteins from membranes using sodium carbonate (pH 11.5) was essentially that described by Fujiki et al. (1982). P2 pellets containing male gonad membranes were resuspended by brief homogenization in 30 mL of ice-cold 0.1 M sodium carbonate (pH 11.5) and incubated for 30 min on ice. Each sample was subsequently layered on a 3 mL cushion of 0.3 M sucrose, 10 mM Tris-HCl, pH 7.4 in an ultracentrifuge tube and the membranes were pelleted by centrifugation for 60 min at 150,000 × *g* to generate high-speed P3 pellets (containing intrinsic membrane proteins) and S3 supernatants (containing extrinsic membrane proteins) (Fig. 1). These were stored at 4°C. P3 pellets were resuspended by brief homogenization in ASW (25 ppt) immediately prior to bioassay.

In some experiments, P3 pellets were heated at either 68°C (30 min) or at 100°C (10 min) prior to bioassay.

Reduction, Alkylation, and Trypsin Digestion

To break the disulfide bonds in sperm intrinsic membrane proteins present in the P3 pellets, a procedure was used for denaturation, reduction and alkylation of proteins with 4-vinylpyridine (4-VP) that was essentially that described by Coligan et al. (1997).

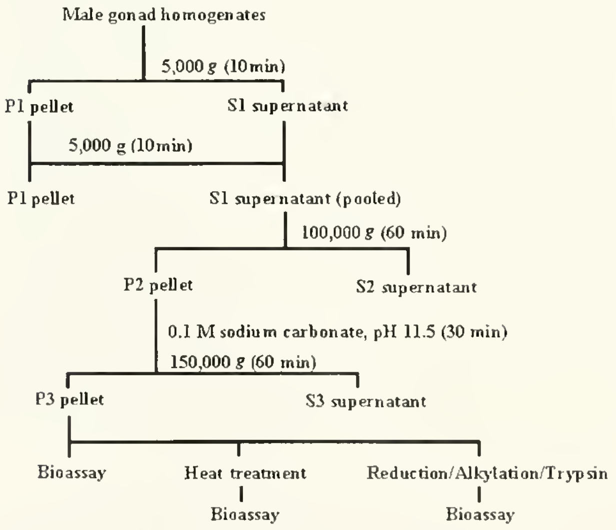

Figure 1. Flow diagram of the extraction and differential centrifugation procedures used to purify membrane fractions from male *Crassostrea virginica* gonads. The P3 pellets that were enriched in sperm membranes were either bioassayed directly or treated further and then bioassayed.

P3 pellets containing male gonad membranes were gently homogenized in 10 mL of ice-cold denaturing buffer (6 M guanidine-HCl, 0.5 M Tris-HCl, 2 mM EDTA, pH 8.2) to obtain a well-dispersed suspension, and the solution was bubbled with N_2 gas at room temperature (20–22°C) for 15 min. Following addition of 2-mercaptoethanol and incubation for an additional 30 min, 4-VP was added to the solution and incubated in the dark for 60 min. The reduced and alkylated samples were then dialyzed (Slide-A-Lyzer Dialysis Cassette; 10 kD MW cutoff; Pierce) against 0.5 M NaCl overnight at 4°C. The dialysate was subsequently digested with trypsin (Type III; Sigma; 37°C for 24 h) by established methods (Coligan et al. 1997) and bioassayed.

Bioassays

Due to the brevity of the reproductive season, most shell closure assays were performed when oysters were either partially or completely spawned out. Shell closure assays were performed on oysters whose valves were open and mantles were extruded, suggesting that filtration was occurring. Beaker lids were carefully removed to permit sample addition, and aeration was maintained throughout the assays. P3 pellets were resuspended by brief homogenization in 10 mL ASW (25 ppt), and 1.0-mL aliquots (i.e., one-tenth of one resuspended P3 pellet) were injected proximal to the incurrent siphon of oysters, and each vigorous closure of oyster valves was visually monitored during the subsequent assay period.

Additional experiments performed outside of the reproductive season specifically examined whether P3 pellets also stimulated at least minimal spawning in addition to shell closure. Oysters were initially observed for 5 min prior to injection of samples and the number of shell closures was recorded; oysters that exhibited large numbers of spontaneous shell closures prior to bioassays were not used. Following addition of sample (resuspended P3 pellet fractions), the number of shell closures was recorded, and material that was ejected during the 20-min period was examined microscopically for the presence of eggs or sperm. Since sperm are released through the excurrent siphon whereas eggs are released through the incurrent siphon, the direction of gamete release was noted. When spawning was detected visually and microscopically, oysters were subsequently sacrificed and the sex was confirmed by examining gonad smears for the presence of sperm or eggs.

Statistics

For each bioassay animal, the number of shell closures was determined, and the mean (± $S_{\bar{x}}$) for all replicates was calculated. The null hypothesis of equal means ($H_0 = \mu_1 = \mu_2 = \ldots \mu_5$) was tested using an F statistic generated during an analysis of variance (ANOVA) to determine whether a comparison of treatment sample means was valid. If the null hypothesis was rejected, then a multiple comparison of the treatment sample means was accomplished using a Fisher's protected least-square-difference (Fisher's PLSD) post-hoc analysis.

RESULTS

To confirm and extend previous observations by Galtsoff (1938b), freshly isolated sperm were diluted in ASW and found to stimulate a series of shell closures (shell clapping events; ≥4 in 5 min) in all cases (n = 3/3), as expected. Furthermore, when freshly isolated sperm were snap-frozen on dry ice, thawed, and assayed, the thawed sperm were still capable of stimulating a series of shell closures (range: 2–4 in 5 min) in all cases (n = 4/4).

Importantly, these observations suggest that the factor(s) that stimulate shell closure activity during spawning are resistant to at least one cycle of freezing and thawing. This suggests that male gonads could be dissected and stored at −70°C during the reproductive season and thawed for experiments at a later date. After selective removal of extrinsic membrane proteins from freshly isolated sperm, aliquots of resuspended high-speed P3 pellets containing intrinsic membrane proteins were assayed. The P3 material (1 mL = 10% of total sample) stimulated shell closure activity (range: 3–4 in 5 min) in 7 of 9 oysters (78%), whereas aliquots of high-speed S3 supernatants (10% of total volume) containing extrinsic membrane proteins stimulated shell closures in only 2 of 11 assays (18%). When male gonad tissue that had been frozen at −70°C for more than six months was subjected to the same purification procedure, that is, extraction and selective removal of extrinsic membrane proteins followed by differential centrifugation, similar results were obtained: resuspended P3 pellets (1 mL = 10% of total) stimulated vigorous, repetitive shell closures (range: 3–10 in 5 min) in 8 of 9 assays (89%). The data indicated that the activity was due to an intrinsic membrane factor(s) and not to extrinsic membrane proteins. In control experiments, the solution that was used to strip extrinsic proteins off of membranes (0.1 M sodium carbonate, pH 11.5) did not stimulate shell closure responses in any oysters ($n = 21$).

A 20-minute bioassay period was used in all subsequent shell closure bioassays. The number of shell closures in control assays using ASW was 1.2 ± 0.35 (mean ± $S_{\bar{x}}$; $n = 32$ oysters tested; Fig. 2A). After selective removal of extrinsic membrane proteins from male gonad membranes, 1-mL aliquots of resuspended P3 pellets (12 mg protein/mL; 10% of total sample) were found to stimulate an average of 10.8 ± 3.8 (mean ± $S_{\bar{x}}$; $n = 60$ oysters tested) shell closures within 20 min (Fig. 2B). After resuspended P3 pellets (12 mg protein/mL) were heated at 68°C for 30 min, the number of shell closures was not significantly different (11.4 ± 3.4; mean ± $S_{\bar{x}}$; $P = 0.82$; $n = 5$ oysters tested; Fig. 2B). However, when resuspended P3 pellets (12 mg protein/mL) were heated at 100°C for 10 min, the number of shell closures decreased by 41% (6.4 ± 1.7; mean ± $S_{\bar{x}}$; $P < 0.05$; $n = 10$ oysters tested; Fig. 2B). To test whether the active factor(s) was a protein, P3 pellets were resuspended in denaturation buffer, reduced and alkylated, dialyzed, and digested with trypsin; in this case, the number of shell closures decreased by 68% (3.5 ± 0.8; mean ± $S_{\bar{x}}$; $P < 0.0001$; $n = 58$ oysters tested) (Fig. 2B).

Additional experiments that were performed outside of the reproductive season, when the majority of animals had presumably already released most or all of their spawn, examined whether P3 pellets also stimulated detectable spawning in addition to shell closure. After selective removal of extrinsic membrane proteins from male gonad membranes, aliquots of resuspended P3 pellets were observed to stimulate spawning in three males ($n = 3$ of 80 experiments). In each case, sperm were released through the excurrent siphon and were visualized microscopically. These results suggested that an intrinsic sperm membrane protein(s) stimulated spawning as well as shell closure activity.

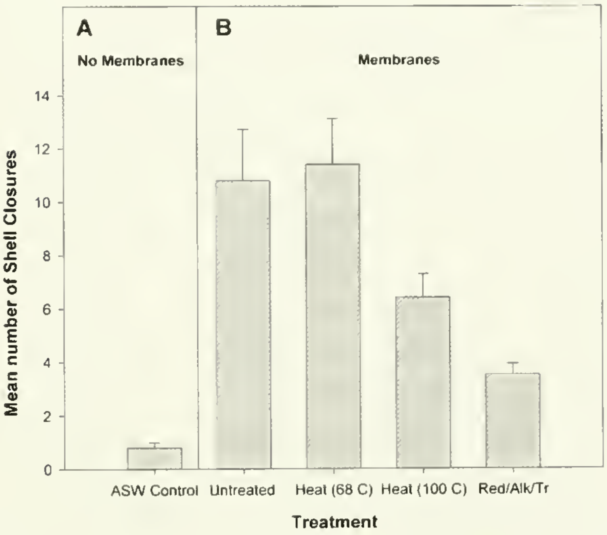

Figure 2. An intrinsic factor in sperm membrane stimulates spawning in *Crassostrea virginica*; spawning is accompanied by a series of shell closures (shell clapping) that have been used to assess the presence of the activity. The graph shows the average number of closures (± $S_{\bar{x}}$) in animals during a 20-min period following exposure to either: artificial seawater (ASW) alone; or ASW to which treated or untreated membrane fractions (resuspended P3 pellet) were added. (A) The average number of shell closures is low when oysters are incubated in ASW alone. (B) The average number was increased when untreated sperm membranes were added. Heating the membrane fraction before it was bioassayed reduced the average number. The effect was statistically significant at 100 C ($P < 0.05$), but not at 68°C ($P = 0.82$). Membranes were also reduced and alkylated to break disulfide bonds, dialyzed, and then digested with trypsin. The reduction in the mean number of closures is significant ($P < 0.0001$), supporting the suggestion that the factor is an intrinsic protein in the sperm membrane.

DISCUSSION

Mass spawning of oysters in an aquaculture setting requires at least one male in the tank to stimulate other male and female oysters to spawn. In some instances, the spawning operation is delayed for several hours or is not possible at all due to lack of a single spawning male to trigger general spawning of a tank of brood oysters. In the British Isles, there is a need for a synthetic or recombinant pheromone for routine 48-h exotoxicity tests that are in widespread use there. Presently, commercial hatcheries in the British Isles purify fresh sperm from *C. gigas* Thunberg to stimulate spawning for exotoxicity tests, but would prefer to avoid having sperm from another oyster in the water for the assays (S. Steele, Royal Holloway Univ. of London, pers. comm.). The availability of a synthetic or recombinant oyster spawning pheromone would: (1) avoid introducing sperm into the water during exotoxicity tests; (2) allow for a pathogen-free method for inducing spawning in oysters; (3) allow males and females to be induced to spawn separately, providing pure gametes for use in genetic manipulation for selective breeding of oysters with disease resistance, fast growth, etc.; and (4) simplify and standardize oyster hatchery operations.

As a first step toward characterizing the sperm-associated pheromone responsible for stimulating spawning in oysters, we used a method for selectively stripping extrinsic proteins off membranes that does not affect the disposition of integral components such as transmembrane and lipid-anchored proteins (Fujiki et al. 1982). It is the procedure most widely used to extract extrinsic (or adsorbed) proteins from organelle membranes, and has enabled investigators working on integral membrane proteins to efficiently remove soluble contaminating proteins, and to distinguish peripheral membrane proteins (those desorbed in the presence of sodium carbonate) from integral membrane proteins (those that remain membrane-associated following treatment) (Coligan et al. 1997).

Following sodium carbonate extraction (Fujiki et al. 1982) of freshly isolated oyster sperm membranes, the purified membranes (P3 pellet) enriched with intrinsic membrane proteins caused vigorous, repetitive shell closures in 78% of oysters tested; in contrast, the S3 supernatant, which was enriched with extrinsic membrane proteins, stimulated shell closures in only 18% of oysters tested. Similar results were obtained using male gonad tissue stored at −70°C for more than 6 mo. These initial data suggested that the sperm-associated pheromone was retained in sperm membranes, and that sodium carbonate treatment did not remove the shell closure-stimulating activity from sperm membranes.

More extensive experiments were conducted to test the heat and trypsin sensitivity of the candidate oyster sperm pheromone (Fig. 2). After removing extrinsic proteins from sperm membranes, vigorous and repetitive shell closure activity was still present in purified sperm membranes (P3 pellet fractions). Incubation of these P3 membranes at 100°C resulted in a significant reduction in this activity. Likewise, breaking disulfide bonds in intrinsic sperm membrane proteins followed by trypsin digestion resulted in a significant reduction of this activity, suggesting that the active factor(s) is a transmembrane or lipid-anchored membrane protein in the sperm membrane.

Little is known about the identity of any invertebrate or vertebrate water-borne peptide/protein pheromones or their receptors. Painter and colleagues (1998, 1999, 2000) were the first to determine the structure and biologic activity of a water-borne peptide pheromone in invertebrates; the 58-residue attractant ("attractin") in *Aplysia* has been cloned (Fan et al. 1997) and its 3-D structure predicted (Schein et al. 2001). Water-borne peptide pheromones (e.g., E*r*-1, E*r*-2) and their receptors have been best characterized in the protozoan *Euplotes raikovi*; the E*r* receptors, which probably arise by alternative splicing from the same gene for the E*r* pheromones, each contain a copy of a pheromone sequence secreted by the cell (reviewed in Luporini et al. 1996).

In contrast with soluble peptide/protein pheromones, oyster spawning presumably involves the direct binding of an intrinsic sperm membrane-associated protein(s) with membrane-associated receptors in neighboring oysters. This is based on the observation that oysters initiate a series of strong, rhythmic contractions of the adductor muscle in response to sperm entering the mantle cavity via the incurrent siphon (Galtsoff 1938a, Galtsoff 1938b). Detailed information regarding the nature of the sperm membrane protein(s) and its receptor are lacking, however. Therefore, a complete understanding of the molecular mechanisms underlying spawning activation will not be achieved until the sperm-associated membrane protein(s) and its receptor are structurally characterized and cloned.

A molecular mechanism for spawning has been proposed for other mollusks. It has been demonstrated in abalone that hydrogen peroxide causes gravid male and female abalones to spawn and that this effect may result from a direct activation of the enzyme-catalyzed synthesis of prostaglandin endoperoxide. The latter is a direct precursor of prostaglandins and thromboxanes; all three may be involved in regulating spawning in abalone (Morse et al. 1977). Hydrogen peroxide also induces synchronous spawning in male and female mussels (*Mytilus*) and in other molluscan species (Morse et al. 1977).

Lastly, regardless of the molecular mechanisms underlying sperm-induced spawning in oysters, the response of female oysters to sperm is not species-specific: sperm from *C. virginica*, *C. gigas*, or *C. angulata* Lamarck have been shown to stimulate spawning in females of all three species (Nelson 1931, Galtsoff 1932, Galtsoff & Smith 1932). This suggests that a synthetic or recombinant *C. virginica* spawning pheromone could be used in the aquaculture industry to induce spawning in several commercially important species.

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FACTORS AFFECTING SPERM MOTILITY OF TETRAPLOID PACIFIC OYSTERS

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ABSTRACT Factors such as osmotic pressure, extender solution, addition of caffeine, and pH have been shown to affect sperm motility in aquatic species. We evaluated the effects of 18 osmotic pressures, two extender solutions, seven caffeine concentrations, and a pH range of 3 to 14 on motility of sperm from tetraploid Pacific oysters, *Crassostrea gigas*. Motility was highest at 1000 mOsmol/kg (mean ± SD: 83 ± 14%). Calcium-free Hanks' balanced salt solution yielded significantly higher sperm motility than did artificial seawater. Sperm motility increased with caffeine concentrations to 20 mM (81 ± 12%) and decreased when concentrations were higher than 50 mM (55 ± 20%). Highest motility was obtained at a pH range of from 4 to 12; values outside this range yielded no motility. Addition of 10 mM caffeine to the different pH treatments also enhanced motility significantly. Overall, calcium-free Hanks' balanced salt solution at 1,000 mOsmol/kg, the addition of 10 mM caffeine, and a pH of around 10 could be used to enhance sperm motility of tetraploid Pacific oysters. Our findings would assist the use of motility assays to evaluate the effectiveness of various refrigeration or cryopreservation procedures, especially outside of the peak spawning season, when sperm motility can be low and variable.

KEY WORDS: *Crassostrea gigas*, sperm motility, pH, caffeine, osmotic pressure, tetraploid, cryopreservation

INTRODUCTION

Factors such as osmotic pressure (Bates et al. 1996), extender composition, pH, temperature (Lahnsteiner et al. 1997, Sunitha and Jayaprakas 1997), and additives such as caffeine (Scheerer and Thorgaard 1989, Tiersch et al. 1998) have been shown to affect sperm motility in aquatic species. Most studies have been conducted on teleosts (e.g., Morisawa et al. 1983a, Morisawa 1983b), and studies on invertebrates mainly focus on sea urchin (Morisawa et al. 1990), polychaetes (Pacey et al. 1994), and ascidians (Yoshida et al. 1992, Yoshida et al. 1994). Little is known about the effects of these factors on oyster sperm, especially on sperm from tetraploid oysters, which possess four sets of chromosomes instead of the normal diploid two sets.

Tetraploid oysters create opportunities for genetic improvement, including direct production of triploid (sterile) seedstocks by crossing with normal diploids. Refrigerated and frozen storage of tetraploid oyster sperm will be a critical tool for commercial-scale application of tetraploid stocks and for developing tetraploid breeding programs. Although subjective, motility estimation is the technique used most commonly to assess sperm quality of fish and shellfish (Piironen 1993, Tiersch et al. 1994, Koupal et al. 1995). Motility has been used to assess the sperm quality of oysters (Paniagua-Chavez et al. 1998), but its application with tetraploid oyster sperm is unexplored. The objective of this study was to develop procedures for evaluation of sperm quality to assist the overall goal of sperm storage for tetraploid Pacific oysters, *Crassostrea gigas*. Specifically, we evaluated the effects on sperm motility of: (1) osmotic pressure; (2) extender solution; (3) caffeine, and (4) pH. Our findings indicate that these factors can alter the motility of tetraploid oyster sperm collected late in the spawning season. To our knowledge, this is the first study to systematically characterize sperm motility of tetraploids of an aquatic species.

MATERIALS AND METHODS

Tetraploid oysters were obtained in September and October 2001 from Whiskey Creek Shellfish Hatchery (WCSH) (Tillamook, Oregon) and were shipped chilled by overnight delivery to the Louisiana State University Agricultural Center, Aquaculture Research Station (ARS). Water samples from WCSH had an osmolality of 873 mOsmol/kg as measured by vapor pressure osmometry (model 5500, Wescor Inc., Logan, UT) at the ARS. Sperm were collected by dry stripping of the gonad (Allen & Bushek 1992). Undiluted nonmotile sperm were equilibrated in 30 μL of test solutions at 23°C for 2 min before assessment of motility. Sperm motility was estimated at 200× magnification using darkfield microscopy (Optiphot 2, Nikon Inc., Garden City, NY) and was expressed as the percentage of cells actively moving in a forward direction.

Throughout the experiments, two extender solutions were used: artificial sea water (ASW) (Fritz Super Salt, Fritz Industries, Inc. Dallas, TX) and calcium-free Hanks' balanced salt solution (C-F HBSS) (Paniagua-Chavez et al. 1998). All chemicals (except ASW) were of reagent grade (Sigma Chemical Corporation., St. Louis, MO). Osmolality was measured with a vapor pressure osmometer.

In our first study, the effect on sperm motility of ASW of 18 different osmolalities ranging from 30 to 1400 mOsmol/kg was evaluated with a total of 20 oysters in four trials, for which oysters were received on August 24, August 30, September 19, and September 26. The second study compared ASW and C-F HBSS at 13 different osmolalities with five oysters, which were received on October 16. In the third study, the effect of caffeine was evaluated at seven concentrations (2 to 100 mM) with eight oysters from two shipments received on October 10 and October 16. In the fourth study, a pH range of from 3 to 14, with and without caffeine, was evaluated with three oysters that were received on October 10. Sperm from individual oysters was used for all studies (samples were not pooled). Within this manuscript, extender solutions at specific osmolalities such as ASW at 1,000 mOsmol/kg are abbreviated as ASW1000.

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Data were analyzed using one-way or two-way analysis of variance. Tukey's honestly significant difference procedure was used to test for differences ($\alpha = 0.05$) among results for osmolalities, caffeine concentrations and pH levels (SAS Institute 1991).

RESULTS

Among the osmolalities tested, sperm from tetraploid Pacific oysters remained immotile when diluted with ASW below 500 mOsmol/kg (Fig. 1). Motility increased from 12 ± 12% (mean ± SD) in ASW500 to 50 ± 11% in ASW670. There was no significant difference among sperm motilities activated with ASW at 670, 700, 750, and 800 mOsmol/kg ($P > 0.05$). Although sperm motility was not significantly different among ASW at 900, 950, 1,000, and 1,100 mOsmol/kg, ASW1000 elicited the highest motility 83 ± 14% (Fig. 1). Motility decreased significantly when osmolality was greater than 1,100 mOsmol/kg.

Motility in C-F HBSS was significantly higher than that in ASW ($P < 0.0001$) (Fig. 2). Each extender showed the highest motility at 1,000 mOsmol/kg, which agreed with the results of the previous experiment. But motility in C-F HBSS1000 (81 ± 9%) was double that in ASW1000 (40 ± 22%). Other osmolalities of C-F HBSS also enhanced sperm motility. For example, motility in C-F HBSS670 (50 ± 10%) was six times greater than that in ASW670 (8 ± 13%) (Fig. 2). Because the oysters used in this experiment were received later in the spawning season than those used for the first study, lower motilities overall were observed for ASW.

Based on the results of the second experiment, C-F HBSS at 1,000 mOsmol/kg was used for caffeine assessments. Motility increased with caffeine concentrations of from 2 mM (60 ± 13%) to 20 mM (81 ± 12%) and decreased when concentrations were higher than 50 mM (55 ± 20%) (Fig. 3). The lowest motility (49 ± 10%) was in the control treatment: C-F HBSS1000 without the addition of caffeine, but additions of 2, 4, and 6 mM caffeine were not significantly different from the control ($P > 0.05$). Motilities in 10 and 20 mM caffeine were significantly higher than in other concentrations ($P < 0.05$), but they were not different from each other ($P > 0.05$) (Fig. 3).

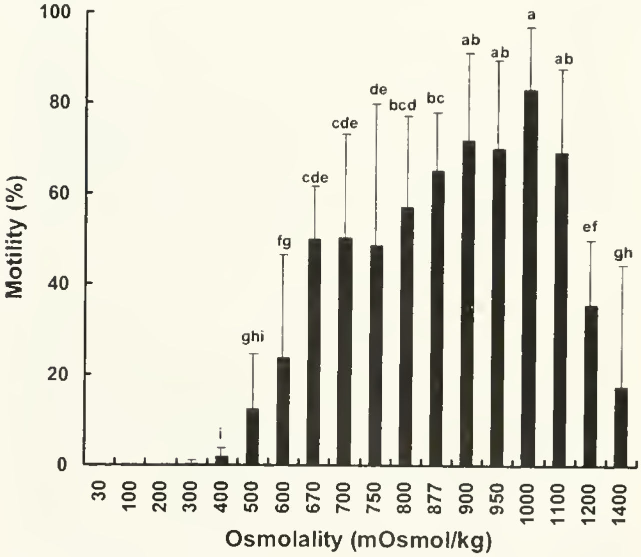

Figure 1. Percent motility (mean ± SD) of tetraploid Pacific oyster sperm activated in artificial seawater at 18 different osmotic pressures. Bars sharing a letter were not significantly different ($P > 0.05$).

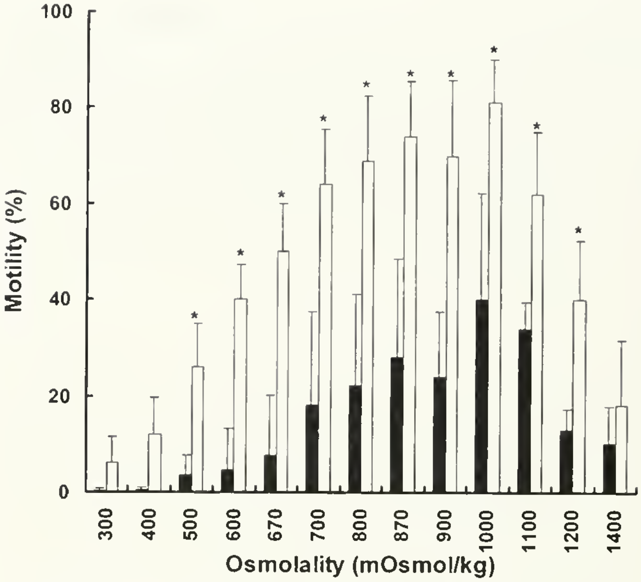

Figure 2. Percent motility (mean ± SD) of tetraploid Pacific oyster sperm activated in ASW (filled bars) and C-F HBSS (open bars). Bars with an asterisk indicate a significant difference ($P > 0.05$) between ASW and C-F HBSS.

Based on these results, C-F HBSS1000 with and without 10 mM caffeine were used for pH assessment. Motility was highest at a pH range of from 4 to 12; values outside this range yielded no motility (Fig. 4). The highest motility was in pH 10.5 (67 ± 6%), followed by pH 10 (63 ± 6%). The addition of 10 mM caffeine to these pH treatments enhanced motility significantly ($P < 0.001$)

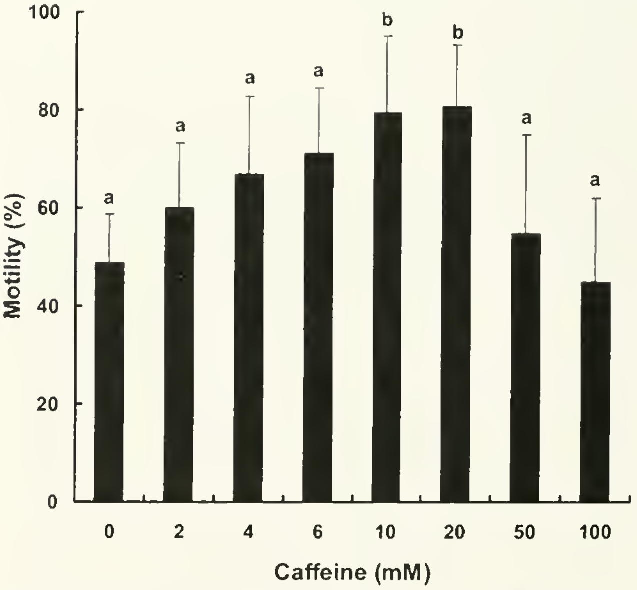

Figure 3. Percent motility (mean ± SD) of tetraploid Pacific oyster sperm activated with the addition of caffeine into C-F HBSS at 1,000 mOsmol/kg. Bars sharing a letter were not significantly different ($P > 0.05$).

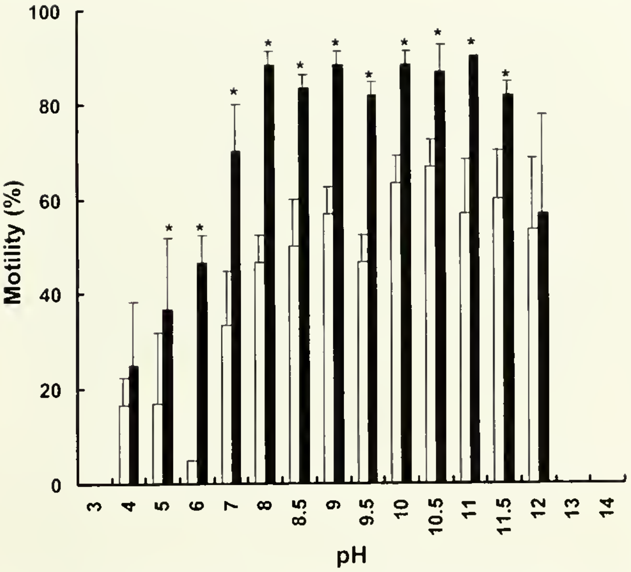

Figure 4. Percent motility (mean ± SD) of tetraploid Pacific oyster sperm in C-F HBSS at 1,000 mOsmol/kg with different pH treatments. Open bars, C-F HBSS without addition of caffeine; filled bars, C-F HBSS with 10 mM caffeine. Bars with an asterisk indicate a significant difference ($P > 0.05$) between presence and absence of caffeine.

(Fig. 4). Motility was 90 ± 0% in pH 11, 87 ± 6% in pH 10.5, and 88 ± 3% in pH 10. However, there was no significant difference in sperm motility across the pH range of from 7 to 12 ($P > 0.05$).

DISCUSSION

Sperm activation is a complex process in which roles are played by many factors, including osmotic pressure, extender composition, membrane structural changes, and extracellular and intracellular pH. Previous studies have suggested that different species have different sperm activation mechanisms. Basically, in fish there seem to be two major factors influencing sperm activation: changes of osmotic pressure and changes of ionic concentration. For instance, studies on muskellunge *Esox masquinongy* (Lin & Dabrwoski 1996), channel catfish *Ictalurus punctatus* (Bates et al. 1996), and Asian catfish *Clarias macrocephalus* (Tan-Fermin et al. 1999) suggested that sperm motility was initiated by a reduction of osmotic pressure. However, activation of sperm from salmonids and cyprinids was caused by changes in concentrations of ions such as potassium and calcium (Morisawa et al. 1983a, Billard & Cosson 1992). The mechanism of initiation of sperm motility has been most studied in salmonids although much remains unknown, especially at the intracellular level.

Motility studies in other taxa such as invertebrates are limited and the associated mechanisms remain largely unknown. There are less than 40 references addressing oyster sperm motility in any way since 1970. Most use motility as a criterion to evaluate fertilization or in toxicological assays to evaluate waste effluents or heavy metals. Factor that affect sperm motility such as osmolality, extender composition, pH, and temperature are briefly mentioned in these studies and others on sperm cryopreservation, and therefore the information is fragmented and dispersed. The present study was designed to address factors affecting the sperm motility of tetraploid Pacific oysters and provided a more detailed and systematic approach.

Osmolality, as mentioned above, plays an important role in the activation of fish sperm. Osmolalities of ~1000 mOsmol/kg (the full-strength salinity of sea water, 32 ppt) and 670 mOsmol/kg were previously used for sperm activation and extender solutions for *C. gigas* (Yankson & Moyse 1991, Kurokura et al. 1990). Other species were studied with sea water of ambient environmental salinity. The first investigation of sperm motility across a wide range of osmolalities was with the diploid eastern oyster, *C. virginica* (Paniagua-Chavez et al. 1998). High sperm motility (90%) was observed across a range of 600 to 1,500 mOsmol/kg (Paniagua-Chavez & Tiersch 2001). In the present study, sperm motility of tetraploid Pacific oysters was also observed across a wide range of osmotic pressures (500–1400 mOsmol/kg), but the highest motility was limited to 1,000 mOsmol/kg, although the oysters were conditioned in seawater at 873 mOsmol/kg. Whether osmotic pressure plays the major role in oyster sperm activation remains unknown; however, motility was suppressed in tetraploid sperm of *C. gigas* at osmolalities of lower than 500 mOsmol/kg. Suppression was reported to occur at less than 22 mOsmol/kg in *C. virginica* (Paniagua-Chavez et al. 1998). This might correspond to the habitat of these species in natural environments. *Crassostrea gigas* prefers higher and more stable salinities (15 to 33 ppt) (Kusuki 1991) than does *C. virginica*, which normally occurs from 5 to 40 ppt (Galtsoff 1964, Wallace 1966).

Natural seawater and ASW are most commonly used as extender solutions for sperm of marine organisms including estuarine organisms like oysters. Other extenders such as DCSB4 (Bougrier & Rabenomanana 1986), HBSS1990 (Zell et al. 1979), and C-F HBSS640 (Paniagua-Chavez et al. 1998) with specific ionic compositions have also been successfully used for oyster sperm. The removal of calcium from HBSS was found to enhance motility in sperm of diploid eastern oysters (Paniagua-Chavez et al. 1998) and tetraploid Pacific oysters (this study) and was also superior to ASW. Contrary to the situation in salmonids, in which sperm motility is initiated by a decrease in potassium concentration upon release into fresh water (Morisawa et al. 1983a), an increase of potassium concentration (200 mM) was found to have an activating effect on diploid Pacific oyster sperm (Faure et al. 1995). The same study showed no effect of the increase of potassium on sperm of the king scallop *Pecten maximus*, but an increase in motility was observed with media lacking sodium. These results may indicate a species-specific response for ionic effects on bivalve sperm motility. Also, changes in ion concentration, rather than absolute concentration, may act as the trigger of initiation of sperm motility.

Caffeine has been used as motility stimulant to optimize the recovery and quality of thawed spermatozoa in mammalian species (Correa & Zavos 1996, Park & Sirard 1996). Few studies have addressed use of this chemical in sperm of aquatic species and there are no reports for oysters. The present experiment showed a significant increase in sperm motility with the addition of 10 mM caffeine, while concentrations above 50 mM reduced motility. A previous study in the razorback sucker *Xyrauchen texanus* showed increased motility in refrigerated sperm after the addition of 5 mM caffeine, but not in thawed sperm (Tiersch et al. 1998). We did not evaluate the addition of caffeine with thawed sperm in this study, but fertilizing capacity of cryopreserved semen of rainbow trout *Oncorhynchus mykiss* was improved when eggs were fertilized in a buffered saline solution containing 5 mM theophylline, a chemical relative to caffeine used to prolong and intensify sperm motility (Scheerer & Thorgaard 1989).

Alkaline pH has been found to be conducive to sperm activation in aquatic species (Thorogood & Blackshaw 1992, Sunitha &

Jayaprakas 1997, Ciereszko et al. 2001), and pH values between 7 and 9 have been used in most studies for oyster sperm (e.g., Zell et al. 1979, Paniagua-Chavez et al. 1998). Study of the king scallop (Faure 1996) found that gonadal pH was more acidic than seawater pH and suggested that the acidity of the genital tract maintained the spermatozoa in a quiescent state. A reduction of sperm motility was reported in *P. maximus* and *C. gigas* upon decrease of pH in seawater (Faure 1996). In the present study, pH values below 7 induced a significant reduction in sperm motility although there was variation among individual oysters. The highest sperm motility was observed at a pH of 10, which agrees with findings for the Japanese pearl oyster *Pinctada fucata* (Yu et al. 1998), although that study found an interaction between salinity and pH. In that study, sperm motility could not be activated by seawater at a salinity of 30 ppt and a pH of 8.0, but motility was greater than 80% when pH ranged from 9.0 to 11.5. Sperm from tetraploid Pacific oysters showed motility over a wider pH range in the present study. It appears that relative influence of most factors affecting sperm motility of bivalves are species-specific.

Finally, the main practical interest in tetraploidy is for the production of triploids by mating with diploids. The induction of tetraploidy in oysters was first reported in 1994 (Guo and Allen, 1994). Studies of the factors affecting sperm motility of tetraploid oysters have only now begun as reported here. Our experiments indicate that use of C-F HBSS at 1,000 mOsmol/kg as an extender, the addition of 10 mM caffeine, and a pH of around 10 can be used to enhance sperm motility of tetraploid Pacific oysters. This would assist the use of motility assays to evaluate the effectiveness of various refrigeration or cryopreservation procedures, especially outside of the peak spawning season when sperm motility can be low and variable such as in this study. The effectiveness of these conditions in improving fertilization rates requires further study. Future research is required to evaluate differences between sperm of diploid and tetraploids within and among aquatic species.

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AN IMPROVED PROCEDURE TO COUNT *PERKINSUS MARINUS* IN EASTERN OYSTER HEMOLYMPH

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ABSTRACT *Perkinsus marinus* infection intensity in *Crassostrea virginica* can be quantified without killing of oysters by determining parasite density in hemolymph samples incubated in fluid thioglycollate medium (FTM). The goal of this study was to improve existing protocols for counting of *P. marinus* in oyster hemolymph. Specifically, the objectives were to examine the effects on parasite number and diameter of: 1) adding supplements to FTM such as lipid and oyster extract; 2) incubating with various FTM preparations with and without agar or beef extract; 3) incubating with various hemocyte densities (10^5, 10^6, and 10^7 hemocytes/mL of FTM) in a constant FTM volume; 4) incubating with different volumes of FTM (0.2 mL, 1.0 mL, 5.0 mL, and 25.0 mL); and 5) sodium hydroxide digestion of cellular debris. From these results, an improved hemolymph protocol was developed. The diameters and numbers of enlarged parasites or hypnospores in hemolymph of 20 oysters measured by the improved protocol and the standard FTM hemolymph assay of Gauthier and Fisher were compared. Finally, the standard and improved protocols were compared with the FTM body burden assay. The diameter of hypnospores from samples processed with the improved protocol (26 ± 13 μm) was significantly greater than the diameters from samples processed with the standard protocol (10 ± 4 μm). The number of hypnospores in samples processed with the improved protocol ($8.6 \times 10^3 \pm 3.3 \times 10^3$) was significantly greater than the numbers in samples processed with the standard protocol ($1.9 \times 10^3 \pm 3.4 \times 10^3$). Results of the body burden assay were significantly correlated with results of the standard hemolymph assay and with results of the improved hemolymph assay. The coefficient of determination ($r^2 = 0.7602$) and slope (0.91189) of the regression of the FTM body burden assay against the improved FTM hemolymph assay was improved from the coefficient of determination (0.5543) and slope (0.61257) of the regression of the FTM body burden assay against of the standard FTM hemolymph assay.

KEY WORDS: dermo, *Perkinsus marinus*, FTM hemolymph diagnostic assay, *Crassostrea virginica*

INTRODUCTION

Use of a protocol to quantify the presence of the pathogenic protozoan *Perkinsus marinus* by determining the number of enlarged parasites, or hypnospores, in hemolymph samples of eastern oysters, *Crassostrea virginica*, allows estimation of infection intensity without killing oysters (Gauthier & Fisher 1990). Therefore, infection intensity in the same animal can be monitored over time. In the existing protocol, hemolymph from the adductor muscle sinus is removed, centrifuged, and the cell pellets containing oyster hemocytes and parasites are incubated in fluid thioglycollate medium (FTM) for 1 week. After incubation, cellular debris is digested with sodium hydroxide (NaOH). The hypnospores are stained with Lugol's solution and their numbers per mL determined. This FTM hemolymph assay has been useful to follow the progression of the disease in individual oysters under different environmental conditions (Fisher et al. 1992, Ragone Calvo & Burreson 1994).

Gauthier and Fisher (1990) proposed additional advantages of the FTM hemolymph assay, including the detection of early infections and the measurement of systemic infections rather than localized infections. In their initial study, the FTM hemolymph assay detected many infections misdiagnosed as negative by Ray's FTM tissue assay. In Ray's FTM tissue assay, a piece of oyster tissue (e.g., mantle tissue in Gauthier and Fisher's 1990 study) is incubated in FTM for about a week, the tissue is then smeared on a slide, and the parasites stained with Lugol's solution and the intensity of infection are estimated using a semi-quantitative scale (Ray et al. 1953, Ray 1954a, Ray 1954b). Bushek et al. (1994), however, found no evidence that the FTM hemolymph assay was more sensitive than the FTM tissue assay in detecting low *P. marinus* infections when both mantle and rectal tissues were used in the tissue assay. Moreover, correlations between FTM tissue and FTM body burden assays were always higher than correlations between FTM hemolymph and FTM body burden assays. The body burden assay measures the number of parasites in the whole oyster and is considered the most sensitive and accurate diagnostic assay for *P. marinus* (Bushek et al. 1994, Fisher & Oliver 1996, Oliver et al. 1998).

A quantitative competitive polymerase chain reaction (QCPCR) assay for *P. marinus* was recently shown to be more sensitive than the FTM hemolymph assay, detecting infections in 24 oysters compared with 22 infections with the FTM hemolymph assay (Yarnall et al. 2000). Diagnosis of *P. marinus* in oyster hemolymph by QCPCR detected as many infections as with the FTM body burden assay, suggesting that hemolymph is suitable for determining *P. marinus* infection in oysters. Whereas QCPCR is an effective assay and specific for *P. marinus*, it is also expensive and requires technical expertise and equipment in molecular biology to perform as indicated by Yarnall et al. (2000). The FTM hemolymph assay in contrast is easy and inexpensive to perform. There is thus a need to improve the sensitivity and accuracy of the FTM hemolymph assay.

Potential problems with FTM assays have been previously noted and include insufficient parasite enlargement, parasite clumping, and parasite adherence to the walls of centrifuge tubes, each of which can lower parasite counts (Bushek et al. 1994, Fisher & Oliver 1996). Poor parasite enlargement may be caused

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by exhaustion of necessary nutrients in FTM or tissue (Ray 1954a, Bushek et al. 1994, Fisher & Oliver 1996). In addition, the protocols used in FTM hemolymph assay varied greatly among researchers in sample preparation (e.g., centrifugation speed), sample processing, and in parasite counting protocols (Gauthier & Fisher 1990, Ragone Calvo & Burreson 1994, Bushek et al. 1994, Oliver et al. 1998). It is likely that these recognized problems and differences in protocols account for the poor performance (i.e., sensitivity and accuracy) of the FTM hemolymph assay.

The goal of this study was to improve the protocol for counting of *P. marinus* in oyster hemolymph through systematic examination of components of the standard protocol described by Gauthier and Fisher (1990). The objectives were to examine the effects of: (1) adding supplements to FTM, including lipid and oyster extract; (2) adding various FTM preparations (with and without agar or beef extract); (3) incubating with various hemocyte densities (10^5, 10^6, and 10^7 hemocytes/mL of FTM) in a constant FTM volume; (4) incubation of hemocytes in different volumes of FTM (0.2 mL, 1.0 mL, 5.0 mL, and 25.0 mL); and (5) NaOH digestion of cellular debris on hypnospore diameter and number.

MATERIALS AND METHODS

Oysters

Eastern oysters were collected from Hackberry Bay (29°23′54″N, 90°28″W) in the spring of 1999, placed in 16 mm mesh shellfish cages (Aquatic Eco-Systems, Inc., Apopka, FL), and hung in the water from docks at the Louisiana Sea Grant Oyster Hatchery (29°12′30″N, 90°02′30″W) in Grand Isle, Louisiana, an area enzootic for *P. marinus*. In the summer and fall of 1999, oysters were transported to the Department of Veterinary Science at Louisiana State University, Baton Rouge, and were placed in an indoor recirculating system equipped with 1 μm and 10 μm cotton filters in polypropylene filter cartridges and an ultraviolet light. Water was maintained at 15 ppt with hw-Marinemix *Professional* sea salts (Hawaiian Marine Imports Inc., Houston, TX) and 25°C.

General Procedures

All chemicals were from Sigma Chemical Co. (St. Louis, MO) unless otherwise indicated. A notch was ground into the edge of oyster shells near the adductor muscle and 3 mL of hemolymph were withdrawn from the adductor muscle sinus. Hemolymph samples were transferred to 3 mL snap-cap tubes and immediately placed on ice to prevent hemocyte clumping. The number of hemocytes per mL of hemolymph was determined using a Neubauer Bright-Line hemocytometer (Reichert, Buffalo, NY). Hemolymph samples from each oyster, containing 10^6 hemocytes, were added to 1.5 mL microcentrifuge tubes, centrifuged at 800 × g for 10 min, and the supernatant was discarded. All samples were prepared in triplicate. Pellets were resuspended in 1 mL of alternative fluid thioglycollate medium (AFTM) (Sigma number A 0465) prepared according to the manufacturer's instructions and supplemented with 16 g/L of hw-Marine *Professional* sea salts and 50 μg/mL chloramphenicol. This solution, referred to as AFTM throughout this report, did not contain agar, unlike FTM (Sigma number T 9032), which contains 0.75 g/L of agar. Samples were layered with 10 μL of nystatin (5,000 Units/mL) to prevent fungal growth. Test tubes were stored in the dark at room temperature for seven days, allowing parasites within hemocytes to enlarge to hypnospores. After incubation, samples were centrifuged at 1500 × g for ten min and the AFTM supernatant was discarded. Pellets were resuspended in 1 mL of 2 N NaOH and incubated in a 60°C water bath for 1 to 2 h to digest hemocyte debris. Samples were centrifuged to remove NaOH and hypnospores were rinsed three times with 0.1 M phosphate-buffered saline (PBS) containing 0.5 mg/mL bovine serum albumin (BSA). In a previous study, it was found that BSA decreased parasite clumping and improved parasite recovery by reducing the number of parasite that adhered to the test tube walls (Coates et al. 1999). Samples were stored at 4°C in 1 mL of PBS supplemented with BSA and 2 mg/mL of sodium azide. At the time of counting, samples were centrifuged, and 900 μL of supernatant were removed. Each 100-μL sample was transferred to a separate well of a 96-well tissue culture plate. Fifty microliters of each sample was transferred to wells containing 50 μL of PBS with BSA to form a 1:1 dilution. Samples were serially diluted in this manner until a 1:64 dilution was reached. Lugol's solution (50 μL; 0.012 g/mL of potassium iodide and 0.008 g/mL of iodine in water) was added to each well to stain the samples. Tissue culture plates containing samples were centrifuged at 200 × g for 5 min to form a monolayer of hypnospores on the plate bottoms to facilitate counting. Numbers of hypnospores were recorded at 200-X magnification using an inverted microscope (Carl Zeiss, Inc., Thornwood, NY) from wells containing 100 to 400 hypnospores. Cells were counted at 400× when they were too small to identify at 200×. The diameter (μm) of 50 hypnospores from each sample was measured with an ocular micrometer, and the number of hypnospores per 10^6 hemocytes was calculated.

Experiments

Effects of Supplemented AFTM on Hypnospore Diameter and Number

Hemolymph was collected from five oysters as described above. AFTM, supplemented with 5% lipid concentrate (Gibco, Gaithersburg, MD), 5% oyster extract, or 5% oyster saline, was added to 10^6 hemocytes from each oyster. Oyster extract was prepared by homogenizing whole oyster tissue in oyster saline at a concentration of 0.2 g wet tissue per mL, centrifuging at 10,000 × g for 15 min, removing the oyster extract (supernatant), and storing it at −20°C. Oyster saline (0.95 g/L $CaCl_2{\cdot}2H_2O$, 1.46 g/L $MgSO_4$, 2.18 g/L $MgCl_2{\cdot}6H_2O$, 0.67 g/L KCl, 11.61 g/L NaCl, and 0.35 g/L $NaHCO_3$) was included as a control. Samples were incubated, processed, counted, and measured as described above. Lipid was found to increase hypnospore diameter and was added to FTM in all further experiments.

Effect of FTM Types on Hypnospore Diameter and Number

Hemolymph was collected from 15 oysters as described above and five formulations of FTM, supplemented with 16 g/L of hw-Marine *Professional* sea salts, 50 μg/mL chloramphenicol, and 5% lipid concentrate, were added to 10^6 hemocytes samples. The formulations compared were Bacto fluid thioglycollate medium (Becton Dickinson and Company, Franklin Lakes, NJ, Difco number 0256154), Bacto thioglycollate medium with K agar (Difco number 0607178), Bacto fluid thioglycollate medium with beef extract (Difco number 0697179), alternative fluid thioglycollate medium (Sigma number A 0465), and thioglycollate medium (Sigma num-her T 9032) (Table 1). Samples were incubated, processed, measured, and counted as described above.

Effect of Hemocyte Density on Hypnospore Diameter and Number

Hemolymph was collected from 15 oysters as described above and 10^5, 10^6, and 10^7 hemocytes from each oyster were incubated in 1 mL of AFTM supplemented with 5% lipid concentrate. For this experiment, the number of hypnospores per hemocyte was calculated, and hypnospore diameter was measured as described above.

Effect of AFTM Volume on Hypnospore Diameter and Number

Hemolymph was collected from 15 oysters as described above. Hemocytes (10^6) were incubated in 0.2, 1.0, 5.0, or 25.0 mL of AFTM supplemented with 5% lipid concentrate. Samples were processed, measured, and counted as described above.

Effect of NaOH Digestion of Samples on Hypnospore Diameter and Number

Hemolymph samples from 15 oysters were processed with and without NaOH digestion after incubation in AFTM supplemented with 5% lipid concentrate. Samples processed without NaOH digestion were centrifuged at 1500 × g for 10 min to remove the AFTM supernatant. Cell pellets were rinsed three times with sterile artificial seawater (hw-Marinemix *Professional*) at 15 ppt and containing 0.5 mg/mL BSA. Samples processed with NaOH digestion were treated as described in the General Procedures section.

Effect of Protocol on Hypnospore Diameter and Number

From the results of the above experiments, an improved protocol for counting of *P. marinus* in oyster hemolymph was developed and compared with the 'standard' protocol of Gauthier and Fisher (1990). Major differences between the standard and improved protocols are summarized in Table 2. Briefly, hemolymph samples were collected from 20 oysters infected with *P. marinus*. Hemolymph samples were divided into two equal aliquots, one aliquot to be processed with the standard protocol and the other to be processed with the improved protocol. Hemocytes (10^6) from samples processed with the standard protocol were incubated for seven days in 1 mL of FTM (Sigma number T 9032) supplemented with 16 g/L of hw-Marinemix *Professional* sea salts, 50 μg/mL of chloramphenicol, and layered with 10 μL of nystatin (5000 Units/ml). Hemocyte debris was digested with 1 mL of 2 N NaOH, and hypnospores were rinsed three times with 1 mL of distilled water. Samples were centrifuged, 900 μL of supernatant were removed, and each 100-μL sample was transferred to a separate well of a 96-well plate, where it was serially diluted in distilled water and stained with Lugol's solution. Standard protocol centrifugation speeds were increased to the centrifugation speeds of the improved protocol to exclude their effects on parasite recovery. Hemocytes (10^6) from samples processed with the improved protocol were incubated for seven days in 1 ml of AFTM (Sigma number A 0465) supplemented with 5% lipid, 16 g/L of hw-Marinemix *Professional* sea salts, 50 μg/mL of chloramphenicol, and layered with 10 μL of nystatin (5000 Units/mL). Hemocyte debris was digested with 1 mL of 2 N NaOH and hypnospores were rinsed once with 1 mL of distilled water supplemented with 0.5 mg/mL BSA and twice with 1 mL PBS supplemented with 0.5 mg/mL BSA. Samples were centrifuged, 900 μL of supernatant were removed, and each 100-μL sample was transferred to a separate well of a 96-well plate, where it was serially diluted in PBS supplemented with BSA and stained with Lugol's solution. After processing samples with both protocols, the number of hypnospores was counted 200× magnification in wells containing 100 to 400 hyp-

TABLE 2.

Differences between the standard protocol and the improved protocol to count *Perkinsus marinus* in oyster hemolymph.

Component	Standard	Improved
Type of FTM	FTM (with agar)	Alternative FTM (without agar)
FTM Supplement	None	5% lipid concentrate
Rinsing solution	Distilled water	Phosphate-buffered saline (0.1 M) with 0.5 mg/mL bovine serum albumin

TABLE 1.

Components of different brands of fluid thioglycollate media.

	Difco[a]			Sigma[a]	
Component	Bacto FTM	Bacto FTM With Potassium Agar	Bacto FTM With Beef Extract	Alternative FTM	FTM
Beef extract	—	—	5 g	—	—
Yeast extract	5 g	5 g	5 g	5.00 g	5.0 g
Casein digest	15 g	15 g	15 g	15.00 g	15.0 g
Dextrose	5.5 g	5 g	5.500 g	5.50 g	5.5 g
Sodium chloride	2.5 g	—	2.5 g	2.50 g	2.5 g
Potassium chloride	—	2.5 g	—	—	—
L-Cystine	0.5 g	0.5 g	0.5 g	0.50 g	0.5 g
Sodium thioglycollate	0.5 g	0.3 mL[b]	0.5 g	0.50 g	0.50 g
Agar	0.75 g	0.45 g	0.75 g	—	0.75 g
Resazurin	0.001 g	0.001 g	0.001 g	—	0.001 g

[a] Decimal places reported as suggested by manufacturer.

[b] Thioglycollic acid.

nospores after dilutions. The diameter of 50 hypnospores was measured from each hemocyte sample.

Comparison of Standard and Improved FTM Hemolymph Assay Against FTM Body Burden Assay

The standard and improved FTM protocols for counting *P. marinus* in oyster hemolymph (as described above) were compared with the FTM body burden assay for determining the number of parasites in whole oyster and a regression line was calculated for each comparison. The regression lines were compared with determine if infection intensities obtained from the improved hemolymph protocol were more closely correlated with infection intensities obtained from the body burden assay than those obtained from the standard hemolymph protocol.

The 20 oysters from which hemolymph was removed for comparison of the standard and improved hemolymph protocols, were processed using modification of the body burden assay of Fisher and Oliver (1996) (Coates et al. 1999). Briefly, each oyster was removed from the shell, blotted dry, and homogenized with a hand-held Biohomogenizer, a stainless-steel rotor/stator emulsifying instrument, (Biospec Products, Inc., Batlesville, OK, catalog number 1281) in 20 mL of sterile artificial seawater prepared at 15 ppt with hw-Marine *Professional* sea salts. One milliliter of homogenate was transferred to 9 mL of AFTM (Sigma number A 0465) supplemented with 16 g/L of hw-Marinemix *Professional* sea salts, 50 μg/mL of chloramphenicol 5% lipid, and layered with 100 μL of nystatin (5000 Units/mL), and incubated for seven days. Samples were centrifuged, the supernatant removed, and 10 mL of 2 N NaOH added for 4 to 5 h at 60°C to digest the oyster tissue. Hypnospores were rinsed once with 10 mL of distilled water supplemented with 0.5 mg/mL BSA and twice with 10 mL PBS supplemented with 0.5 mg/mL BSA. Samples were centrifuged, 5 mL of supernatant were removed, 50 μL of each sample were transferred to a separate well of a 96-well plate, where it was serially diluted in PBS supplemented with 0.5 mg/mL BSA and stained with Lugol's solution. The number of hypnospores was counted (200× magnification) in wells containing 100 to 400 hypnospores after dilutions. The number of hypnospores per gram of oyster tissue was calculated for each oyster and compared with the number of hypnospores per 10^6 hemocytes determined by the standard and improved hemolymph protocols using linear regression analysis.

Statistical Analysis

Statistical analysis was performed using SAS Version 8.0 software (SAS Institute, Inc. Cary, NC). Only hemolymph samples containing at least 50 hypnospores (per triplicate) were used data analysis. Data were log transformed and analyzed with a randomized block design (blocked by oyster). Tukey's post-ANOVA test was used to examine differences among treatments. Differences were considered significant at $P < 0.05$. All data were reported as mean ± standard deviation. To compare the standard and improved FTM hemolymph assays against the FTM body burden assay, two linear regressions were calculated using log-transformed data.

RESULTS

Effects of Supplemented AFTM on Hypnospore Diameter and Number

The diameter of hypnospores from samples incubated in AFTM supplemented with lipid concentrate (27.3 ± 11.5 μm) was significantly greater ($P < 0.0001$) than the diameter of hypnospores from samples incubated in AFTM supplemented with oyster extract (18.8 ± 9.4 μm) or with saline (16.5 ± 10.2 μm) (Fig. 1). No significant difference in hypnospore diameter was found between samples incubated in AFTM supplemented with oyster extract or with saline. No significant difference was found in the hypnospore numbers between samples incubated in AFTM supplemented with lipid concentrate ($1.9 \times 10^5 \pm 7.1 \times 10^4$), oyster extract ($1.8 \times 10^5 \pm 4.7 \times 10^4$) or the saline control ($2.2 \times 10^5 \pm 6.6 \times 10^4$).

Effect of FTM Types on Hypnospore Diameter and Number

No significant differences were found for hypnospore diameter or hypnospore number among samples incubated in different formulations of FTM (Table 3). The absence of agar in the Sigma alternative fluid thioglycollate medium facilitated sample processing because the viscous layer that generally collected over hypnospore pellets after centrifugation when samples were incubated in FTM with agar was eliminated.

Effect of Hemocyte Density on Hypnospore Diameter and Number

The diameter of hypnospores from samples that received 10^5 hemocytes per ml of AFTM (19.9 ± 8.8 μm) was significantly greater ($P < 0.001$) than the diameter of hypnospores from samples that received 10^6 hemocytes per ml (15.3 ± 8.7 μm) and 10^7 hemocytes per ml (15.2 ± 12.1 μm) (Fig. 2). No significant difference was found in hypnospore diameters between samples that received 10^6 hemocytes per ml and 10^7 hemocytes per ml. No significant difference was found in the number of hypnospores per hemocyte among samples that received 10^5 hemocytes per ml (0.06 ± 0.15 hypnospores/hemocyte), 10^6 hemocytes per ml (0.67 ± 2.26 hypnospores/hemocyte) and 10^7 hemocytes per ml (0.03 ± 0.07 hypnospores/hemocyte).

Effect of AFTM Volume on Hypnospore Diameter and Number

The diameter of hypnospores from samples that were incubated in 5.0 mL (24 ± 12 μm) and 25.0 mL (28 ± 13 μm) of AFTM was significantly greater ($P < 0.0001$) than the diameter of hypnospores from samples incubated in 0.2 mL (13 ± 6 μm) and 1.0 mL (16 ± 8 μm) of AFTM (Fig. 3A). However, the number of hypnospores in samples incubated in 25.0 mL of AFTM ($4.5 \times 10^4 \pm 6.9 \times 10^4$) was significantly lower ($P < 0.0002$) than the number of hypnospores in samples incubated in 0.2 mL ($9.3 \times 10^4 \pm 1.1 \times 10^5$), 1.0

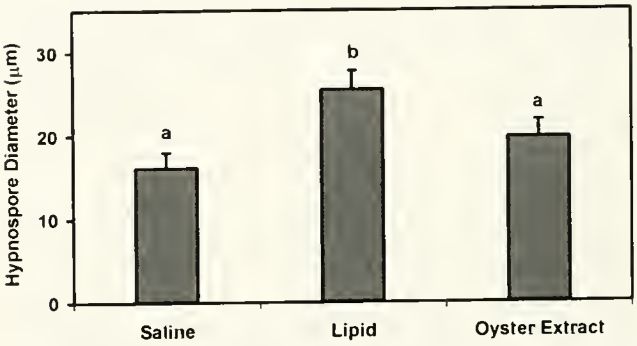

Figure 1. Hypnospore diameter (mean ± SD, $n = 5$) after incubation in AFTM supplemented with lipid concentrate, oyster extract, and saline (control). Hemocytes (10^6) were incubated for 7 days in 1 mL of supplemented AFTM. Treatments sharing a letter were not significantly different.

TABLE 3.

Average values (mean ± SD, $n = 9$) of hypnospore diameter and number of hypnospores per 10^6 hemocytes after incubation in different types of FTM.

Type of FTM	Hypnospore Diameter (μm)	Number of Hypnospores
Difco Bacto FTM	21 ± 11	$8.3 \times 10^4 \pm 1.1 \times 10^5$
Difco Bacto FTM with K agar	20 ± 12	$1.8 \times 10^5 \pm 3.3 \times 10^5$
Difco Bacto FTM with beef extract	19 ± 11	$1.2 \times 10^5 \pm 1.7 \times 10^5$
Sigma alternative FTM	18 ± 11	$2.0 \times 10^5 \pm 4.0 \times 10^5$
Sigma FTM	22 ± 13	$1.8 \times 10^5 \pm 3.1 \times 10^5$

mL ($1.8 \times 10^5 \pm 2.7 \times 10^5$), and 5.0 mL ($1.2 \times 10^5 \pm 1.5 \times 10^5$) of AFTM (Fig. 3B). No significant difference was found in the number of hypnospores among samples incubated in 0.2, 1.0, and 5.0 mL of AFTM.

Effect of NaOH Digestion of Samples on Hypnospore Diameter and Number

No significant difference was found in hypnospore diameter between samples processed with NaOH digestion (24 ± 17 μm) or without it (19 ± 9 μm). No significant difference was found in the number of hypnospores between samples processed with NaOH digestion ($1.8 \times 10^4 \pm 2.6 \times 10^4$) or without it ($2.6 \times 10^4 \pm 3.8 \times 10^4$). Sodium hydroxide facilitated counting by reducing cellular debris.

Effect of Protocol on Hypnospore Diameter and Number

Hypnospore diameter from samples processed with the improved protocol (26 ± 13 μm) was significantly greater ($P < 0.0001$; $n = 20$) than the diameter of hypnospores from samples processed with the standard protocol (10 ± 4 μm). Hypnospore numbers in samples processed with the improved protocol ($8.6 \times 10^3 \pm 3.3 \times 10^3$) were significantly greater ($P < 0.0001$) than the number of hypnospores in samples processed with the standard protocol ($1.9 \times 10^3 \pm 3.4 \times 10^3$). Hypnospores from hemocytes processed with the improved protocol readily settled to the plate bottom and could be easily counted. Hypnospores processed with the standard protocol were difficult to count because they remained suspended in the wells and the media column had to be scanned vertically as a consequence (Fig. 4).

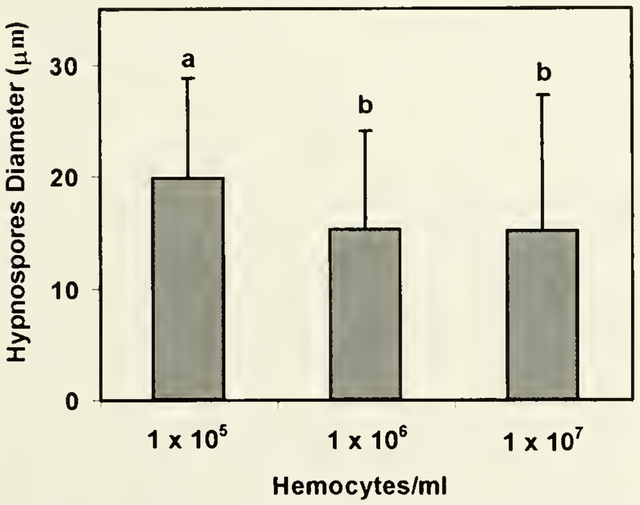

Figure 2. Effect of hemocyte density (10^5, 10^6, or 10^7 hemocytes per ml of AFTM) on hypnospore diameter (mean ± SD, $n = 13$). Hemocytes were incubated in 1 mL of AFTM supplemented with 5% lipid for seven days. Means sharing a letter were not significantly different.

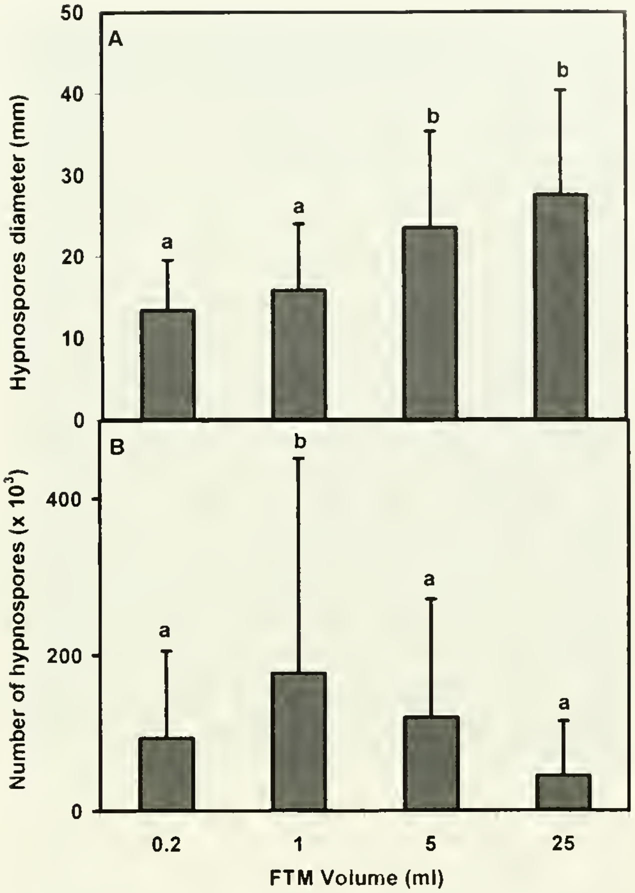

Figure 3. Hypnospore diameter (A) and number (B) (mean ± SD, $n = 10$) after incubation in 0.2, 1.0, 5.0, and 25.0 mL of AFTM. Hemocytes (10^6) were incubated for seven days in 1 mL of AFTM supplemented with 5% lipid. Means sharing a letter in each panel were not significantly different.

Comparison of Standard and Improved FTM Hemolymph Assays with FTM Body Burden Assay

Using linear regression, comparison of the infection intensity determined by the body burden assay (log of hypnospores per gram of oyster tissue) and the infection intensity determined by the standard hemolymph assay (log of hypnospores per 10^6 hemocytes) showed that the correlation was highly significant ($P < 0.0001$) with a coefficient of determination (r^2) of 0.5543 and a slope for the regression line of 0.6126 (Fig. 5A). Using linear regression, comparison of the infection intensity determined by the body burden assay and the infection intensity determined by the improved hemolymph assay showed a highly significant correlation ($P < 0.0001$) with an improved coefficient of determination ($r^2 = 0.7602$) and slope (0.9119) (Fig. 5B). This increased coefficient of determination indicated a reduction in variance with the improved FTM hemolymph assay.

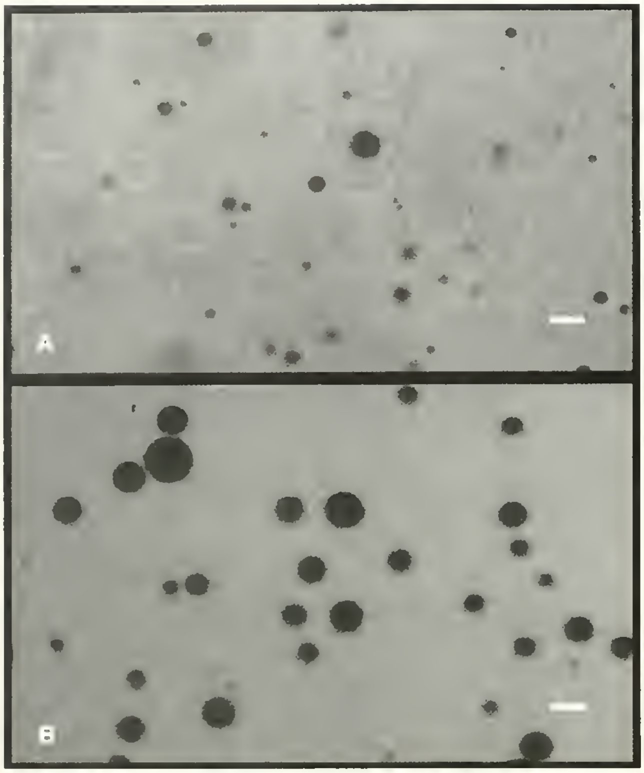

Figure 4. Light micrographs of *Perkinsus marinus* hypnospores after incubation and processing with the standard and improved protocols at 200× magnification. Bar represents 30 μm. A, Hypnospores were small (7–13 μm) and not clearly visible after incubation and processing with the standard protocol. B, Hypnospores were larger (15–44 μm) and more visible after incubation and processing with the improved protocol.

DISCUSSION

Use of the improved protocol to count *P. marinus* in hemolymph resulted in a 167% increase in hypnospore diameter and a 358% increase in hypnospore number over the standard protocol. The improved protocol also facilitated sample processing and counting. These improvements were caused by modifications of the standard hemolymph protocol, such as the addition of lipid concentrate to AFTM, the use of FTM without agar, and the addition of BSA to rinsing solutions. These modifications resulted in larger hypnospores, decreased sample viscosity, increased hypnospore recovery, and reduced cellular debris. Because processing, identification, and counting of hypnospores were facilitated, the improved protocol was simpler and more accurate than the standard protocol for counting of hypnospores in hemolymph. As a result, the coefficient of determination (r^2) of the linear regression between *P. marinus* $\log_{10}$ numbers in oyster body and *P. marinus* $\log_{10}$ numbers in hemolymph increased from 0.554 to 0.760 when the improved hemolymph protocol instead of the standard hemolymph protocol was used to count *P. marinus* in hemolymph.

Results from previous studies have suggested that nutrients from FTM and oyster tissues are used by enlarging parasites and that the ratio of FTM to oyster tissue may be critical for optimal hypnospore enlargement (Ray 1954a, Bushek et al. 1994, Fisher & Oliver 1996). The failure of parasites in highly infected oyster tissue to fully enlarge in FTM was proposed to be a result of exhaustion of necessary substances in medium or tissue (Ray 1954a). Hypnospore enlargement was also reported to be greatest in the FTM tissue assay, intermediate in the FTM hemolymph assay and least in the FTM body burden assays and therefore related to the volume of oysters sampled per ml of FTM (Bushek et al. 1994). Moreover, parasite enlargement was inversely related to infection intensity. Using *P. marinus* cultured *in vitro*, parasite enlargement in FTM can be significantly increased by addition of various nutrients including lipids (Wagner et al. 2001). Despite these observations, the effects of supplementing FTM with nutrients such as lipid or oyster extract and the effects of the ratio of FTM to oyster nutrients (e.g., from hemocytes) on parasite enlargement and numbers had not been studied and therefore needed to be investigated to improve the FTM hemolymph assay.

Addition of lipids to AFTM significantly increased hypnospore diameter by 66% over the saline control, and facilitated sample processing and counting because larger hypnospores were more easily pelleted during centrifugation. Lipids were added because the accumulation of numerous lipid droplets in hypnospores is characteristic of this life stage and may be critical to enlargement (Perkins & Menzel 1966, Perkins 1996). It was recently confirmed that lipids are taken up and stored in hypnospores (Soudant et al. 2000). This study found that fluorescent lipid analogs were primarily stored in cytoplasmic lipid droplet after 24 h of incubation but that after 24 h, fluorescence appeared in the membrane and cytosol of hypnospores.

The mean diameter of hypnospores in AFTM supplemented with 5% oyster extract was only 14% greater than the diameter of hypnospores in AFTM supplemented with oyster saline. It was surprising that the addition of oyster extract did not induce significant cell enlargement over saline as oyster extract would be expected to contain high concentrations of nutrients. Although the components of the oyster extract were not examined, it is likely that the water-soluble extract contained only small amounts of oyster lipids, which may account for the minimal enlargement of hypnospores.

Determining the effects of the ratio of infected hemocyte number to AFTM volume on hypnospore formation helped formulate the optimal combination of nutrients, from oyster and AFTM, to promote maximal parasite enlargement. In this study the number of infected hemocytes incubated in a constant volume of AFTM was adjusted, as was the volume of AFTM that had a constant number of infected hemocytes. Hypnospores from the lowest hemocyte density (10^5 cells per ml of AFTM) had the greatest enlargement indicating hypnospores in this treatment had more nutrients available from AFTM. Unfortunately, this low density may not provide an adequate sample size for determining parasite infection intensity. Using a higher number of hemocytes while maintaining hemocyte density (e.g., 10^6 per 10 mL) requires a larger volume of FTM, which in turn may affect the assay. To address this potential effect of volume on the assay, 10^6 hemocytes were incubated in various volumes of AFTM. Hypnospores from 10^6 hemocytes incubated in the largest AFTM volumes (5 and 25 mL) had the greatest enlargement, but there was also a significant decrease in the number of hypnospores from samples incubated in 25 mL of AFTM, indicating that smaller cells may be lost during processing of these volumes. Moreover, large volumes of AFTM requires the purchase of larger amounts of media and antibiotics, which increases cost. The most practical and optimal ratio was set at 10^6 hemocytes to 1 mL of AFTM. At this ratio of infected hemocytes to AFTM volume, parasites had adequate nutrients available for

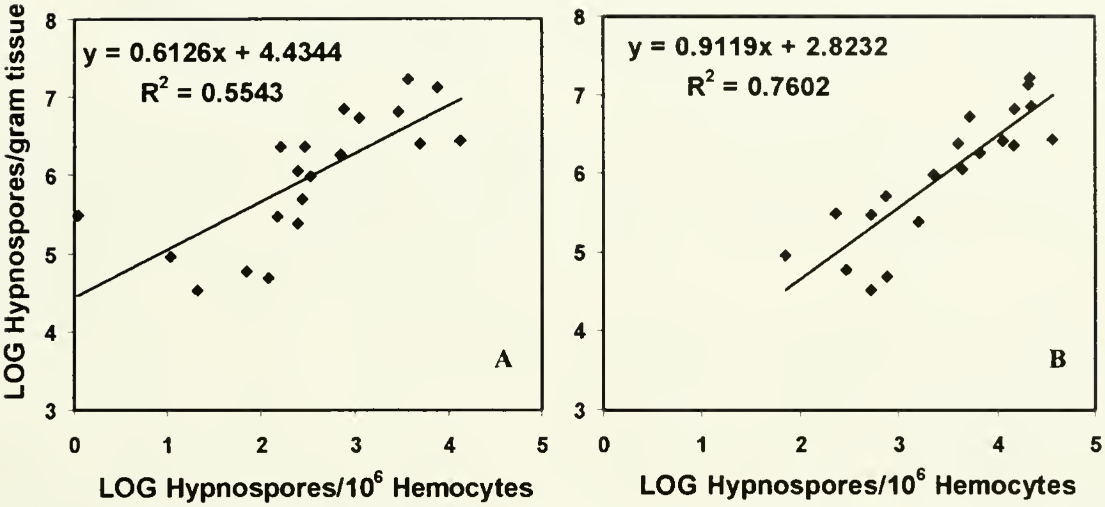

Figure 5. Linear regression lines, formulas, and coefficient of determinations for the comparison of the log of the number of hypnospores per 10^6 hemocytes determined by the standard FTM hemolymph protocol and the log of the number of hypnospores per gram of oyster tissue determined by the body burden assay (A), and for the comparison of the log of the number of hypnospores per 10^6 hemocytes determined by the improved FTM hemolymph protocol and the log of the number of hypnospores per gram of oyster tissue determined by the body burden assay (B).

enlargement in a small AFTM volume while minimizing protocol cost and hypnospore loss.

Perkinsus marinus in hemolymph has exclusively been reported in numbers of parasite per ml of hemolymph (Gauthier & Fisher 1990, Bushek et al. 1994, Oliver et al. 1998, Yarnall et al. 2000). The volume of hemolymph collected per oyster for use in the assay in these past studies varied by as much as a factor of 10, affecting the assay performance. Most parasites, however, are found within the phagosomes of hemocytes (Ray 1954a, Perkins 1996) and the density of hemocytes in hemolymph of bivalves can vary considerably with factors such as temperature, disease and feeding (Feng et al. 1977, Ford & Tripp 1996). *Perkinsus* may, therefore, be better expressed in number of parasites per number of hemocytes (e.g., per 10^6 hemocytes).

The coefficient of determination (r^2) of the linear regression of *P. marinus* $\log_{10}$ numbers in oyster body on *P. marinus* $\log_{10}$ numbers expressed per 10^6 hemocytes was 0.760 (n = 20). When the numbers of *P. marinus* for the same hemolymph samples were expressed per ml of hemolymph instead of 10^6 hemocytes, the coefficient of determination was 0.776 (n = 20) and higher than all, except for one previously reported r^2. An r^2 of 0.53 (n = 12) for Texas oysters sacrificed 24 h after collection and an r^2 of 0.89 (n = 12) for animals held for ten days at high temperature and salinity before sampling were reported by Gauthier and Fisher (1990). An r^2 of 0.675 (n = 25) was reported by Yarnall et al. (2000). An r^2 of 0.241 (n = 100) for oysters from Apalachicola Bay, Florida and an r^2 of 0.771 (n = 100) and 0.738 (n = 100) for oysters from Virginia and New York were reported by Oliver et al. (1998).

The use of FTM hemolymph assay is not recommended for predicting the number of parasites in whole oyster with light *P. marinus* infections (<1,000 parasite/g tissue) (Bushek et al. 1994, Oliver et al. 1998). In our study, the intersects (i.e., y when x = 0) of the linear regression for the number of parasites in whole oyster was 665 ($\log_{10}$ 665 = 2.823) or 1834 ($\log_{10}$ 1834 = 3.2635) depending on whether hemolymph parasite number was expressed per hemocyte or per ml. Hence, parasite numbers per g tissue in whole oyster would need to be greater than these numbers for the hemolymph FTM assay to be able to detect any parasite. This is in agreement with a Bushek et al. (1994) study in which a high percentage of oysters with parasite numbers below 1,000 *P. marinus* per g (wet) tissue were diagnosed as negative with the FTM hemolymph assay. The limitation in sensitivity of the FTM hemolymph assay is less of a concern for Gulf coast oysters than for oysters from the Northeast and central Atlantic coast because *P. marinus* prevalence in Gulf oysters in most sites is close to 100% as determined by the FTM tissue assay. This assay typically detects infection intensities when they are greater than 1,000 parasite/g wet tissue (Bushek et al. 1994, Soniat 1996, Fisher et al. 1996).

No statistical differences in hypnospore diameters or numbers were found among the FTM formulations tested, indicating that none of the FTM formulations provided more nutrients for parasite uptake than others. After incubation of parasites in media composed of individual FTM components, Ray (1954a) found that yeast extract combined with dextrose or casitone were the nutrients responsible for parasite enlargement. All FTM formulations tested in the present study contained approximately equal concentrations of yeast extract (5.0 g/L), dextrose (5.5 g/L), and casitone (15.0 g/L), which explains why no increase in hypnospore diameter or number was found among the FTM formulations tested. Incubation of hemocytes in FTM without agar, or AFTM, did simplify the processing and counting of samples. A viscous layer often forms over the cell pellets during rinsing steps before and after NaOH digestion when FTM with agar is used to enlarge *P. marinus* cells (La Peyre, personal observation, Oliver et al. 1998). This layer is made of indigestible agar because the use of AFTM eliminated this layer, thereby simplifying sample processing and reducing the risk of losing hypnospores in the discarded layer. Sample counting was also greatly simplified because 1) debris including indigestible agar that can interfere with hypnospore counting was eliminated, and 2) hypnospores readily settled on the bottom of wells of tissue culture plates forming monolayers and could be easily counted. In contrast to earlier studies, the need to scan for parasites vertically through the column was eliminated (Choi et al.1989, Gauthier & Fisher 1990, Bushek et al. 1994). Moreover, parasite suspensions could be serially diluted in 96-well plates and counted at the appropriate dilution faster than they could be prepared at the appropriate dilution and counted on filter paper by the technique of Oliver and Fisher (1996) or counted with hemacytometers as used by Choi et al. (1989). Although agar is traditionally used in the microbiological medium FTM to preserve anaerobic conditions and keep bacteria suspended throughout the media for maximizing the use of nutrients (Hitchens 1921), it was clear from our study that the conditions produced by this viscosity are not necessary to enlarge *P. marinus* parasites. AFTM was therefore selected for parasite enlargement in the improved FTM hemolymph protocol.

In an evaluation of methods to diagnose *P. marinus*, NaOH digestion was used to process hemolymph and oyster tissue. It was found that NaOH digestion facilitated the counting of hypnospores from oyster tissues and from hemolymph, but it was noted that NaOH may not be needed for the hemolymph technique because it adds time and labor to the assay (Bushek et al. 1994, Fisher & Oliver 1996). In our study, NaOH digestion of hemolymph samples simplified and reduced the time spent counting. By eliminating debris, cells formed a monolayer on cell culture plate bottoms eliminating the need to scan vertically for hypnospores caught in debris. In past studies, it was noted that hypnospores became sticky forming clumps and adhering to the sides of test tubes after NaOH digestion and upon rinsing (Choi et al. 1989, Bushek et al. 1994, Fisher & Oliver 1996). In our study, the loss of hypnospores as a result of clumping and adherence to the side of test tubes was alleviated by adding BSA to rinsing solutions as recommended by Coates et al. (1999).

In conclusion, simple modifications to the standard protocol for counting of *P. marinus* in oyster hemolymph improved the technique. The addition of lipid to AFTM, the use of FTM without agar, and the use of BSA in rinsing solution facilitated sample processing and counting. The availability of an improved protocol to count *P. marinus* in hemolymph will be useful in monitoring the dynamic change of parasites in hemolymph in infected individual oysters under various conditions. The role of hemocytes in the infection process or in the elimination of the parasites (e.g., hemocyte killing assay) can also be investigated more accurately with this assay.

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OYSTER BIOMASS, ABUNDANCE, AND HARVEST IN NORTHERN CHESAPEAKE BAY: TRENDS AND FORECASTS

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ABSTRACT We applied time series of fishery-dependent and fishery-independent data to develop indices of relative biomass and estimates of absolute abundance and biomass for the Maryland oyster population. The principal objectives for this work were to specify a baseline and determine trends relative to the Chesapeake Bay Program's goal to achieve a 10-fold increase in the standing stock of oysters in the Bay from a 1994 baseline. Population biomass varied by 3-fold from 1986–2001, with most of the variation caused by changes in the stock of market-sized oysters. There were also important spatial differences in population structure and trends over a gradient of salinity. Lagged correlations between small oyster biomass and either market-sized oyster biomass or landings were not statistically significant but suggested that most of the market-sized oysters and those harvested were four to five years old. The index of relative biomass for market-sized oysters proved to be a useful predictor of annual harvests in Maryland. We used this relationship to estimate absolute abundance and biomass of oysters for the time series. The latter estimates may be biased negatively with respect to true abundance and biomass because of biases in both the fishery and the fishery-independent monitoring program. The index of relative biomass will be a suitable measure of population status relative to the oyster restoration goal only so long as the population remains at relatively low levels. Because the index has a theoretical maximum of about 75% of the 10-fold goal, a quantitative estimator will be required for higher population levels.

KEY WORDS: oysters, *Crassostrea virginica*, stock assessment, biomass, Chesapeake Bay

INTRODUCTION

Oysters once supplied the most valuable fishery in Chesapeake Bay (Christmas & Jordan 1991), but after the mid-1980s, a declining oyster stock could no longer support historical levels of harvest. The principal reasons for reduced harvests from 1987 to 2001 were (1) high mortality rates of oysters caused by the parasitic diseases MSX (*Haplosporidium nelsoni*) and Dermo (*Perkinsus marinus*) and (2) low rates of spat settlement throughout most of this period (Krantz undated, Maryland DNR 2001). In addition to economic losses to the fishery, the depletion of oyster standing stocks has been thought to (1) reduce the quantity and quality of shell-bar habitats for oysters and associated epifauna (MacKenzie 1996) and (2) impair water quality by loss of the oyster population's capacity to filter particulate matter (Jordan 1987, Newell 1988, MacKenzie 1996).

In June 2000, the interjurisdictional Chesapeake Bay Program established a goal to increase oyster populations 10-fold by 2010, from a 1994 baseline. Our objectives for the work presented here were to (1) quantify the 1994 baseline, (2) compare the current population with recent and historical populations, (3) develop methods to measure consistently the status of populations with respect to the goal, and (4) examine relationships between long-term fishery-independent surveys and annual harvests. In addition to meeting these objectives, we have developed methods for estimating absolute abundance, biomass and fishing mortality from these data. Only data from the Maryland portion of Chesapeake Bay were used in our analysis. We are working with scientists in Virginia on a combined, bay-wide assessment of the oyster population.

MATERIALS AND METHODS

Oyster Surveys

Size frequency, disease, mortality, and spat density data were collected from oyster dredge samples at 43 sites (Fig. 1) in October to November from 1990 to 2000 (in a few cases, 42 or 44 sites were sampled). The standard dredge has an opening 80 cm wide and a capacity of approximately 2.5 bushels (the volume of a Maryland oyster bushel is ≈46 L, or about 475 oysters at the minimum market size of 76 mm). Dredge tows were taken from areas of the oyster bars with relatively high concentrations of shell, as determined by the sampling crew with a sounding pole. Our observations indicated that tow times and boat speed (hence the area swept) tended to be consistent, but these variables were not formally controlled. From 1990 to 1996, five replicate dredge tows were made at each site; data were collected from a 0.2 bushel subsample of all material in each dredge sample. From 1997 to 2000, two tows were made at each site, with a 0.5 bushel subsample taken from each tow. All live oysters in the subsamples were counted and measured, with measurements recorded in 5 mm size classes. Boxes (articulated oyster shells without tissue remaining) also were counted and measured. All spat were counted in each sample. Sample data were standardized to a fixed volume (one bushel, as defined above) of all material retained by the dredge. A complete description of this survey, known as the Modified Fall Survey (MFS) can be found in Smith and Jordan (1993).

A more extensive survey of 300 to 400 sites has been conducted in the fall of each year since the 1970s. In this survey, typically only one dredge tow is made; all live oysters, spat, and boxes from 0.5 bushel of material are counted. The oysters are not measured individually but are classified as markets (>76 mm), smalls (age 1+ and older oysters <76 mm), or spat. Means and ranges of shell heights for each class are estimated visually by an experienced technician and recorded (Homer et al. 1996).

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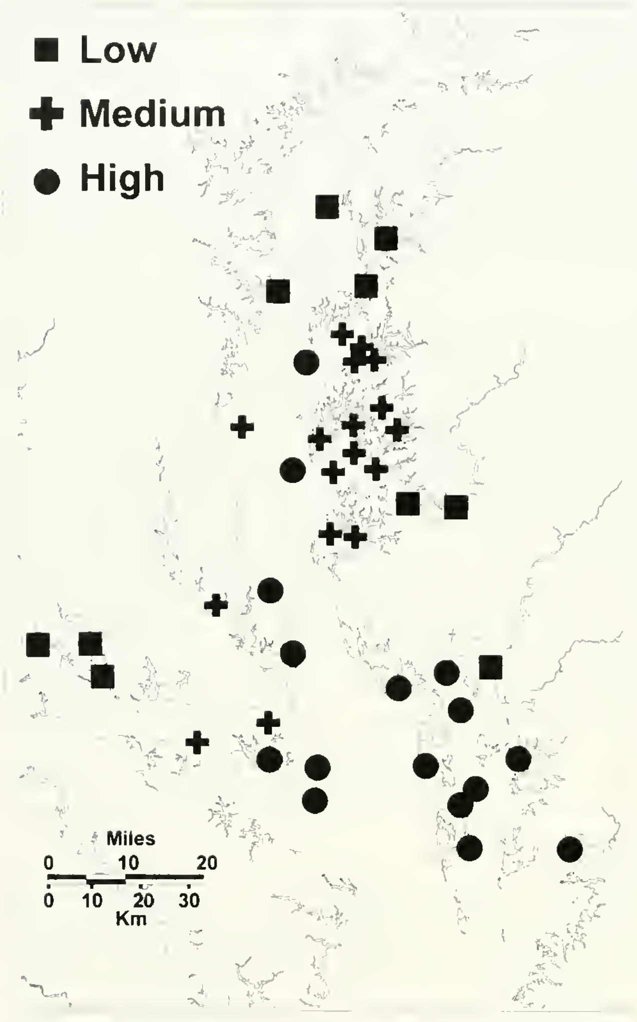

Figure 1. Maryland MFS sites in Chesapeake Bay and tributaries. Symbols identify classification of sites by long-term mean salinity: Low <12 ppt, Medium 12 to 14 ppt, High >14 ppt.

Length-Weight Analysis

A relationship between dry tissue weight and shell height was developed to convert size-frequency data to biomass. During the 1999 fall survey, 10 oysters were collected from each of 42 sites (one of the 43 survey sites did not have sufficient live oysters) and returned to the laboratory for processing. The oysters were selected to be representative of the size range encountered at each site. In the laboratory, the oysters were measured (bill to umbo curved shell height in mm), then shucked carefully, retaining all meat and shell liquor. Each oyster was put into a tared aluminum weighing boat, weighed, dried to constant weight at 85°C, and then reweighed. Wet and dry tissue weights were recorded in g, to the nearest mg.

The relationship between dry tissue weight and shell height was quantified by linear regression of the $\log_{10}$-transformed variables. Indices of relative biomass were calculated by applying the length-weight regression equation to each 5 mm size class of live oysters (excluding spat), then multiplying the number of oysters in that size class by the predicted weight. The midpoint in mm of the size class was used as the nominal shell height for the estimated weight. The weights were summed over all size classes within each replicate subsample, resulting in 1629 individual observations of the index over 43 sites and ten years. Indices of relative abundance were calculated similarly using total counts of oysters (excluding spat) within each subsample. Indices of biomass and abundance were calculated separately for small (<72 mm) and market (≥72 mm) oysters, and for all oysters combined. The legal minimum size for harvested oysters in Maryland is 3 inches (76 mm), but the nearest size class in which survey data are recorded is 72 to 77 mm. We examined the underlying variation in the indices by two-way analysis of variance, accounting for the fixed effects of site, year, and site-by-year interaction.

Relationships Between Fishery-Dependent and Fishery-Independent Data

We used linear regression to determine whether the fishery-independent data could predict commercial harvests. Harvest data were obtained from the Maryland Department of Natural Resources. Because the oyster season is open from October to March, landings are reported for the year the season closes, whereas the MFS data applicable to the annual harvest are collected in October through November of the previous year. For this reason, the analyses reported here are indexed to the harvest year, that is, the 1985 MFS corresponds to the 1986 harvest, and the biomass index for 1986 is derived from data collected in the fall of 1985. However, when referring to the fishery-independent data per se, we use the year in which the data were collected. Annual harvest totals, recorded in bushels, were regressed against the annual mean biomass index for market oysters and the annual abundance index for market oysters. Nine years of data, from harvest years 1991–1999, made up the initial calibration data set. We then used dredge survey data from 1985–1989 for the bars sampled by the MFS to calculate the biomass indices and perform a hindcast test of the harvest prediction model. The 1985–1986 harvest was 1.56 million bushels, nearly 20 times greater than the 1993–1994 harvest. Also, the index previous to 1990 was based on mean sizes for market oysters rather than individual measurements. Prediction of the 1985–1986 and subsequent harvests, therefore, would provide a stringent test of the robustness of this simple model. Once the model was calibrated and validated by hindcasting, we recalibrated over the entire time series, and forecast landings for the 1999–2000 and 2000–2001 harvest seasons.

Quantitative Estimation of Standing Stocks

The success of the market oyster biomass index in predicting annual landings suggested that we could use this relationship to quantify the oyster population. We made the key assumption that differences between predicted and reported landings were entirely caused by differences in rates of fishing mortality. Smith and Jordan (1993) estimated a mean exploitation rate of 0.53 (53% of the market stock harvested) for Maryland oysters for the 1990 to 1991 season. They projected size-frequency data from the 1990 MFS to 1991, assuming a mean growth rate of 20 mm per year and subtracting 1991 observed natural mortality, estimated from box counts as [boxes/(boxes + live oysters)], from each size class. The exploitation rate then was estimated by difference between the projected relative abundance (without exploitation) and that observed from the 1991 survey. Because the ratio of reported to predicted harvest for that year was approximately unity, we computed the instantaneous rate of fishing mortality (F) for the time series as

$$F = -\log e\left[1 - 0.53\left(\frac{H}{\hat{H}}\right)\right],$$

where H = reported harvest and $\hat{H}$ = predicted harvest. Estimates of harvestable stock size (S) for each year were computed as $S = H \cdot e^{F}$ (in units of bushels). Estimates of total population abundance (N, excluding spat) were computed as

$$N_t = N_m + N_m\left(\frac{R_s}{R_m}\right),$$

where the subscripts t, m, and s indicate total, market, and small oysters, respectively, R refers to the relative abundance estimates from the fishery-independent survey, and

$$N_m = \sum_{1}^{i}\left[a_i S\left(\frac{R_i}{R_t}\right)\right],$$

where $a_i = 10^{3.5-0.0113(L_i)}$, R_i is the relative abundance of market oysters in each size class, R_t is the total relative abundance of market oysters, and L_i is the nominal length for each size class of market oysters. The last equation estimates the number of oysters of a given size per bushel, derived by linear regression from tabulated counts (unpublished data, Maryland Department of Natural Resources). Absolute population biomass was estimated by multiplying the proportion of oysters in each size class from fishery-independent size frequency distributions by total absolute abundance (N_t, calculated as above) and the nominal weight for each size class:

$$B_t = \sum_{1}^{i}\left[N_t W_i\left(\frac{R_i}{R_t}\right)\right],$$

where B_t is total population biomass, W_i is the nominal weight in g for each 5 mm size class, R_i is the relative abundance in each size class, and R_t is the total relative abundance over all size classes.

Recruitment

Spat (young of the year oysters) are counted in each sample taken during fall oyster surveys. Although annual mean spat counts have been used for many years as an indicator of year class strength (Meritt 1977, Homer et al. 1996, Krantz undated), they are not always reliable indicators of subsequent recruitment to the population because of high, variable rates of early juvenile mortality (Newell et al. 2000) and the impacts of diseases on submarket oysters. Counts of small oysters (>1 y old and <76 mm in shell height) may be better indicators of recruitment to adult and exploitable stocks than spat counts. Therefore, we examined the potential of small oyster counts and biomass to predict landings, as well as biomass of market oysters in subsequent years, by regressing landings and market oyster biomass against 1- to 5-y lags of the small oyster indices.

Influence of Salinity

Recruitment of oysters to the population and disease-related mortality are positively correlated with salinity in northern Chesapeake Bay (Jordan 1995, Calvo et al. 1996). To examine the effects of salinity on the biomass index, we assigned each of the MFS sites to one of three salinity zones based on 1990 to 2000 mean salinity measured during the fall at the time oyster samples were taken (Fig. 1). These zones were defined as low (mean salinity <12 ppt), medium (12–14 ppt), and high (>14 ppt). At salinity <12 ppt, *H. nelsoni* infections occur rarely, if ever; *Perkinsus marinus* infections, although chronic in this zone, are associated with low to moderate mortality rates; and recruitment rates of both small and market oysters are typically very low, except in areas where natural recruitment has been supplemented by transplanted seed oysters. In the medium salinity zone, *H. nelsoni* epizootics are sporadic, occurring only in drought years, mortality associated with *P. marinus* is moderate to high, and recruitment is variable. In the high-salinity zone, *H. nelsoni* infections tend to be enzootic, mortality rates associated with *P. marinus* infections are consistently high, and recruitment, although variable, tends to be higher than in the lower salinity zones.

RESULTS

Length-Weight Relationship

The linear relationship between the log of dry tissue weight and the log of shell height was quantified as $\log_{10}$(dry tissue weight) = 2.06[$\log_{10}$(shell height)] − 3.76, with tissue weight in g and shell height in mm; n = 410, r^2 = 0.50, $P < 0.0001$ (Fig. 2). Removal of a few outlying observations or weighting the regression for uneven distribution of the dependent variable on the independent variable made only trivial differences in the parameter estimates and the coefficient of determination. Therefore, population-scale estimates of biomass should be accurate, even though the relationship does not have satisfactory precision for estimates at smaller scales (individuals or small samples).

Variability of Biomass Indices

In the two-way analysis of variance models, sites, years, and site-by-year interactions explained 85 to 91% of the variation in index values for small oysters, market oysters, and the total population; all effects were significant (Table 1). The residual 9 to 15% of overall variation was caused by differences between replicate samples.

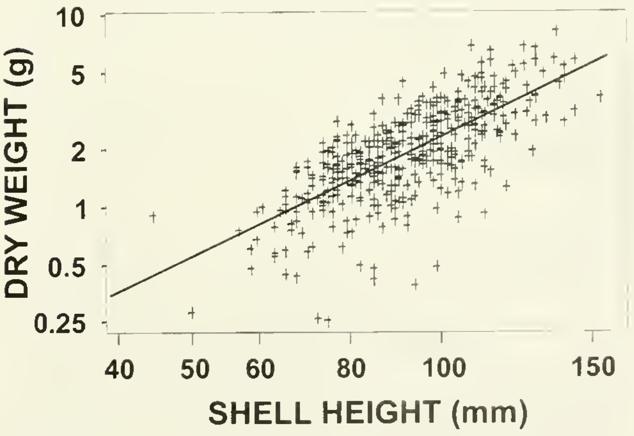

Figure 2. Relationship of oyster dry tissue weight to shell height.

TABLE 1.

Analysis of variance results for examining sources of variability in biomass indices of total, market, and small oysters, 1990 to 1999.

Source of Variation	Degrees of Freedom	F	P Value
Total Biomass			
(Mean = 114.4 g dry weight per bushel, r^2 = 0.91, n = 1629)			
Site	43	85.96	<0.0001
Year	9	89.10	<0.0001
Site × year	378	18.73	<0.0001
Total	1628	28.10	<0.0001
Market Oyster Biomass			
(Mean = 63.7 g dry weight per bushel, r^2 = 0.85, n = 1631			
Site	43	46.54	<0.0001
Year	9	94.46	<0.0001
Site × year	378	8.88	<0.0001
Total	1630	16.24	<0.0001
Small Oyster Biomass			
(Mean = 49.8 g dry weight per bushel, r^2 = 0.89, n = 1631)			
Site	43	64.60	<0.0001
Year	9	23.31	<0.0001
Site × year	378	15.81	<0.0001
Total	1630	22.74	<0.0001

Relationships Between Fishery-Dependent and Fishery-Independent Indicators

There was a significant linear relationship between the mean relative biomass of market oysters sampled in October through November of each year and the quantity of oysters harvested during the commercial season (generally October 1 through March 31, with minor variations). The resulting regression equation was $\log_{10}(y) = 1.64(\log_{10}x) + 2.39$, where y = predicted annual harvest in bushels and x = mean relative biomass of market oysters (r^2 = 0.89; Fig. 3). This regression was developed from 10 y (harvest years 1991–2000) for which we had replicate samples and size frequency data. A hindcast of this model for 1986 to 1990 landings, using only counts and mean sizes of market oysters, yielded reasonable estimates. Over the 15 harvest years from 1986 to 2000, the mean absolute difference between predicted and reported landings was 18.5% (minimum 3.7%, maximum 57.6%, standard deviation 17%). The poorest prediction was for 1987, a period of sharply declining relative biomass associated with an epizootic of MSX disease. An earlier validation of the model used a regression of nine years of data along with biomass from the 1999 Fall Survey to predict 1999 to 2000 oyster landings at 379,000 bushels; reported landings were less than 1% greater (380,000 bushels). The prediction for 2000 to 2001 landings was 331,000 bushels, about 5% less than reported (348,000 bushels). With the model validated by hindcasts and forecasts, it was appropriate to use the entire time series to estimate model parameters (Fig. 4).

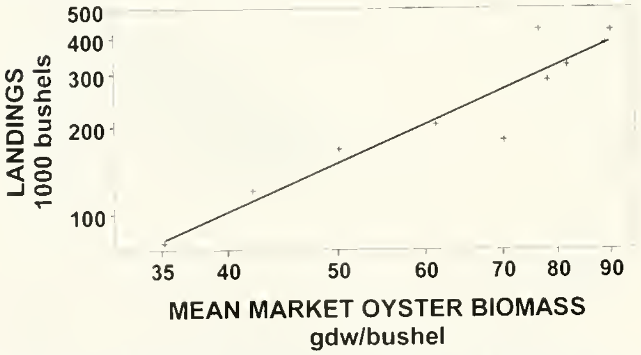

Figure 3. Relationship between annual reported oyster landings and annual mean market oyster biomass index (g dry weight of oyster tissue per bushel of dredged material). Both axes are logarithmic. r^2 = 0.89.

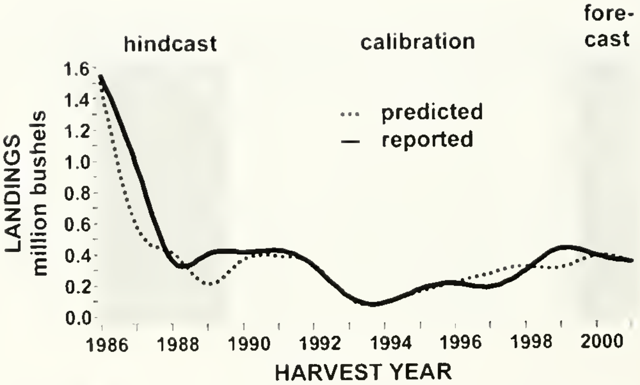

Figure 4. Predicted and reported oyster landings, 1986 to 2001.

Biomass as an Indicator of Population Size

The biomass index for the total population showed more than 3-fold variation over a 15-y period (Fig. 5). Most of this variation was caused by changes in the market oyster index, which varied by more than 6-fold. Small oyster biomass was more stable, varying by a factor of 2.4. The 1994 baseline index for the total population

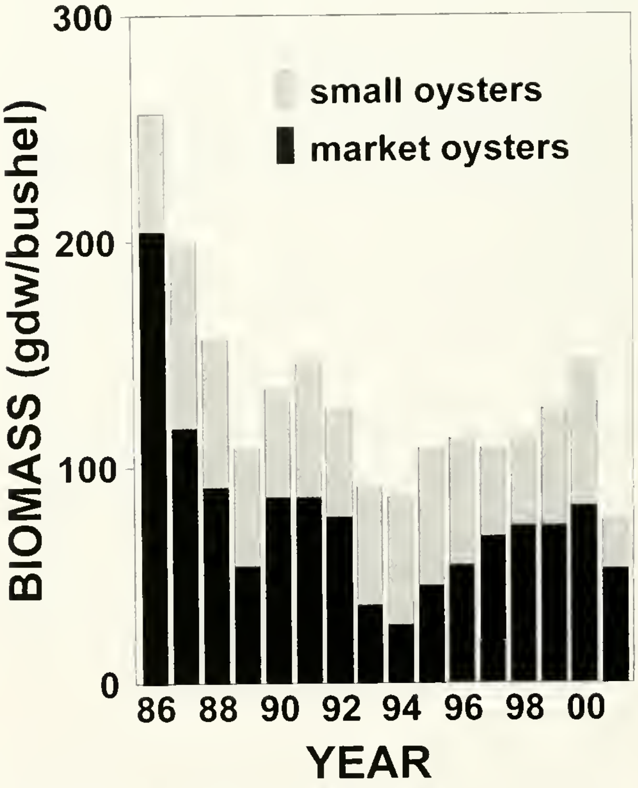

Figure 5. Biomass indices (g dry weight of oyster tissue per bushel of dredged material) for small, market, and total oysters, 1986 to 2001.

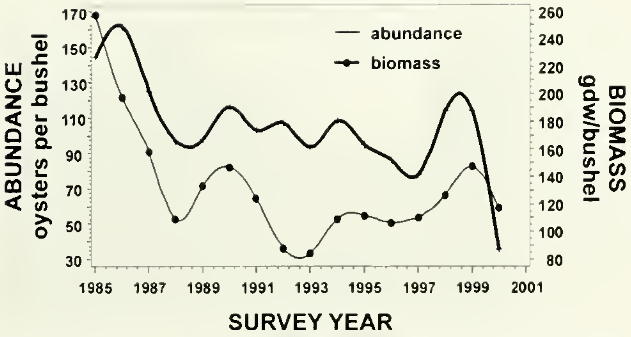

Figure 6. Annual means of relative biomass and relative abundance, 1985 to 2001.

(84.4 g dry weight of oyster tissue per bushel of dredged material) was the lowest in the time series, largely because of a very low index of market oysters (34.8), yet small oyster biomass in 1994 (49.6) was close to the 15-y mean (51.4).

Although there were similarities between trends in relative biomass and relative abundance from 1986 to 2001, there were important divergences (Fig. 6). The sharp rise in abundance from 1997 to 1998, for example, reflects the large spat set observed in 1997, followed by a sharp increase in the abundance of small oysters in 1998. The biomass also increased from 1997 to 1999, but more slowly. Sharp declines in biomass from 1985 to 1989 and from 1990 to 1992 were associated closely with disease epizootics.

Trends in biomass varied considerably over three salinity zones (Fig. 7). In the low-salinity zone, population biomass was moderate and relatively stable, whereas in the midsalinity zone, biomass declined almost steadily for nine years, followed by partial recovery from 1995 to 1999. In the high-salinity zone, biomass decreased sharply from 1986 to 1989 and did not show sustained recovery. Differences between the zones were even more evident when expressed as mean biomass per individual over the full time series (Fig. 8).

Estimates of Fishing Mortality, Total Mortality, Absolute Abundance, and Absolute Biomass

Estimated instantaneous annual rates of fishing mortality varied from 0.23 in harvest year 1997 to 1.29 in 1989. Instantaneous annual total mortality for the market oyster stock ranged from 0.57 in 1997 to 1.67 in 1989 (Table 2, Fig. 9). These ranges corresponded to annual exploitation rates of 21 to 72% and annual total mortality rates of 43 to 92% for the market stock.

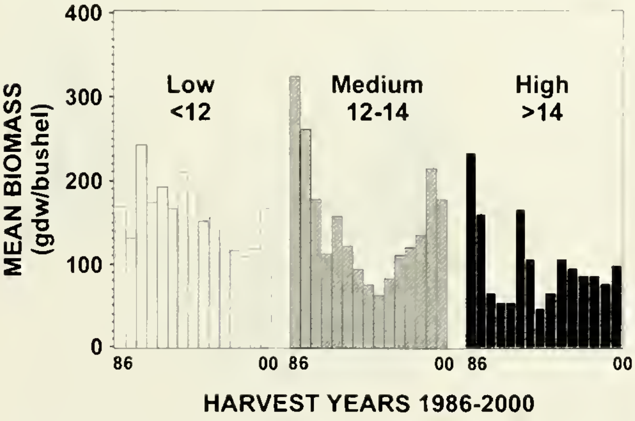

Figure 7. Mean relative biomass by salinity zone, 1986 to 2001.

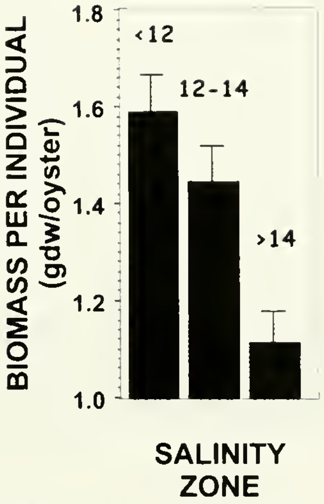

Figure 8. Mean relative biomass per individual oyster by salinity zone. Error bars are standard errors.

Our estimates of total oyster abundance from harvest years 1991 to 2001, for which size-frequency data were available, ranged from 2.66 to 6.29 × 10^8 individuals, with a mean of 4.78 × 10^8. Total population biomass over the same time period ranged from 2.41 to 8.64 × 10^8 g dry tissue weight, with a mean of 5.74 × 10^8 g (Fig. 10). Mean biomass per individual oyster (biomass divided by abundance) was 1.18 g, ranging from 0.82 to 1.42 g over the time series (Fig. 11). For reference, the predicted dry weight of a minimum-sized market oyster (76.2 mm) is 1.31 g.

Recruitment

Lags of small oyster biomass (0–5 y) were not significantly correlated (Spearman r, $P > 0.05$) with market oyster biomass or annual oyster harvests from 1986 to 2001. The strongest positive correlation ($r = 0.58$, $P = 0.06$) was between harvest and a 4-y lag of small oyster biomass (Fig. 12).

DISCUSSION

We estimated total oyster population abundance and biomass for Maryland's portion of Chesapeake Bay from 1986 to 2001. Although these estimates appear to represent population trends accurately, there are several sources of uncertainty and potential bias, which are discussed below. Until these uncertainties can be reduced by further assessment and analysis, we recommend use of the indices of relative biomass reported here as more reliable mea-

TABLE 2.

Summary statistics for instantaneous rates of fishing mortality (F), natural mortality (M), and total mortality (Z) for harvest years 1986 to 2001 ($n = 16$).

Variable	Minimum	Maximum	Mean	Standard Deviation
F	0.23	1.29	0.71	0.25
M	0.11	0.91	0.39	0.22
Z	0.57	1.67	1.11	0.32

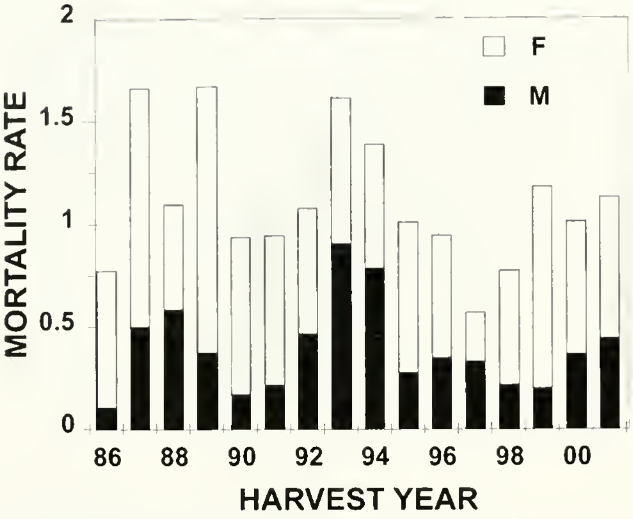

Figure 9. Estimated instantaneous annual mortality rates, 1986 to 2001; F = fishing mortality, M = natural mortality.

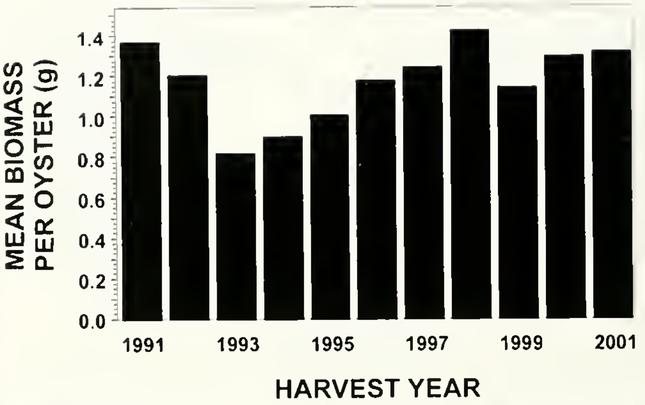

Figure 11. Estimated annual means of absolute biomass per individual oyster, 1981 to 2002.

sures of changes in the oyster population than our estimates of absolute abundance and biomass.

Indices of relative biomass and abundance rely on the principal assumption that the fishery-independent monitoring program accurately represents trends in the oyster population as a whole. The MFS, the source of data for index calibration, was designed specifically for consistent assessment of trends in oyster relative abundance, population structure, mortality and disease status (Smith & Jordan 1993). For these purposes, fixed sites, sampled consistently over time, are appropriate. The monitoring sites are widely distributed throughout the oyster-producing areas of Maryland, and evidence presented here suggests they provide data representative of real trends in the population.

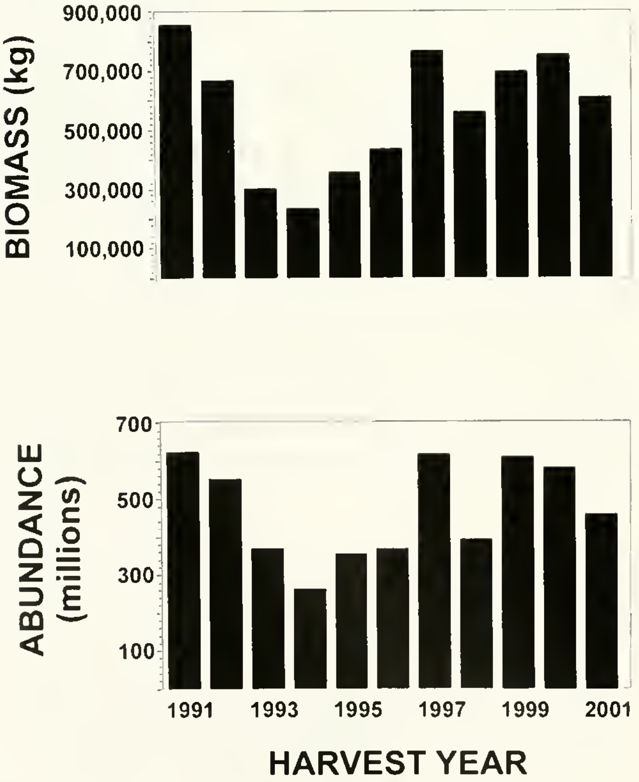

Figure 10. Estimates of absolute biomass and abundance, 1991 to 2002.

Unbiased, quantitative estimates of population size generally require spatially randomized sampling, a condition not satisfied by the data used in our assessment. Our estimates of absolute biomass and abundance also depend on the untested assumption that differences between predicted and reported oyster landings are caused solely by variations in fishing mortality. Both of these considerations introduce biases and inaccuracies of unknown magnitude to the quantitative assessment. Preliminary estimates of population abundance we derived entirely from fishery-independent data sources resulted in higher estimates of population abundance than those derived from fishery-dependent estimates. The fishery-independent estimates ranged from 1.1 to 1.7 × 10^9 oysters in 2000 to 2001, compared with 0.6×10^9 oysters estimated for the same period by the methods reported here. We conjecture that neither the fixed-station monitoring program nor the fishery landings data adequately represent potentially large numbers of oysters scattered at low densities in marginal habitat areas. Areas with low densities of oysters would not be targeted by the fishery because the catch per unit of effort would be very low. Similarly, the fixed monitoring sites were selected to represent typically productive areas of high quality habitat. We plan to collect quantitative data from a variety of habitat types during 2002 to get better

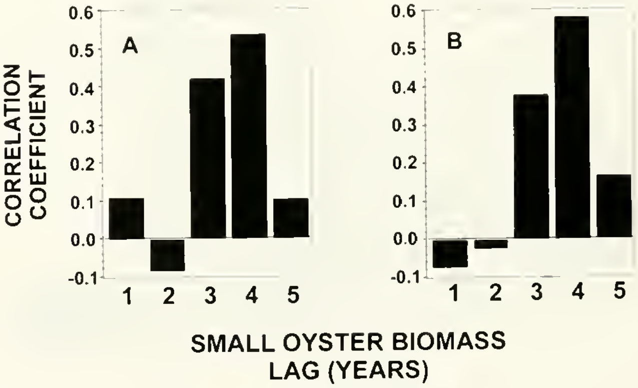

Figure 12. Correlations between lags of small oyster biomass and (A) reported landings and (B) market oyster biomass.

estimates of oyster densities, especially in areas of marginal habitat. Until further refinements can be made to the quantitative assessment, we view it as yielding minimal estimates of oyster abundance and biomass.

The equation relating shell height to dry tissue weight used to compute the biomass index explained 50% of the variation between individual oysters. Morphology, growth, and physiologic conditions can vary greatly in *C. virginica*, depending on specific habitat conditions, health, and other factors (Carriker 1996, Ford & Tripp 1996). The condition of each oyster weighed and measured for this study was assessed both by visual inspection (scored 1–8, "watery" to "fat," according to standard procedures used in our laboratory) and by calculating the percentage of water in the tissues from the difference between wet tissue weight and dry tissue weight. Inclusion of either of these condition indicators as a covariate in the length-weight regression improved the total r^2 to > 0.70. Because oyster condition is not measured routinely in population surveys, we could not account for this source of variation in biomass indices. We conclude that the length-weight equation has low precision for predicting the biomass of individuals or small groups of oysters, but has sufficient accuracy for estimating biomass at population or sub-population scales.

Harvest Predictions, Fishing Mortality, and Recruitment

The close relationship between reported annual landings and the biomass index for market oysters has provided a method for predicting total annual harvest early (mid-November) during each oyster season (October–March). This capability is helpful to managers of the oyster fishery, who are often asked by the press and other interested parties to make these predictions. This relationship also has provided a quantitative link between relative and absolute abundance and biomass of oysters, and a way to estimate fishing mortality rates. Because natural mortality (the ratio boxes:live oysters plus boxes) is observed directly during the annual monitoring program, we now have estimates of total mortality for market-sized oysters. These relationships are being used, along with other parameters, to calibrate a population dynamics model for Maryland oysters.

Ulanowicz et al. (1980) developed a multiple regression model to predict Maryland oyster harvests four years into the future from data on spat settlement and management effort (transplantation of seed oysters) in previous years. The time series of data used in their model was from the 1960s and 1970s, a period when parasitic diseases were not major influences on oyster population dynamics. During the period of the present study, variations in annual landings have depended strongly on oyster mortality rates, which in turn have depended primarily on variations in disease intensity (Gieseker 2001).

Instantaneous annual rates of fishing mortality (F) varied from 0.23 to 1.29 from 1986 to 2001, corresponding to exploitation rates of 21 to 72% of the market oyster stock harvested each year. The mean exploitation rate of market-sized oysters over 16 y was 51% ($F = 0.71$). This value of F contrasts with that estimated by Rothschild et. al. (1994) at 1.3 (73% exploitation rate) for 1990; our estimate of F for that year was 0.76 (53%). Their estimate of F apparently included some natural mortality (M) because their estimated value of M was 0.15, compared with our measured value of $M = 0.22$ for 1990. The resulting total mortality of the market stock for 1990 was 0.94 (61%) in this study, compared with 1.45 (77%) reported by Rothschild et al. (1994). Besides underestimating natural mortality, Rothschild et al. (1994) estimated mean age at first capture at 2.6 y (legal minimum length 76 mm), and the mean age of the harvest (85 mm mean length) at 3.0 y. Growth curves we derived from size-frequency data for thousands of Maryland oysters collected during surveys from 1990 to 2000 predicted that the average 76 mm oyster was 4.0 y old, and the average 85 mm oyster was 4.5 y old. This disparity in age-at-length determinations could explain the higher rates of total mortality and fishing mortality estimated by Rothschild et al. (1994) using a modified Beverton-Holt formulation (Ehrhardt & Ault 1992), because overestimation of the growth coefficient would yield a higher rate of total mortality. Our recruitment analysis suggested that oysters are most likely to be harvested at ages of four to five years in Maryland, consistent with our growth-based predictions of age at first capture and mean age of capture, and with findings by Ulanowicz et al. (1980).

Our estimates of total mortality assume that instantaneous rates of fishing mortality and natural mortality are additive, that is, these sources of mortality do not occur simultaneously within a harvest year. This assumption cannot be strictly true, but mortalities associated with *P. marinus* and *H. nelsoni* dominate the natural mortality term and generally occur from May to October (Ford & Tripp 1996). Because the fishery operates from October to March, interaction between the two mortality terms should not be a major source of error, but probably is a source of positive bias in the total mortality estimates. We plan to test this assumption more rigorously in our continuing stock assessments. Among the uncertainties in estimating mortality, we note the questionable accuracy of determining annual rates of natural mortality from box counts. For example, experimental data presented by Christmas et al. (1997) indicated that roughly 50% of boxes remained intact for more than two years in northern Chesapeake Bay, suggesting that our surveys overestimated annual mortality. Counteracting biases might involve (1) mechanical disarticulation of boxes by the dredge used for sampling, or (2) pre-mortem deterioration of the hinge ligament in diseased and dying oysters, which could hasten disarticulation compared with the chemically-killed oysters used in the Christmas et al. (1997) study.

Effects of Salinity on Population Structure and Biomass

The sharp differences between trends in oyster biomass in high-, medium-, and low-salinity zones partially reflect different rates of recruitment and natural mortality. In high salinity areas, infections with *H. nelsoni* and *P. marinus* are enzootic, with chronically high rates of mortality. Relatively few oysters survive to market size in this zone, but higher rates of spat settlement and recruitment to the sub-market stock tend to maintain populations of small oysters. Populations in the medium salinity zone undergo epizootics of *H. nelsoni* and attendant mortality episodes during extended dry periods, when salinity rises to within the tolerance range for the parasite (13–15 ppt; Paraso et al. 1999). Infections with *P. marinus* are enzootic in this zone except during very wet periods; mortality can be high even in the absence of *H. nelsoni*, but oysters generally survive to larger sizes than in the high-salinity zone. Spat settlement is moderate and episodic in these mid-salinity areas. In the low-salinity zone, *H. nelsoni* is absent or very rare, and although *P. marinus* can reach high prevalence in these populations, oyster mortality rates are lower than in high- and mid-salinity zones. Spat settlement and natural recruitment are sporadic and must be supplemented by transplants from other regions of the Bay or from hatcheries to sustain these harvested subpopulations. Occasionally, freshets cause significant oyster mortality in the areas of lowest salinity. The net long-term results of these dynamics are as follows: (1) relatively stable subpopulations of larger oysters in the low-salinity zone; (2) subpopulations that fluctuate in size and abundance in the mid-salinity zone; and (3) subpopulations in the high-salinity zone that are typically comprised mostly of submarket oysters, which can reach high abundance in some areas. Jordan (1995) used multivariate analysis to classify Maryland oyster bars into six groups based on several population attributes; salinity was the most important independent variable associated with population structure in that analysis.

Oyster Biomass and Abundance Relative to the Chesapeake Bay Restoration Goal

In terms of the relative biomass index, the 1994 baseline Maryland oyster population (from which a 10-fold increase is to be gained by 2010) was 84.4 g dry tissue weight per bushel of dredged bottom material. An index of 844, therefore, would indicate achievement of the goal. In 2001, the index was 117, about 1.4 times the baseline, or 14% of the goal. By combining the length-weight equation and the equation for estimating the number of oysters per bushel, we estimate that a Maryland bushel cannot contain more than about 640 g dry weight of oyster tissue. For this reason, the index of relative biomass cannot realize the desired value of 844 with current sampling methods. Although the biomass index is a satisfactory indicator of trends as long as the oyster population remains at low levels, quantitative assessment will be essential for determining attainment of the goal. Working with Virginia scientists, we expect to establish a bay-wide baseline and annual assessments of absolute abundance and biomass by the end of 2002.

These analyses would not have been possible without relatively long time series of consistent monitoring data. Earlier publications based on MFS data (Smith & Jordan 1993, Jordan 1995) characterized the first 2-4 y of the data set (1990—1993), but relationships such as those depicted in Figures 3, 4 and 12, along with our estimates of absolute oyster abundance and biomass, would be difficult to elicit without data spanning many years. Significant temporal trends in ecosystems as large and variable as Chesapeake Bay can be difficult to detect without decades of consistent data (Vaas & Jordan 1991, Jordan & Vaas 2000), an important consideration in the design and maintenance of large-scale monitoring programs. In turn, such monitoring programs, including the resources to manage, analyze and interpret the data, are essential wherever society has established specific goals for rehabilitation of natural resources and ecosystems.

ACKNOWLEDGMENTS

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EXPERIMENTAL EFFECTS OF WATER TEMPERATURE ON THE GAMETOGENIC DEVELOPMENT OF BROODSTOCK IN THE OYSTER, *OSTREA CHILENSIS*

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ABSTRACT A protocol for reliably conditioning broodstock of the Chilean oyster, *Ostrea chilensis*, is required because the commercial aquaculture of this species has been constrained by the low and variable number of naturally spawning adults. Therefore, the gametogenic response of hatchery conditioned *O. chilensis* broodstock under differing temperature regimes (10°C, 15°C, and 20°C) was investigated, and compared with changes in a wild population over the same time. The overall loss of gametes, particularly ova, through spawning and phagocytosis was significantly higher in oysters held at 20°C and these oysters tended to increase early oogenesis and decrease early spermatogenesis. There was also greater variability in the abundance of reproductive products, particularly female products, among oysters at the higher experimental temperature. At 10°C and 15°C, oysters had similar levels of gametogenic products to wild oysters. Over the experimental period when natural water temperatures remained low the wild oysters also increased early spermatogenesis and oogenesis, but there was little loss of gametes or phagocytosis. The results indicate that warmer water temperatures may be involved in synchronizing and initiating oogenesis, and could be a useful tool for resetting the gametogenic cycle in broodstock. Unlike many other species of cultured oysters, it appears that conditioning broodstock of *O. chilensis* may best be undertaken at low water temperatures.

KEY WORDS: Chilean oyster, *Ostrea chilensis*, conditioning, gametogenesis, reproductive cycle, New Zealand

INTRODUCTION

The lack of a large and reliable supply of larvae is a major impediment to commercial aquaculture of the Chilean oyster, *Ostrea chilensis* (Philippi 1845), in a number of countries including South America and New Zealand (Lépez 1983, Utting 1987, Valencia Camp 1990, Hickman 1992, Jeffs 1995). The development of hatchery techniques for artificially conditioning and inducing larval production in broodstock at any time of the year would overcome this obstacle. However, the factors influencing the reproductive cycle of the Chilean oyster are poorly understood and consequently the development of effective hatchery techniques remains in their infancy (Jeffs & Creese 1996). There are only three published reports of preliminary attempts in Chile to condition broodstock of this oyster, all of which have produced mixed results (Ramorino 1970, Chaparro 1990, Wilson et al. 1996). The first unsuccessful attempts to condition and spawn Chilean oysters in a laboratory were made with minimal control over water temperature and feeding (Ramorino 1970). A preliminary study by DiSalvo et al. (1983a), DiSalvo et al. (1983b) induced some oysters to spawn earlier than in the wild by using artificially raised temperatures. Similar results were obtained with control over both water temperatures and feeding by Chaparro (1990). He found that some broodstock held at 17°C and 20°C matured and spawned spermatozoa and eggs, however, oysters held at 14°C matured and spawned spermatozoa, but did not spawn eggs. Broodstock held at 14°C and given a higher feeding allocation was the only experimental treatment in which oysters maintained their reproductive condition.

Field research in New Zealand has suggested that low winter water temperatures (<12°C) play an important role in increasing adult gonad condition and subsequent larval production (Westerskov 1980, Jeffs et al. 1996, Jeffs & Hickman 2000). The aim of this research is to experimentally assess the role of water temperature on the gametogenic cycle of the Chilean oyster while attempting to control other variables, such as food availability, that may also affect reproductive activity. The broodstock for the research came from a wild population of oysters in the Manukau Harbour, in northwestern New Zealand that has previously been identified as having high natural fertility (Jeffs et al. 1996).

MATERIAL AND METHODS

Experimental Setup

On June 20, 1993, 216 oysters of a size known to be capable of brooding larvae (≥50 mm shell height; Jeffs et al. 1996, Jeffs et al. 1997a) were randomly collected from a natural bed of oysters in the Manukau Harbour (see Fig. 1) and transported to the laboratory. The oysters were scrubbed to remove debris and then randomly assigned to six 300-l rectangular plastic conditioning tanks. Oysters were suspended in the tanks in plastic trays and each tank was filled with 5 μm filtered seawater at the ambient temperature of the Manukau Harbour that was 13.1°C. Over the next 48 h the water temperature of three sets of two tanks was adjusted slowly to each of the three experimental temperatures of 10°C, 15°C, and 20°C. Every 24 h the seawater in the tanks was changed with 5 μm filtered seawater at the experimental temperature. The water temperatures in the tanks were monitored using maximum to minimum mercury thermometers that were reset at each water change. Tanks were aerated continuously via an airstone. With each water change, the tanks of oysters were all fed an equal quantity of mixed monoalgal cultures of *Chaetoceros gracilis*, *Isochrysis galbana*, *Pavlova lutheri*, and *Thalassiosira pseudonana* that were in excess of daily requirements. This was judged by the presence of remaining food in the water at the end of the 24-h period. If the water was cleared prior to this time additional food was added to all of the tanks. On five occasions when live algal cultures were insufficient Celsys™ Algal 161 microfeed consisting of dried *Tetraselmis suecica* resuspended in seawater was also fed. All of these microalgal species are known to be nutritious to *Ostrea chilensis* or bivalves generally (Walne 1970, Brown et al. 1989, Laing 1991). After 50 days the broodstock from each tank were harvested and processed for histology.

Wild Population Samples

Oysters were collected from the population in the Manukau Harbour to provide a comparison with the results for the experi-

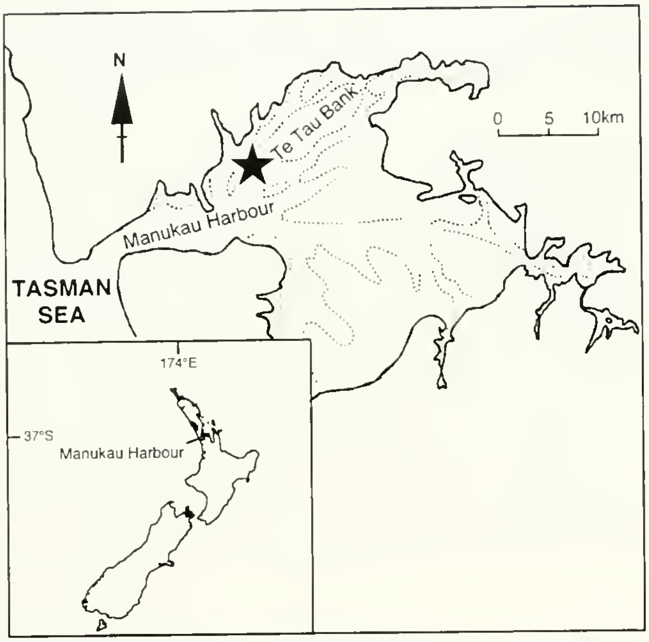

Figure 1. Map showing location of Chilean oyster population used for this study.

mental broodstock. At the start (day 1), mid-point (day 28) and conclusion (day 50) of the experiment about 70 oysters (≥50 mm shell height) were collected randomly from the Manukau Harbour and processed for histology. Ambient water temperature in the harbor was recorded at each sampling time.

Histologic Processing

Each oyster was carefully opened and a note made of the presence of larvae in the brood chamber. A 5-mm thick section of tissue was excised from each oyster parallel to the anterior-posterior axis between the labial palps and the posterior adductor muscle and then fixed in Bouin's solution. Tissue sections taken in this manner for this species are known to contain gametes representative of the whole gonad (Jeffs 1998). Permanent microscope slide mounts were made of the gonad material once it had been stained with haemotoxylin and eosin to differentiate the reproductive tissues.

Histologic Analysis

Each slide was examined under a compound microscope at up to ×100 magnification and the presence of different types of reproductive products were analyzed by a semi-quantitative method developed and verified specifically for this oyster (Jeffs 1998, Jeffs 1999). The method relies on assigning a score of 0–3 (0 = absent, 3 = abundant) to the abundance of reproductive features found that are associated with different aspects of the gametogenic cycle. Five stages of spermatogenesis (spermatogonia, primary and secondary spermatocytes, spermatids, and spermatozoa) and three stages of oogenesis (oogonia, oocytes, and ova) were scored. Additionally, the release of ova and spermatozoa from the lumen of the follicles (= gamete loss or spawning) and the abundance of reabsorption of unspawned ova and spermatozoa (= phagocytosis or reabsorption) were scored in the same manner that has previously been verified (Jeffs 1998, Jeffs 1999). The amount of connective tissue surrounding the follicles was also scored, providing an inverse measure of the overall quantity of reproductive material present in the follicles (Jeffs 1998, Jeffs 1999). Finally, a visual estimate was made of the percentage of male reproductive products over the entire gonad section.

Statistical Analyses

Statistical comparisons were made for the scores of the gametogenic attributes of oysters among the three wild sampling events, the three temperature treatments and between the replicate pairs of treatment tanks using Kruskal-Wallis tests (Sokal & Rohlf 1995). The 12 response variables consisted of those reproductive attributes measured under the microscope. Bonferroni corrected significance levels were used for the tests to control for inflated type 1 errors caused by multiple significance testing.

RESULTS

Water Temperatures

Recorded water temperatures in Manukau Harbour were 13.1°C at the initial collection of broodstock, 11.6°C at the day 28 of the experiment, and 11.9°C at the completion of the experiment. After the initial water temperature change over 48 h the water temperatures in the experimental tanks did not fluctuate more than ±1°C from the treatment temperatures for the remaining duration of the experiment.

Brooding

At the completion of the experiment five oysters (6.9%) were brooding larvae in the 10°C treatment (3 in one tank and 2 in the other). Three oysters (4.2%) were brooding in the 15°C treatment (3 in one tank and none in the other). No oysters were brooding in the 20°C treatment.

Samples of oysters taken from the wild population found 5 out of 70 oysters (7.1%) were brooding larvae at the outset of the experiment, 5 out of 73 oysters (6.8%) were brooding at day 28 and 6 out of 73 oysters (8.2%) were brooding at the end of the experiment.

Statistical Analyses

Analyses showed that there was no difference in the reproductive attributes of oysters held in the two tanks used in each experimental treatment. Therefore, the results for the two tanks were pooled for each experimental treatment. Statistical comparisons were made of the scores for the reproductive attributes of oysters among the three experimental temperature treatments, and among those sampled from the wild over the duration of the experiment (Fig. 2 and Fig. 3). For the experiment, significant differences were found among the scores for connective tissue ($P < 0.000001$) and phagocytosis ($P < 0.000005$). For oysters sampled from the wild, significant differences were found among the scores for percentage male ($P < 0.001$), spermatogonia ($P < 0.0005$), primary spermatocytes ($P < 0.0001$) and oocytes ($P < 0.0001$). The overall trends in the data were explored by plotting mean values for the variables with their standard errors (Figs. 2 and 3).

Spermatogenesis

Over all of the oysters examined the male reproductive products were generally more abundant than female reproductive prod-

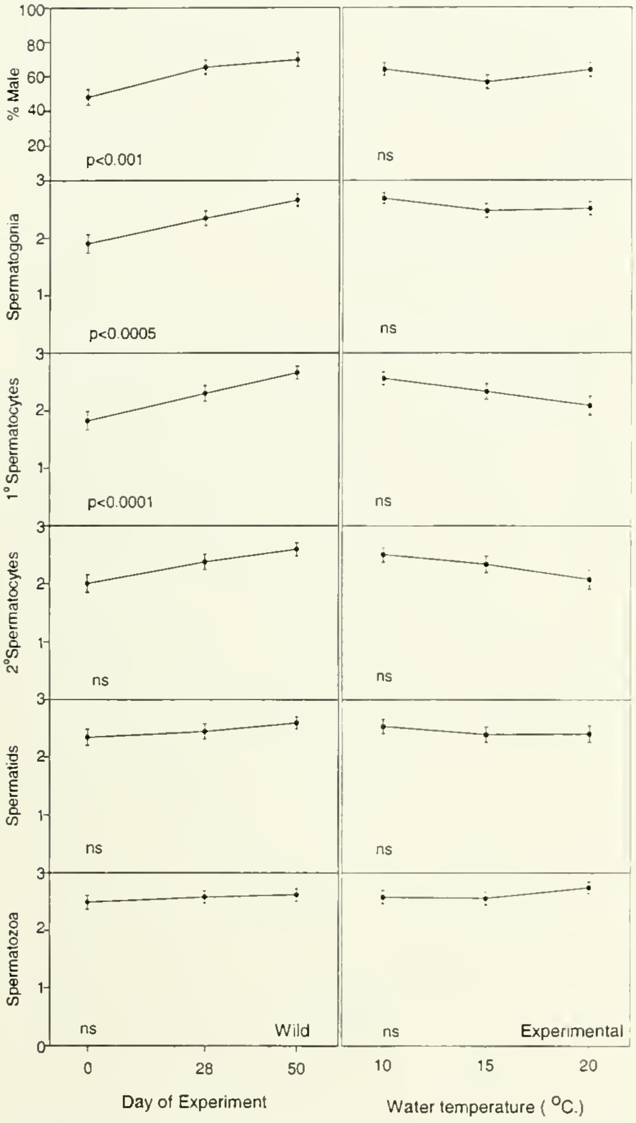

Figure 2. Mean scores (± standard error) of male gametogenic characteristics and percent male of Chilean oysters plotted against either time of sampling (wild population), or experimental water temperature (experimental population). Significant differences between scores as identified by Kruskal–Wallis tests are indicated (ns = not significant).

ucts. In the wild population of Chilean oysters there was a significant increase in the proportion of male reproductive material accumulating in the gonad ($P < 0.001$). This was associated with a trend for male reproductive attributes, especially for the early spermatogenesis stages (spermatogonia ($P < 0.0005$), primary spermatocytes ($P < 0.0001$) and secondary spermatocytes) to increase in abundance over the 50-day experimental period as the natural water temperatures decreased. At the end of the experimental period oysters in the three temperature treatments contained a similar abundance of male reproductive products as oysters in the wild population. However, there was a general trend for the abundance of male reproductive products, especially the early spermatogenesis stages, to decrease slightly and for the variability in the abundance of reproductive products to increase with increasing experimental temperature.

Oogenesis

On average ova were more abundant among all of the experimental and wild oysters than oogonia or oocytes. These earlier stages of oogenesis tended to increase in the wild population of Chilean oysters over the study period. For example there was a significant increase in oocytes among wild oysters over the period ($P < 0.0001$). At the end of the experiment the oysters in three temperature treatments contained a similar abundance of female reproductive products as oysters in the wild population. The abundance of the early stages of oogenesis (oogonia and oocytes) in oysters tended to increase with higher experimental water temperature. The abundance of ova in the 20°C treatment, however, tended to be lower than for the other temperatures, and for the wild population. The variability in abundance of female reproductive products was also greatest in the 20°C treatment.

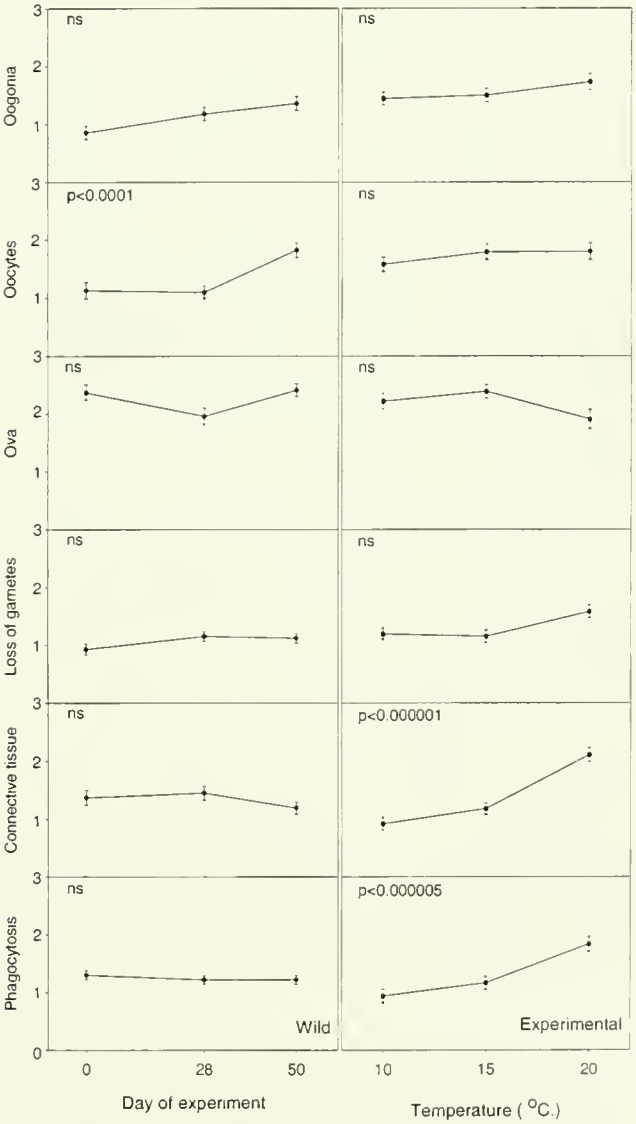

Figure 3. Mean scores (± standard error) of female gametogenic of Chilean oysters plotted against either time of sampling (wild population), or experimental water temperature (experimental population). Significant differences between scores as identified by Kruskal–Wallis tests are indicated (ns = not significant).

Gamete Loss and Phagocytosis

The recent loss of gametes, the amount of connective tissue and phagocytosis of reproductive products was at similar low levels among the three wild samples of oysters suggesting that gamete materials were accumulating in the gonads over this period. However, for the experimental oysters the recent loss of gametes, and especially the amount of connective tissue and the amount of phagocytosis of reproductive products all tended to increase with increasing temperature and especially at 20°C.

DISCUSSION

Low water temperatures have been widely implicated in controlling the production of female gametes in *Ostrea chilensis* (Westerskov 1980, Winter et al. 1984, Jeffs et al. 1997a, Jeffs et al.1997c, Jeffs 1999). Field studies within New Zealand surmised that water temperatures of <12°C acted to increase the gonad condition and the development of ova in local Chilean oyster populations (Westerskov 1980, Jeffs et al. 1997b, Jeffs 1999, Jeffs & Hickman 2000). The results of our laboratory study revealed that the effect of temperature on gonad development was consistent with the patterns observed in wild populations of this oyster (Jeffs & Creese 1996, Jeffs et al. 1997a). However, a considerable amount of variability remained in the gametogenic response of individual oysters to experimental temperatures, a pattern that appears to be a feature of this species (Jeffs et al. 1997b).

In this study male and female gametes were accumulated in the gonads of wild oysters experiencing cold winter water temperatures and there was little overall loss or phagocytosis of gametes. The accumulation of reproductive products in winter/spring preceding a minor spring/summer peak in spawning has been observed in other wild populations of this oyster (Jeffs et al. 1996, Jeffs 1999, Jeffs & Hickman 2000). The experimental oysters exhibited a similar response to lower water temperatures, with oysters held at 10°C and 15°C also accumulating similar amounts of developing reproductive material to wild oysters. In both the wild and experimental oysters at 10°C and 15°C early spermatogenesis had increased. In wild oysters there were signs that early oogenesis also increased. A similar pattern has also been observed in the Olympia oyster, *Ostrea lurida*, where experimental broodstock held at 12°C increased their gonad condition, while those at 18°C and 21°C lost condition through the gonad cycle being rapidly advanced (Santos et al. 1993). Similar results have also been found for Chilean populations of *O. chilensis* maintained in hatcheries over a range of temperatures (Chaparro 1990, Toro & Morande 1998). Such a response is common to many other species of bivalves held at elevated temperatures (Utting & Spencer 1991, Utting & Millican 1997). This rapid advance of the gonad cycle would also explain the absence of brooding oysters among the broodstock oysters held at 20°C in our study because these oysters would have already spawned or reabsorbed their gametes, whilst those at lower temperature were still developing. This was evidenced by the increases in the loss of gametes, connective tissue and phagocytosis observed amongst our experimental oysters at 20°C, and previously identified amongst oysters taken from wild populations during periods of similar warm water conditions (Jeffs et al. 1996, Jeffs et al. 1997a, Jeffs et al. 1997c, Jeffs et al. Jeffs 1998, Jeffs et al. 1999).

The results for these two *Ostrea* species are in contrast to the widely cultivated Pacific oyster, *Crassostrea gigas*, the European oyster, *O. edulis*, and the American oyster, *C. virginica*, which are known to undergo gonad development at warmer water temperatures, (e.g., 18°C to 24°C) and commercial broodstock conditioning relies on using these warmer temperatures (Aboul–Ela 1960, Quayle 1969, Mann 1979, Muranaka & Lannan 1984, Dinamani 1991, Santos et al. 1993, Castagna et al. 1996). Within this temperature range the experimental *O. chilensis* in this study lost condition through increased gamete loss and phagocytosis and decreased early spermatogenesis. There was also a trend for early oogenesis to increase, but this has previously been associated with rapid initiation of early gametogenesis after the extensive loss of gametes and subsequent rapid phagocytosis of remaining gamete material at warm water temperatures (Jeffs 1998, Jeffs 1999, Jeffs & Hickman 2000). All of these patterns of gonad change observed in our study are consistent with those previously observed in populations of wild oysters in warm water temperatures during summer-autumn (Jeffs 1998, Jeffs & Hickman 2000).

The results of this study suggest that manipulating water temperatures has the potential to be an effective method of controlling broodstock development in *O. chilensis*. Unlike other species of commercial oysters, cold-water temperatures appear to be important in stimulating early spermatogenesis and oogenesis and the accumulation of developing gametes much needed for effective conditioning of broodstock. Warm water temperatures appear to rapidly advance the development and loss of developing gametes (through spawning and phagocytosis) already held in the gonads, and this is followed by the rapid increase in oogenesis. Therefore, warm water temperatures have the potential to be used for advancing the development of gametes in broodstock conditioned at cold temperatures toward spawning and for restarting the gametogenic cycle from early gametogenesis in broodstock in attempt to create greater synchrony among individual broodstock.

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HISTORICAL CHANGES IN INTERTIDAL OYSTER (*CRASSOSTREA VIRGINICA*) REEFS IN A FLORIDA LAGOON POTENTIALLY RELATED TO BOATING ACTIVITIES

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ABSTRACT Research in the late 1990s showed that some intertidal eastern oyster (*Crassostrea virginica*, Gmelin) reefs in Mosquito Lagoon within the Canaveral National Seashore, Florida had dead margins consisting of mounded up, disarticulated shells. It was hypothesized that boating activities were the cause of the damage because all the reefs were adjacent to major navigation channels. To investigate this, we characterized the history of the appearance of dead margins and other reef changes using aerial photographs taken between 1943 and 2000. Imagery analyzed included prints (black & white, color, or color IR) from 1943, 1951, 1963, 1975, 1988, and 1995, and digital imagery from 2000 (USGS 1:12,000 digital ortho-quarterquads), at scales from 1:6,000 to 1:24,000. Prints were scanned at a resolution sufficient to yield 1-m pixels. After scanning, each set of images was georeferenced to the year 2000 imagery using ArcView and ArcInfo GIS software. All reefs found to have dead margins based on 1995 and 2000 aerials were visited in 2001 and 2002 to confirm the presence and extent of dead areas. This provided ground-truthing for the "signature" (a highly reflective, light-colored area adjacent to darker-colored live reef) to be used to detect the appearance of dead margins in the historical aerials. The earliest appearance of dead margins was in the 1943 aerials on one reef adjacent to the Intracoastal Waterway (ICW), a major navigation channel. The total number of reefs affected and areal extent of dead margins steadily increased from 1943 through 2000. In 2000, 60 reefs (of a total of ~400 in the Park) had dead margins, representing 9.1% of the total areal coverage by oyster reefs in the Park. Along the ICW, some reefs migrated away from the channel as much as 50 m and in 2000 consisted mainly of empty shells mounded up a meter above the high water mark. In contrast, many reefs in areas away from navigation channels showed little change over the 57-year period. This historical analysis provides strong (although correlative) evidence that boating activity has had dramatically detrimental effects on some oyster reefs in the study area. Ongoing studies are aimed at further testing this hypothesis and elucidating the causal mechanisms involved.

KEY WORDS: eastern oyster, *Crassostrea virginica*, reefs, aerial photographs, remote sensing, boating activities

INTRODUCTION

The eastern oyster, *Crassostrea virginica*, forms extensive reefs subtidally and intertidally along the eastern US coast (Bahr & Lanier 1981, Burrell 1986). Intertidal reefs make up the dominant form from North Carolina to the northern end of the Mosquito Lagoon in east central Florida. Most of Mosquito Lagoon in this area is within the boundaries of Canaveral National Seashore (CANA), the present study area. Here, the intertidal oyster reef constitutes a major habitat type (Grizzle 1990). Within CANA, reefs are managed for their ecological importance and are harvested commercially and recreationally (Walters et al. 2001).

Previous research within CANA characterized reef distribution and abundance patterns (Grizzle 1990) and focused on management issues (Grizzle & Castagna 1995). Based on an analysis of 1995 aerial imagery and subsequent field surveys, it was discovered that numerous reefs had dead margins consisting of disarticulated shells mounded up several decimeters above the adjacent living reef (Grizzle, pers. obs.). This pattern differed from the well-documented, long-term growth pattern of a dead middle area surrounded by living oysters (the "senescent stage" of Bahr & Lanier 1981) because the dead zones were along the margins of the reefs. Also, the dead margins consisted mainly of well-packed shells instead of a shell/sand/mud mixture as typically found in the dead middle area of senescent reefs. Further analysis showed that all reefs with dead margins occurred adjacent to channel areas that were heavily used by boats, including the federally maintained Intracoastal Waterway (ICW) that runs along the western edge of CANA. It was hypothesized that boating activities may be responsible, at least in part, for the dead margins.

This study was initiated as part of a larger project aimed at assessing the potential impacts of boating activities on oysters. Emphasis in the present report is on historical changes in oyster reefs based on aerial imagery, and the results of associated studies will be reported in future publications. The major objectives of this report are: (1) describe the present (2000) distribution of oyster reefs in CANA; (2) characterize historical (1943 to 2000) changes in the CANA reefs based on aerial photographic imagery, with an emphasis on those reefs with dead margins in 2000; and (3) relate historical reef changes to environmental factors, with an emphasis on boating activities.

METHODS

Study Area

The study area was in northern Mosquito Lagoon in east-central Florida (Fig. 1) and restricted to the Canaveral National Seashore (CANA), a National Park unit that is an example of a relatively stable barrier beach/lagoonal ecosystem (Grizzle 1990, Walters et al. 2001). Most of the Lagoon within CANA is a complex system of shallow open water areas and nearly 100 mangrove (*Rhizophora mangle* and *Avicennia germinans*)-dominated islands. Oyster reefs occur in intertidal areas, often adjacent to seagrass (mainly *Halodule wrightii*) beds that are extensive in some areas (Morris et al. 2000). Water depths are <1 m in most areas and the annual salinity range is typically between 25 and 35 psu, occasionally increasing to 40 psu (Grizzle 1990). Mosquito Lagoon is the northernmost body of water in the Indian River Lagoon system (IRL) that has been described as the richest and most diverse estuary in the continental United States (see references in 1995 special issue of *Bulletin of Marine Science*, Vol. 57; also see Walters et al. 2001). The ecological importance of this area has

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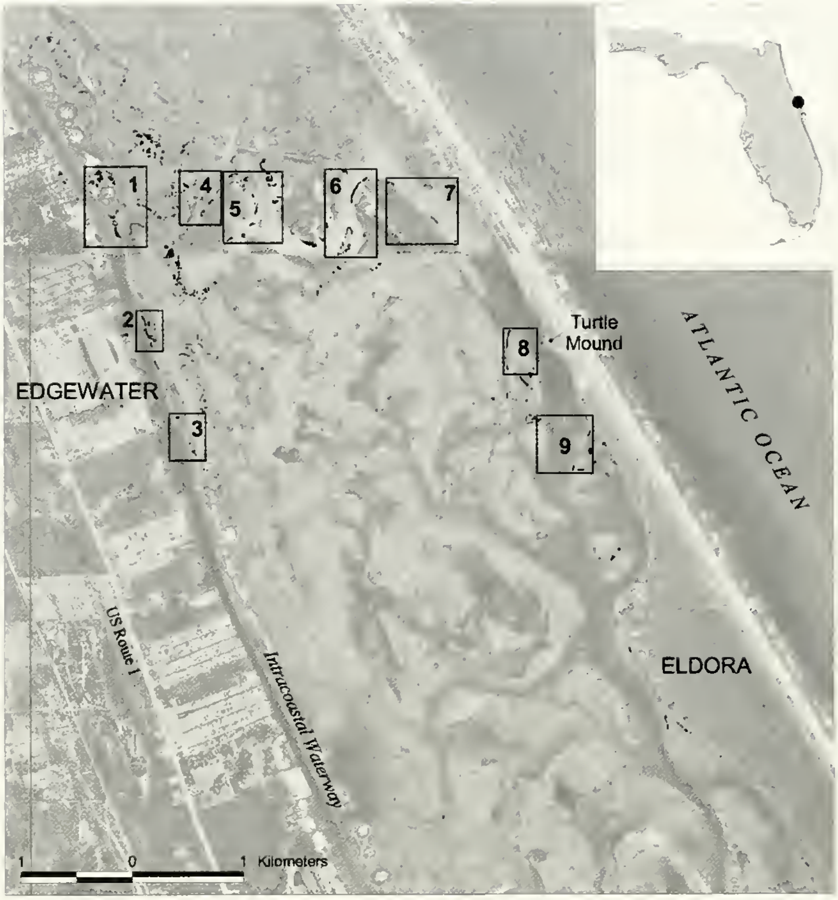

Figure 1. Northern portion of Canaveral National Seashore, showing most of ~400 mapped oyster reefs (each shown as a black polygon exaggerated 2× horizontally) based on year 2000 imagery. Nine study groups used in historical (1943–2000) assessment are indicated. Note that "live reefs" and "dead margins" are combined in this figure.

been demonstrated by the US Environmental Protection Agency in listing the entire IRL as an Estuary of National Significance, and by the State of Florida in classifying it as a Florida Outstanding Waterway and Aquatic Preserve, the highest level of State protection.

Aerial imagery of the types and sources indicated in Table 1 were used in this analysis. Prints were converted to digital format following the "soft-copy photogrammetry" methods outlined in Finkbeiner et al. (2001). Each print was scanned at a resolution sufficient to yield 1-m pixels. After scanning, each set of images was referenced to the year 2000 orthorectified digital imagery using sufficient reference points to remove distortion from the photographs. Year 2000 imagery provided enough detail so that link points could easily be established to all historical photos. The link files were created in ArcView by doing a side-by-side comparison of photos and creating points on each to reference corresponding locations. This method created "from" and "to" points that were used in ArcInfo to warp the historical photos and register them to a UTM coordinate plane. On average, 35 to 40 points were established for each photo pair. This allowed the polynomial order of transformation to be varied enough to compare different levels of warping in ArcInfo. The higher the order of the polynomial, the more distortion can be removed. Warping results from order 1 to 6 were compared and it was determined that third order transformations were sufficient to match these images to their year 2000 counterparts. Such results were anticipated due to the low vertical relief of the target area. Vertical relief is one of the primary sources of distortion in aerial photography.

TABLE 1.

Aerial imagery used in present study.

Year	Type	Source	Scale
1943	Black & white prints	St. Johns River Water Management District	1:16,000
1951	Black & white prints	Volusia County	1:16,000
1963	Black & white prints	Volusia County	1:24,000
1975	Color prints	Volusia County	1:24,000
1988	Color prints	Volusia County	1:24,000
1995	Color infrared prints	University of New Hampshire	1:6,000
2000	Digital	US Geological Survey	1:24,000

Live reefs initially were identified based on the following criteria (i.e., "signature"): dark margins with a lighter middle area, round to irregular in shape, and with a smooth texture (Fig. 2). These criteria were applicable regardless of imagery type. In color and color IR photos, the darker zone of the reef was typically olive to olive-gray in color. As an example of the overall image, round reefs resembled a donut with the center being lighter in color than the outer ring. In stark contrast to the live reef signature, dead

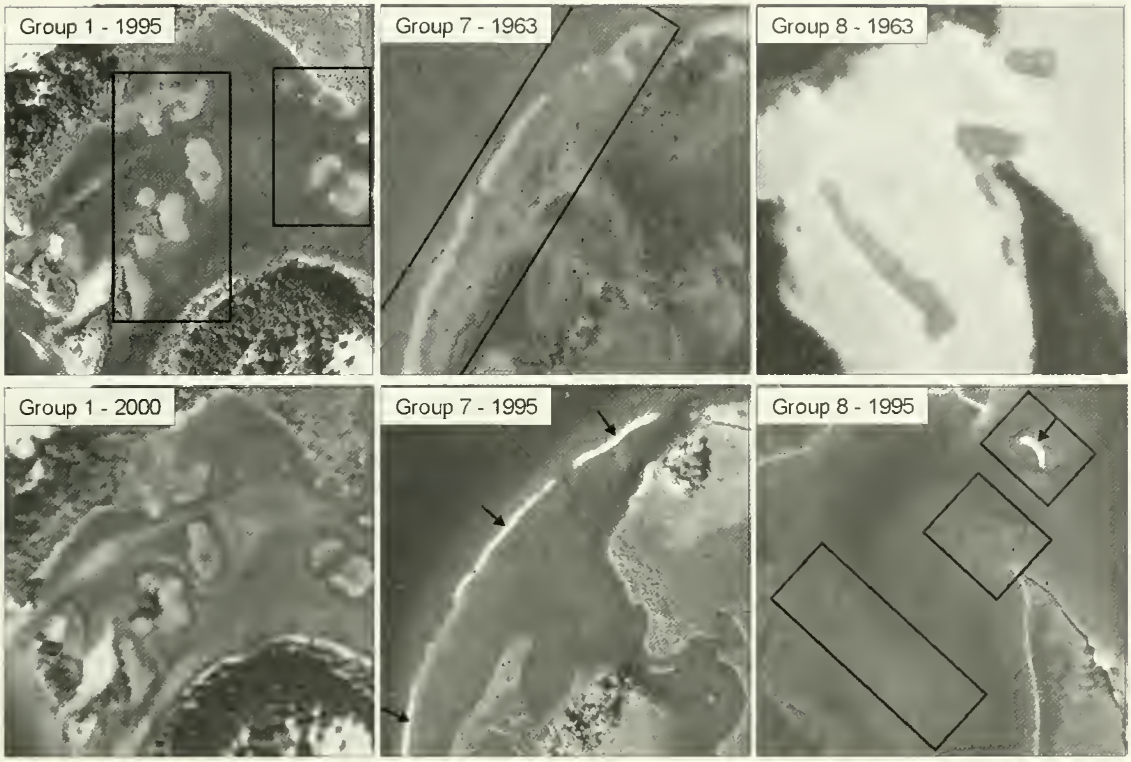

Figure 2. Examples of signatures for live reef (LR; reefs enclosed in boxes for one year only of each pair of aerials) and dead margins (DM; indicated by arrows) from different kinds and qualities of imagery. Group 1—1995: LR signature with dark margin not evident; Group 1—2000: typical LR signature of dark margin surrounding lighter central region, but low quality imagery. Group 7—1963: 100% LR; Group 7—1995: LR with extensive DM (bright white) along outer edge of reef. Group 8—1963: three LR reefs in low quality imagery caused by poor sun angle and surface reflectance; Group 8—1995: three LR reefs as in 1963, but outer reef with extensive DM in 1995.

margins appeared in all types of imagery as a high reflective, light-colored (typically bright white) area adjacent to the darker-colored live reef (Figs. 2 and 3).

Using these interpretation criteria, eight preliminary maps were produced: a basemap showing all oyster reefs (minimum size ~10 m^2) in CANA based on 2000 aerials, and one map for each of the seven aerials from previous years showing only those reefs that had dead margins in 2000. It should be noted that only a subset of all reefs in CANA was mapped using the pre-2000 imagery. This is because emphasis with respect to historical changes was on those reefs that had dead margins in 2000. To make assessment of historical changes more manageable, reefs with dead margins in 2000 were arranged into nine groups based on location (Fig. 1). All nine groups were quantitatively analyzed but only representative aerial imagery is shown here. Due to variations in size of the reefs, a constant map scale could not be used across all nine groups. Each group boundary was positioned to emphasize changes from year to year on an individual group basis and to show sufficient detail. Special care was also taken to include any partial reefs so that area calculations would remain consistent.

Field surveys were conducted in November 2001 and March 2002 to ground-truth the reef maps produced using the 2000 aerials. Ground-truthing included assessment of initial reef signature criteria and mapping accuracy. In November 2001, all reefs initially mapped with dead margins were visited to determine the accuracy of the interpretations of live reef and dead margins. Based on this survey, a revised set of reef maps was produced. A second field survey was conducted in March 2002, to assess the revised maps with an emphasis on mapping accuracy. Following the general recommendations of Congalton and Green (1999) and Finkbeiner et al. (2001), multiple points on several individual reefs were chosen for field inspection. At each point, latitude and longitude (using a differential geographic positioning system, DGPS) were recorded, and bottom type (dead margin, live reef, or non-reef) identified. Each logged point was plotted on the revised maps to determine locational and interpretational accuracy.

RESULTS AND DISCUSSION

Reef Signature and Ground-Truthing

A field survey in November 2001 indicated that the initial reef signature criteria were sufficient for correct identification of nearly all of the reefs based on 2000 aerials. Live reefs surrounded by bottom features (e.g., light-colored sand, dark mud) that contrasted with the reef signature were accurately mapped. Interpretation of reef boundaries was difficult only when bottom features (e.g., some seagrass beds, gray sands and muds) that resembled live reefs occurred adjacent to the reef. It should also be noted that in some areas on some aerials, reefs appeared a uniform gray, either darker or lighter than surrounding areas (e.g., see "Group 1—1995" and "Group 8—1963" reefs in Fig. 2). These departures from the typical signature were apparently a result of the photo being taken during low tide when the reefs were exposed. In some cases, these aerials were also of poor quality caused by excessive glare off the water surface and thus poor light penetration of the water column (e.g., "Group 8—1963" reefs in Fig. 2). Dead margins were dramatically visible in all but the poorest quality aerials because of high reflectivity of the mounded up and sun-bleached

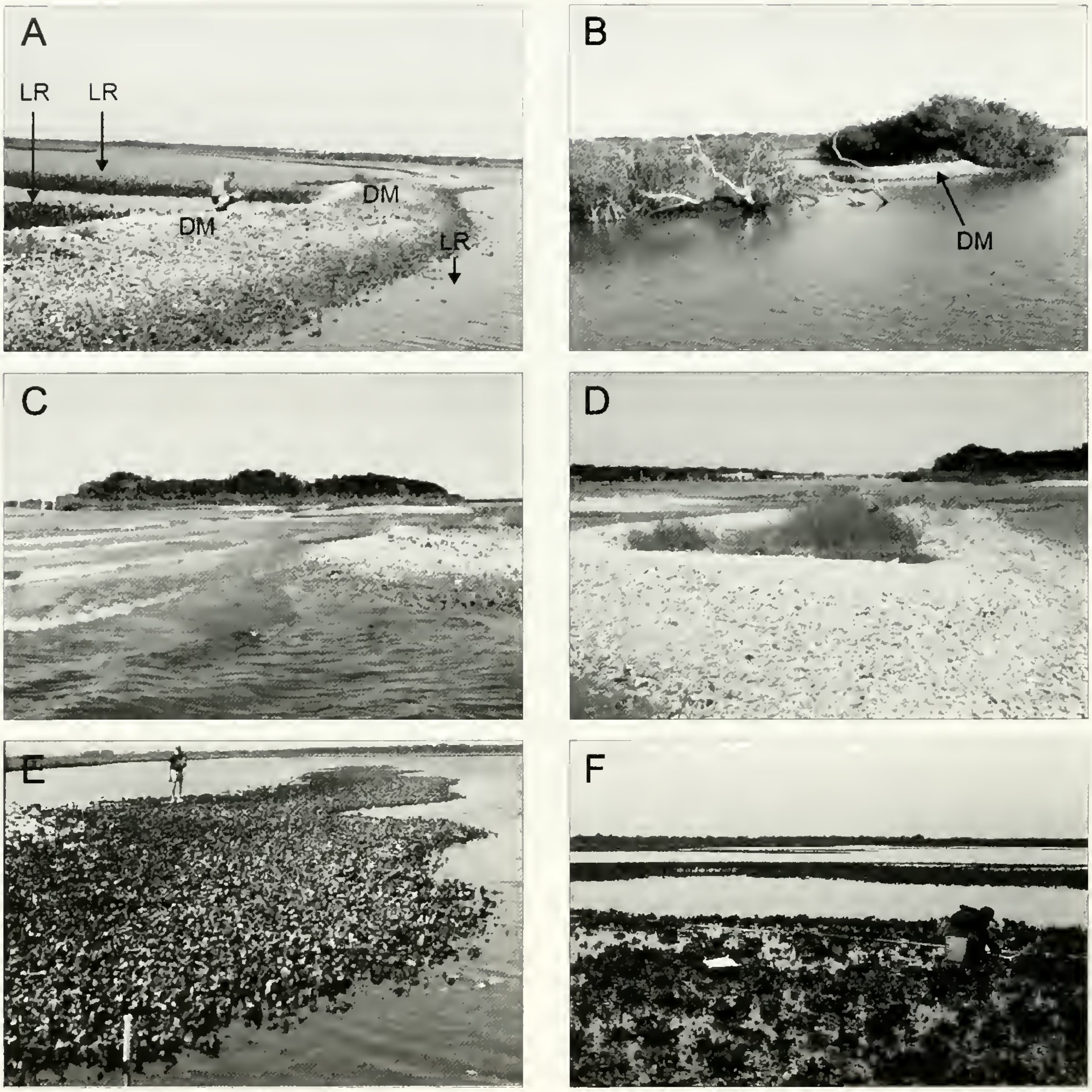

Figure 3. Dead margins (DM) in 2002 showing typical relation to live reef (LR). A) Long, arching reef from Group 6; note presence of LR on both sides of DM. B) Small reef from Group 4 with DM and scattered LR behind it. C) Large cluster of DM in Group 1 resulting from long-term migration away from ICW from 1943–2000. D) DM being colonized in middle by macrophytes; mound of dead shells extended nearly 1 m above the high water line and was adjacent to navigation channels on two sides, resulting in the rounded shape. E) and F) Typical live reefs with no DM.

shells (Fig. 2). Dead margins were only difficult to interpret when they occurred adjacent to light-colored sand or sand/shell mixtures. Hence, the initial reef signature criteria described above were found to be valid.

Remote sensing of various types of coastal benthic habitats using aerial photography is becoming an important tool for detecting environmental change (Finkbeiner et al. 2001). Much of this work has been on seagrasses (e.g., Ferguson et al. 1993, Morris et al. 2000, Virnstein 2000) but research on other habitat types, including oysters, has been published. One of the earliest published studies mapped the reefs in this study area using aerial photographs taken in 1984 (Grizzle 1990). A similar survey was conducted in Georgia in the late 1970s (Harris 1980). There are also ongoing studies involving aerial photography of oyster reefs in other areas of Florida and South Carolina, but we are aware of no published literature on this work. Hence, although aerial imagery has been used in previous oyster reef research, the published literature is meager. Finkbeiner et al. (2001) suggest a general protocol for mapping coastal habitats, but to our knowledge no published description of an interpretive signature for intertidal oyster reefs exists. Such a description is essential for further development of a standard protocol for mapping oyster reefs.

It should be noted that the suggested signature for live reefs includes potential "dead centers" (see Introduction section) as part of the live reef. This study did not discriminate between different densities of live oysters. However, there were discernable differences between dead centers and areas of different densities of live oysters, suggesting that useful information on oyster abundance may be obtained using aerial imagery. The overall pattern of a darker outer region surrounding a lighter-colored middle derives at least in part from the fact that higher densities of live oysters typically occurred at the margins of CANA reefs compared with middle areas (Grizzle & Castagna 1995). This pattern should be expected in general for older reefs in the "senescent stage" described by Bahr and Lanier (1981) but it may also be the pattern for most large reefs. Increased growth at the margins also has been reported for reef-forming blue mussels, *Mytilus edulis* (e.g., Newell 1990; Svane & Ompi 1993). In the only relevant study on oysters we are aware of, Powell et al. (1987) characterized small-scale differences in distributions of oysters on reefs but did not report an "edge effect." Further work is needed on small spatial scale patterns detectable by aerial photographs so that remote sensing techniques can be developed to monitor characteristics reflecting overall reef "health" in addition to just total areal coverage of "live" reef.

To further assess interpretational accuracy as well as locational accuracy, 405 individual points on 56 reefs were logged and plotted in March 2002; the second field visit to the study site. Overall, this assessment again (as did the November 2001 field survey) verified that interpretation accuracy was nearly 100%. It also showed that location accuracy was within expected ranges. Of the 405 points visited, identified and plotted, 94% were within 5 m of the actual DGPS-determined location (Table 2). DGPS is generally considered to have an accuracy of about 5 m with 95% confidence.

Oyster Reef Distribution Within CANA in 2000

Approximately 400 live reefs ranging in size from 10 m^2 to 4180 m^2 were mapped using year 2000 aerials, and nearly all reefs occurred in the northern half of the study area (Fig. 1). Although not quantified, there was a strong decreasing trend in areal coverage by live oyster reefs from north to south, reflecting the amount of tidal influence in the area (see Grizzle 1990 for further discussion). The total bottom area within CANA covered by live reefs in 2000 was 12.3 ha. Live reefs showed a wide range of overall morphologies and spatial orientations, including all three types described by Kennedy and Sanford 1999 (based on Stenzel 1971): string (perpendicular to shore), fringe (parallel to shore), and patch (small, compact form). String reefs and fringe reefs occurred along the edges of many of the major tidal channels. Patch reefs typically occurred away from the channels, sometimes in groups that resulted in dendritic patterns, with reefs separated by winding open water areas only a few meters wide.

In 2000, dead margins occurred on about 60 reefs covering 1.12 ha and representing 9.1% of the total oyster reef areal coverage in the Park (Fig. 1, Table 3). All reefs with dead margins occurred adjacent to major channel areas, including narrower channels probably not affected by wind waves and broader expanses with sufficient fetch for development of waves. On an individual reef basis, the dead margins ranged from <10% to ~100% of each reef's total areal coverage. Hence, some reefs only suffered minor losses while others appear to have been completely killed.

TABLE 2.

Locational accuracy data showing percent of individual plotted points within the indicated (1 m, 2 m, etc.) accuracy level.

Reef type	# points plotted	Accuracy level			
		0 m	1 m	2 m	5 m
live	137	100	110	116	125
dead	161	119	140	145	154
live + dead	298	219	250	261	279
Percent accuracy:		73.4	83.9	87.6	93.6

TABLE 3.

Total coverage in hectares for dead margins and live reef in all nine groups by year, and Park-wide in year 2000 only. See Figure 4 for bar charts of data by group by year.

Year	1943	1951	1963	1975	1988	1995	2000	All of CANA
TOTAL Dead	0.02	0.10	0.26	0.29	0.55	0.85	1.12	1.12
TOTAL Live	6.55	5.28	4.71	5.73	5.39	3.60	4.06	12.28
Live + Dead	6.57	5.38	4.97	6.02	5.95	4.46	5.18	13.39
% Dead	0.3	2.0	5.5	5.1	10.3	23.7	27.6	9.1

Historical Changes in CANA Oyster Reefs

There was a consistent increase in areal coverage by dead margins from their first appearance on a reef near the ICW in 1943 through 2000, when about 60 reefs were affected throughout the study area (Fig. 4, Table 3). In 1951, dead margins covered only 0.10 ha of bottom area, and all but one (Group 5) were located along the ICW. In most other areas away from the ICW, dead margins first appeared in 1975 or 1988 and showed a steady increase in areal coverage over time. As already noted, by 2000, dead margins occupied 1.12 ha of bottom area representing 9.1% of the total oyster reef areal coverage in the Park.

Some reefs were remarkably dynamic over the 57-year study period, with the most dramatic changes occurring in nearly all reefs adjacent to the ICW (e.g., Group 1 in Fig. 5). In these areas, there was a general movement away from the ICW and an increase in areal coverage by dead margins over time. For example, some reefs in Group 1 moved a distance of approximately 50 m away from the ICW between 1943 and 2000. In contrast, none of the reefs in groups located away from the ICW moved by more than a few meters over the 57-year period, including those with dead margins. Also, the general shape, areal coverage, and location remained relatively stable for many reefs over the period (e.g., most reefs in Group 5, Fig. 5).

An obvious and probably the most serious limitation on use of historical imagery is that ground-truthing is limited. For this study, the 2000 aerials were ground-truthed with respect to interpretation and mapping accuracy. Information from this effort was used to infer the level of confidence placed on historical mapping. There was a wide range of overall quality of the historical aerials caused by differences in sun angle, water clarity, tidal stage, and other factors. This variability affected the mapping process but there is no way to quantitatively assess the effect. A technique that seemed to make the mapping process more accurate for the historical imagery was to analyze a single reef or group of reefs beginning with the ground-truthed 2000 maps and proceeding backwards in time. It was also found that for a year with poor quality imagery, examination of the previous and subsequent years made identification of the reefs easier on that imagery. Thus, while there is no way to rigorously assess accuracy of historical imagery there may be ways to improve the process.

Based on 1986 imagery, Grizzle (1990) presented a distribution map of oyster reefs in this study area. This map was revised based on 1995 imagery by Grizzle and Castagna (1995). The maps pro-

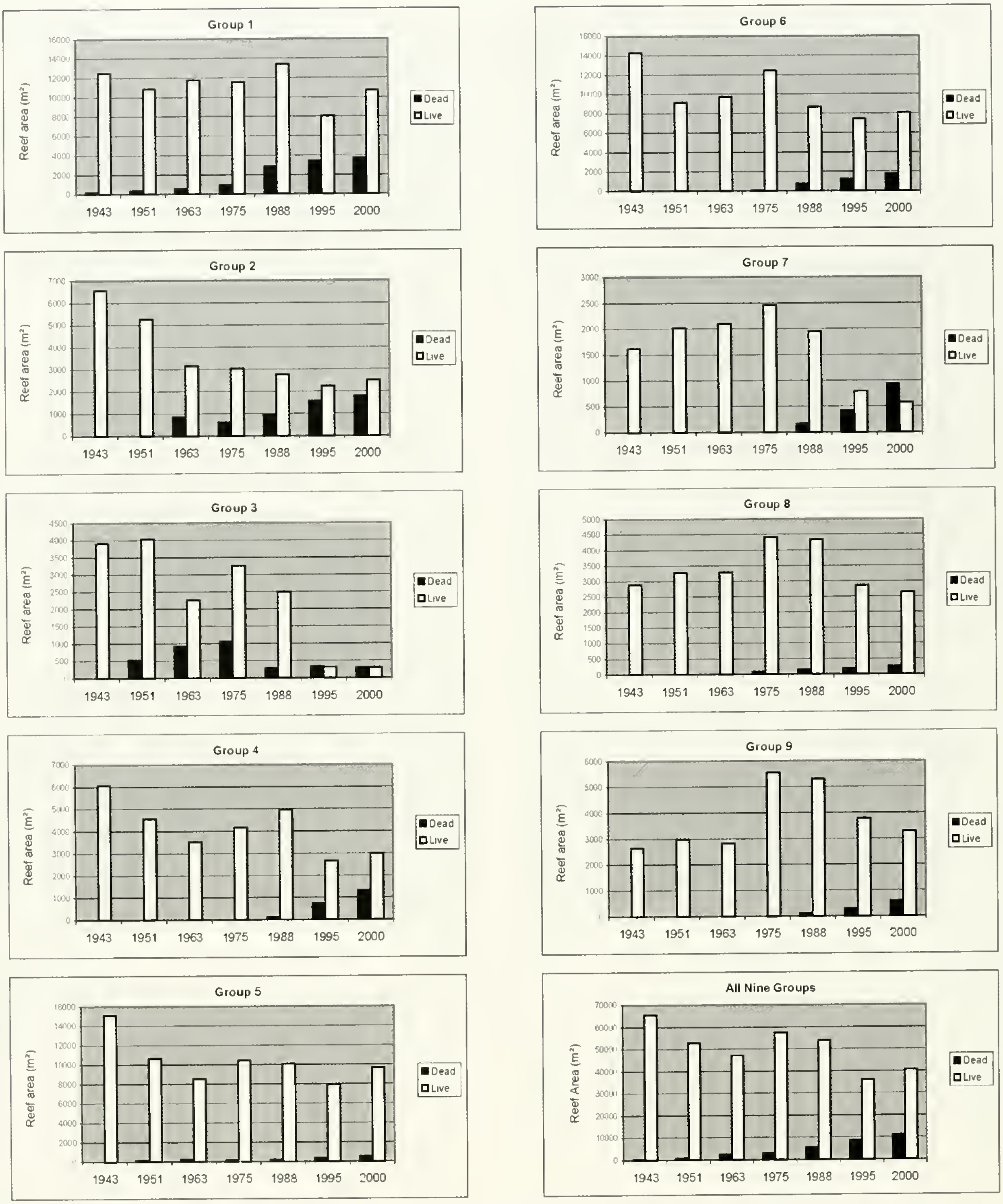

Figure 4. Areal coverage by live reef and dead margins from nine study groups over 57-year period, and all groups combined in last graph.

duced for both studies were very similar with respect to total number of reefs (~400) and overall reef distribution pattern. However, Grizzle and Castagna (1995) noted a wide disparity in reported total areal coverage by oyster reefs: 31.7 ha in 1986/1990, and 14.0 ha in 1995. They further noted that the higher figure was incorrect, and was an artifact of the low-resolution GIS program used in 1990 that assigned all mapped reefs a minimum size of 0.05 ha. Inspection of the 1983 aerials used in this study corroborates this conclusion, and suggests that other than an increase in dead margins there was probably little change in total oyster reef coverage in the Park between 1986 and 1995.

Potential Causes of Historical Reef Changes

This historical assessment was designed to provide a partial test of the hypothesis that boating activities have been a causal factor

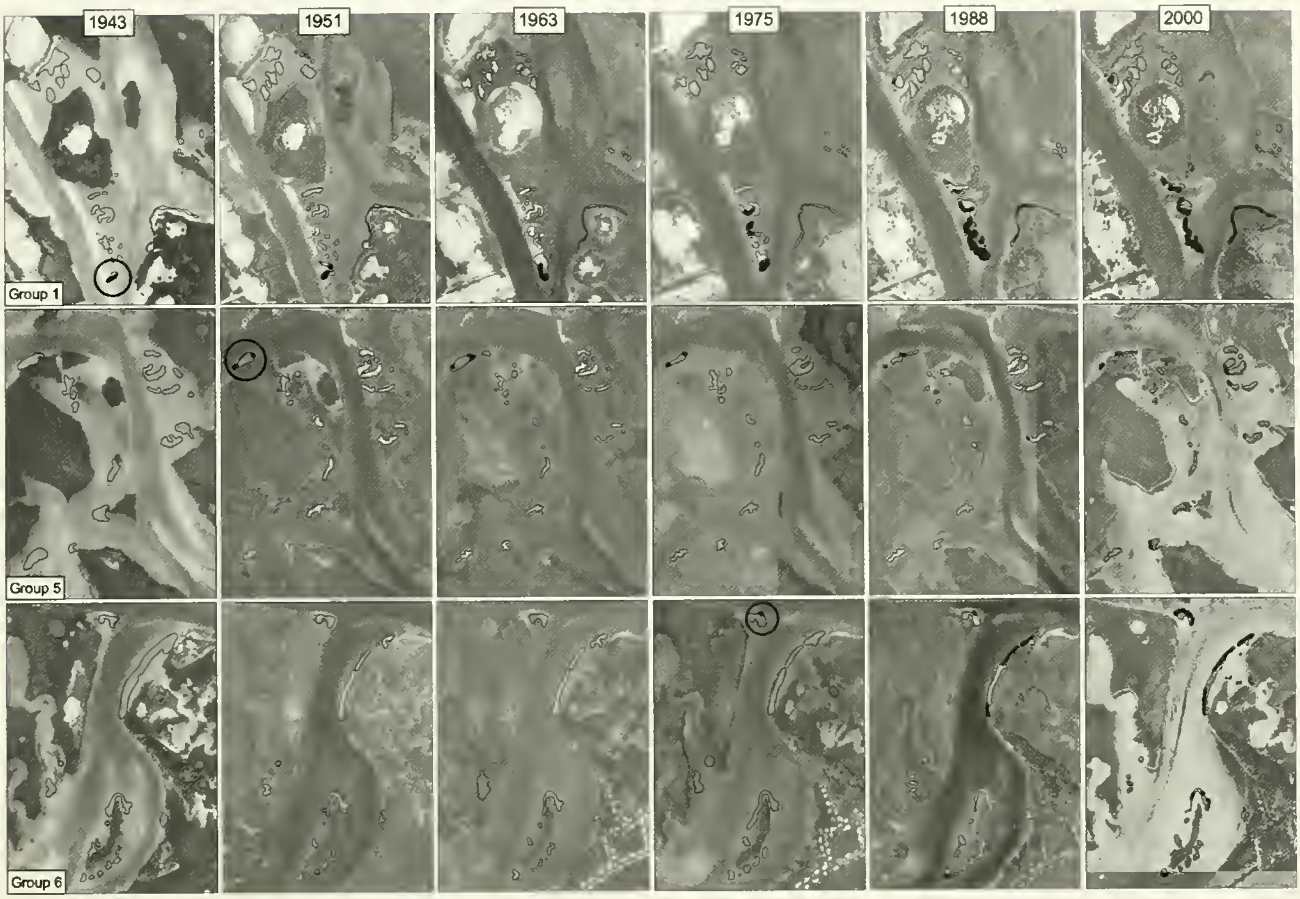

Figure 5. Three of nine reef groups showing differences in reef dynamics over the 57-year study period. Live reefs outlined with narrow black line; dead margins (DM) filled in. Circles show first occurrence of DM for that group (1943 for Group 1, 1951 for Group 5, 1975 for Group 6.

in the occurrence of dead margins on some oyster reefs in the study area. Data presented here support this hypothesis in several ways, and yield no information for rejection of it. Moreover, the present analysis indicates that for some reefs the existing (2000 to 2002) dead margins represent only a vestige of the areal extent of those reefs historically.

The major findings that implicate boating activities are associated with the fact that all of the approximately 60 reefs with dead margins identified for the 57-year study record (1943 to 2000) occurred immediately adjacent to major navigation channels. Data available since 1986 show an average annual increase of about 10% in the number of boats registered in the two counties (Volusia and Brevard) that border the Mosquito Lagoon (Hart et al. 1994), coinciding with an average annual increase of about 9% in bottom area covered by dead margins from 1988 to 1995 (Table 2). There were over 51,000 registered boats in Volusia and Brevard counties in 1998, nearly twice the state average (J. Stewart, pers. com.). The extent of dead margins increased by 16.4% from 1995 to 2000. Such a correlation between registered boats, and thus potential boating activity, and dead margins does not of course require a cause-and-effect relation. However, if boating activities were involved in the observed increases in dead margins, such a correlation would be expected. Assuming boating activities were involved, what might be the actual mechanisms causing the dieoffs at the margins?

The importance of water movement (including tidal currents and waves) to oyster reefs was recognized by Grave (1905), and subsequent research has provided several hypothetical cause-and-effect relations but no quantification of them (see reviews by Bahr & Lanier 1981, and Kennedy & Sanford 1999; see additional discussion later). Bahr and Lanier (1981) suggested that vertical growth rate and morphologic form of intertidal reefs were strongly affected by wave action. Waves (and currents) transport sediment to and from the reef, and in some cases eventually result in sediment buildup and loss of oysters in the central region (see more discussion in the Introduction section). Bahr and Lanier also noted (p. 57) that ". . . a water current or wave energy regimen above a certain threshold level will prevent the development . . ." of a reef. And they showed (p. 34) a photograph of "shell debris characteristic of high energy beach shores . . ." that resembled the mounded up dead margins observed in the present study. Wave energies of some magnitude are clearly capable of destroying an existing reef. However, we are not aware of research that quantifies the relationships involved, nor the actual mechanisms involved.

Hypothesized inhibitory mechanisms for reef development include: vertical accretion of the reef surface to an elevation too high in the intertidal caused by waves (Churchill 1920; Bahr & Lanier 1981), smothering and substrate instability caused by excessive sediment transport (Marshall 1954), and inhibited larval settlement caused by sediment ("grit") movement (Gunter 1979). Analysis of historical aerials for reefs along the ICW in this study also suggest that a reef can be physically moved by wave energies, resulting in a range of responses from slow migration to total destruction, much in the fashion that barrier islands migrate shoreward (termed "transgression") under increased wave energies (Davis 1996). As already discussed, this study was not designed to test particular causal mechanisms for observed reef declines. They are the topic of ongoing studies by Linda Walters and colleagues (e.g., Wall et al. 2002, Walters et al. 2002) and will be reported elsewhere. This study, however, does provide strong (although correlative) evidence that boating activity has had dramatically detrimental effects on some oyster reefs in the study area.

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BIOCHEMICAL COMPOSITION OF *SPONDYLUS LEUCACANTHUS* BRODERIP, 1833 (BIVALVIA: SPONDYLIDAE) AND ITS RELATIONSHIP WITH THE REPRODUCTIVE CYCLE AT ISLA DANZANTE, GULF OF CALIFORNIA, MEXICO

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ABSTRACT The monthly variation of the biochemical composition was studied for one year in relation to the reproductive cycle in a population of *Spondylus leucacanthus*, a commercially important species in the Gulf of California. Carbohydrate, lipid, and protein concentrations were determined in the adductor muscle, gonad, and digestive gland. Protein was the main constituent in the gonad, digestive gland, and adductor muscle. Lipids were important in the digestive gland, whereas in the gonad they were moderately represented and in the muscle they showed low and virtually invariable concentrations during the study period. Carbohydrates were found largely in the adductor muscle and the digestive gland, whereas the gonad showed lower concentrations. *S. leucacanthus* has a seasonal reproductive cycle with a reproductive inactivity period at the end of the autumn and throughout the winter. Gametogenesis starts at the end of the winter, continuing during springtime, and ending in the summer, where 90% of organisms are ripe, coinciding with the highest lipid concentration in the gonad. Spawning takes place in early autumn. It is concluded that this species has a conservative reproductive strategy because it largely uses energy reserves for gamete development, in addition to energy obtained from food, to supplement its energetic expenditure.

KEY WORDS: *Spondylus leucacanthus*, reproductive cycle, biochemical composition, Gulf of California

INTRODUCTION

In the Gulf of California, *Spondylus leucacanthus* and other bivalve species, including *Megapitaria aurantiaca, Argopecten ventricosus, Lyropecten nodosus*, and *Spondylus calcifer* are of great importance because of the high commercial value of soft parts, adductor muscle, and shell. (Villalejo-Fuerte & Muñetón-Gómez 1995, Skoglund & Mulliner 1996, Villalejo-Fuerte & García-Domínguez 1998, Muñetón-Gómez et al. 2001). The exploitation of commercially important bivalve populations in the Gulf of California has fostered a number of studies focused on reproductive and feeding aspects (Baqueiro et al. 1982, Villalejo-Fuerte & Ochoa-Báez 1993, Villalejo-Fuerte & Ceballos-Vázquez 1996, Félix-Pico et al. 1997, Villalejo-Fuerte & García Domínguez 1998, Villalejo-Fuerte et al. 2002).

In general, reproduction in marine bivalves is associated with food availability and nutrient reserve storage in specialized organs (Gabbott 1975, Bayne 1976, Barber & Blake 1981, Boadas et al. 1997, Claereboudt & Himmelman 1997). When organisms reach reproductive maturity, growth slows down as a result of the reproductive effort, and the biochemical composition may change according to the reproductive requirements (Lodeiros et al. 2001). Those species where energy storage and gamete production cycles overlap temporally are considered "opportunistic"; in contrast, those in which both cycles are clearly separated in time because of the use of previously stored energy reserves for gonad development are known as "conservative species" (Bayne 1976).

Gonad development implies an intense metabolic activity, including the storage of large amounts of lipids in the developing eggs, either at the expense of glycogen reserves previously accumulated in storing tissues, at the expense of food supply, or from both sources (Gabbott 1975). A decrease in glycogen and protein contents coupled with a rise in lipid content during gonad development has been described for the pectinids *Chlamys septemradiata* (Ansell 1974), *Pecten maximus* (Comely 1974), *Argopecten irradians* (Hickey 1978, Barber & Blake 1981), *Chlamys opercularis* (Taylor & Venn 1979), *Placopecten magellanicus* (Robinson et al. 1981), and *Hinnites giganteus* (Lauren 1982). The influence of temperature on nutrient-transfer regulation from reserve-storing organs to the gonad has also been described for *A. irradians* and *Patinopecten caurinus* (Sastry & Blake 1971, Barber & Blake 1981, MacDonald & Bourne 1987). The potential relationship between morphophysiologic indices and the storage and use of nutrient reserves during reproduction was described for wild populations of *A. circularis* (Villalejo-Fuerte & Ceballos-Vázquez 1996) and *S. calcifer* (Villalejo-Fuerte et al. 2002).

In the Gulf of California, *S. leucacanthus* banks have been overexploited at depths of less than 15 m (Baqueiro et al. 1982), but beyond 40 m there are banks that have not been affected by commercial fisheries. Studies of this population focused on the reproductive cycle (Villalejo-Fuerte & García-Domínguez, 1998) and gut content have been conducted in relation to reproduction and phytoplankton abundance (Muñetón-Gómez et al. 2001). The lack of information on protein, lipid, and carbohydrate content in storage organs and its potential association with the reproductive period in this species, analyzed from a seasonal perspective, were the reasons for the development of this research.

MATERIALS AND METHODS

An average of 36 specimens of *S. leucacanthus* with a range in shell height from 40 to 94 mm (mean of 60 mm) were collected each month, from June 1996 to May 1997, south of Isla Danzante (26°55′–26°30′N and 112°–111°40′W) in the Gulf of California (Fig. 1), using a net at 40 m of depth.

For the biochemical analysis, 16 specimens were used each month. In the laboratory, specimens were cleaned, eliminating water from the pallial cavity, and were washed with distilled water. Then, soft parts were separated from the shell and weighed, followed by the dissection of the adductor muscle, gonad, and digestive gland. Each organ was weighed separately and oven-dried at 100°C for 24 h. The dry tissue was pooled and homogenized and

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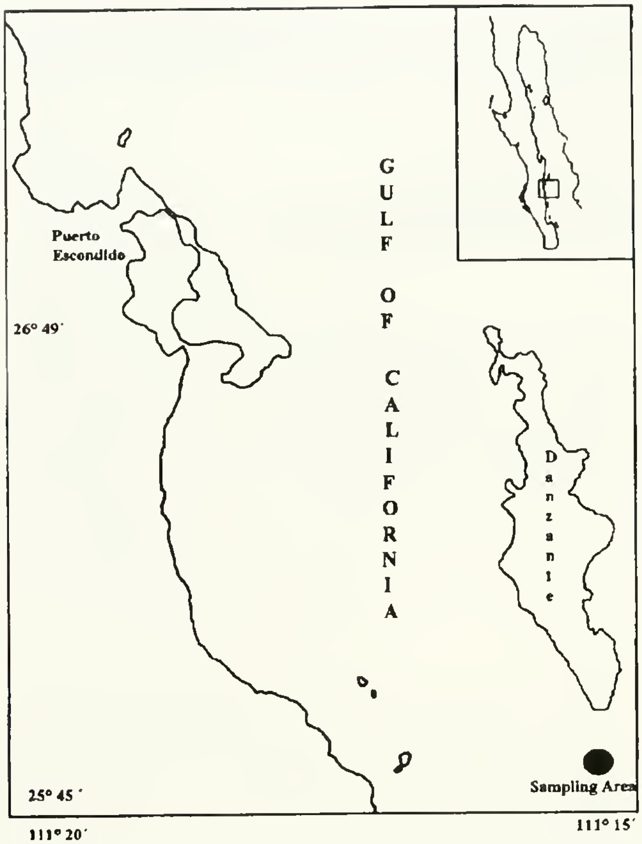

Figure 1. Location of the sampling area in the vicinity of Danzante island, Gulf of California.

then was ground into a powder. Subsamples of this homogenate were taken for the biochemical determination.

Protein determination was conducted using the method by Lowry et al. (1951). Carbohydrates were determined by Hewitt's method (1958). Lipid extraction was performed using the method by Folch et al. (1957) and a modification of Bligh and Dyer's method (1959).

Energetic substrate concentration mg/g dry weight curves were interpreted as follows: An increase was considered as indicative of accumulation and a decline as indicative of substrate use in the corresponding organ.

The reproductive cycle analysis included an average of 20 specimens per month. The degree of gonad development was defined from histologic section (7 μm thick) stained with the hematoxylin and eosin technique (Humason 1979), assigning one of the following categories proposed by Villalejo-Fuerte and García-Domínguez (1998) for this species, undifferentiated, developing, ripe, spawning, and spent. Bottom water temperature was recorded at the time of collections.

A Spearman's rank correlation analysis (r_s) was used to determine the relationship of the gametogenic cycle stages with protein, lipid, and carbohydrate concentrations in the adductor muscle, digestive gland, gonad, and water temperature. Before performing the correlation analysis, logarithmic transformations were performed for energetic substrates, and arc-sin transformation were conducted for percentages of the reproductive cycle stages, aimed at obtaining data normality and homoscedasticity (Sokal & Rohlf 1995). Only those results where a significant correlation was found are reported below.

RESULTS

Biochemical Composition

The trend in the protein concentration curve is similar for the three organs analyzed. During the summer, protein concentration reaches high values and remains relatively unchanged. In autumn, there is a noticeable decline, with low concentrations remaining until the end of this season. In the winter, protein concentration in the gonad and digestive gland shows a recovery, reaching high values in mid-winter and decreasing by early spring. In the muscle, proteins show a steady increase that lasts until mid-spring, reaching high values also in the digestive gland and gonad, then decreasing at the end of the spring (Fig. 2A).

Lipid concentration in the adductor muscle did not show noticeable variations during the study period. In the digestive gland, high concentrations were found at the beginning of the summer, which declined throughout this season and remained low, although with some variations, during the autumn. In winter, there is a clear recovery, reaching the peak value in mid-winter and then declining, with some fluctuations, afterwards and during the spring. In the gonad, the highest lipid concentration was recorded during the summer, with lower values in all other seasons (Fig. 2B).

During the summer, carbohydrate concentration drops in the three organs analyzed (August), with drops being more pronounced in the muscle and gonad. In late summer and early autumn, a considerable rise occurs in both gonad and muscle, remaining unchanged in the gland. During autumn, there is a decline in the muscle and gonad, remaining low in early winter whereas there is a considerable increase in the digestive gland (November); at the end of the autumn, carbohydrates decrease, reaching low values at the beginning of the winter. During the winter, carbohydrates in the muscle and digestive gland rise steadily, reaching peak values during the spring. In the gonad, there is a slight increase in mid-winter, decreasing afterwards until mid-spring, then recovering at the end of this season (Fig. 2C).

Temperature

During the study period, bottom water temperature ranged from 17 to 24.5°C, the highest values recorded in July (24.5°C) and October 1996 (24°C). The lowest temperature occurred in January 1997 (17°C; Fig. 2D). Spawning took place during a temperature rise from 22.5 to 24°C, and gametogenesis occurred when temperature rose from 17 to 18°C (Fig. 2D).

Reproductive Cycle

The bivalve population enters a reproductive inactivity stage at the end of the autumn (undifferentiated stage near 100% in November), high values being maintained (around 70%) during the winter. The reproductive activity (gametogenesis) starts at the end of the winter, and in springtime 100% of the population is undergoing this stage. The first ripe individuals appear at the end of the spring, and in the summer 90% of the population is ripe. The population spawns in the autumn (90% in October). Gamete resorption is rapid because the post-spawning stage only takes place in early winter with low percentages (Fig. 3A–D).

DISCUSSION

In general, proteins were the main constituent of the gonad, digestive gland, and adductor muscle. Lipids were important in the digestive gland, whereas in the gonad they were highest before

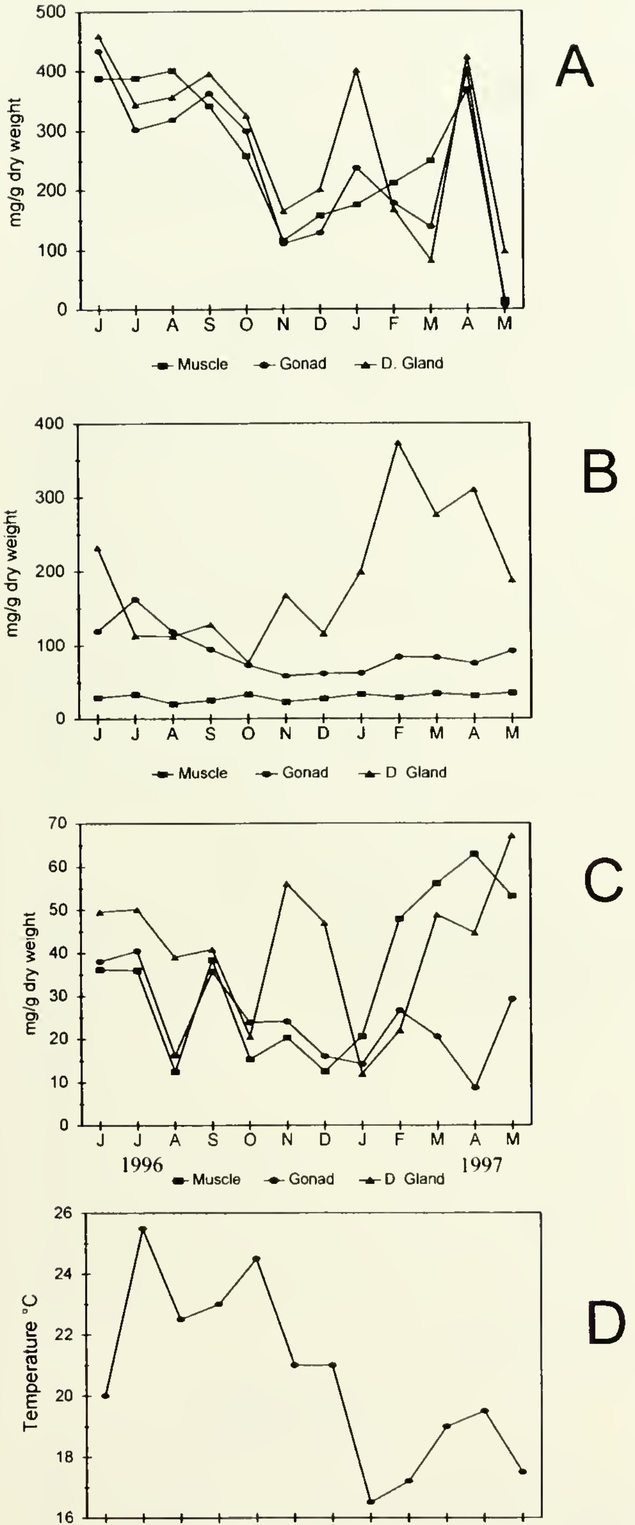

Figure 2. Variation of biochemical components in *Spondylus leucacanthus*. A, proteins; B, lipids; C, carbohydrates in muscle, digestive gland, and gonad; and D, bottom water temperature at Danzante island, Gulf of California.

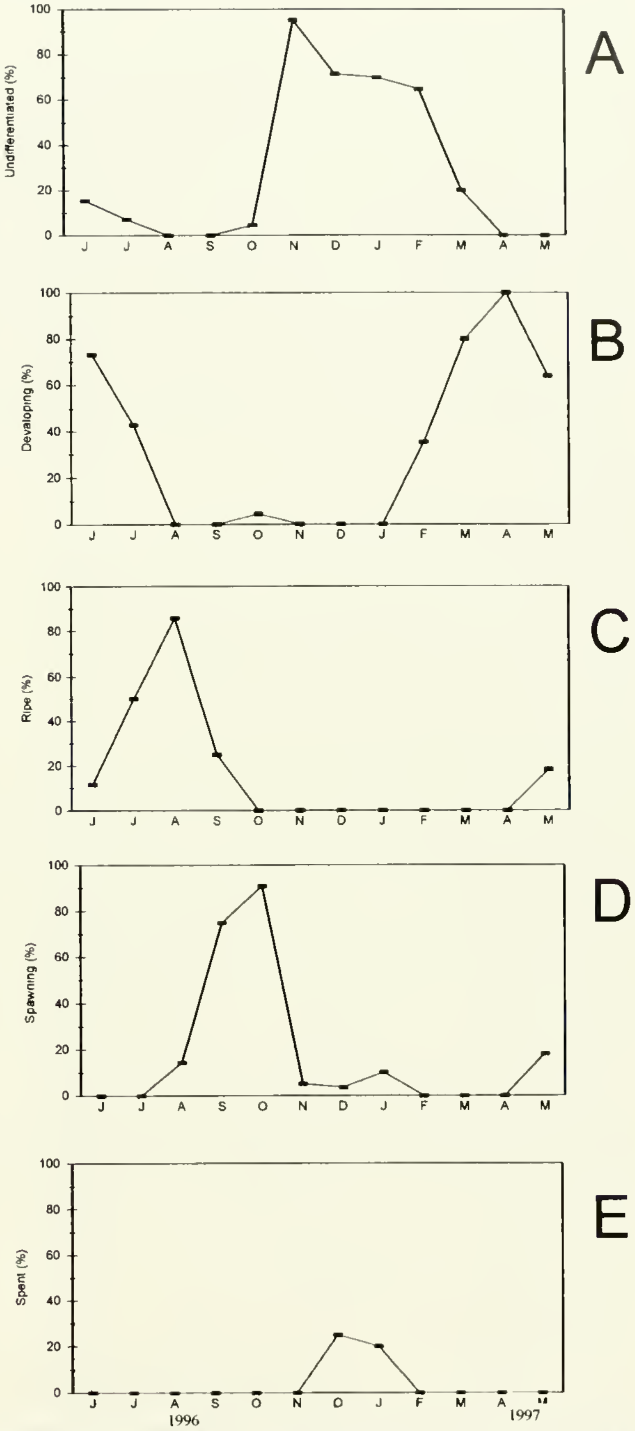

Figure 3. Reproductive cycle stages in *Spondylus leucacanthus*. A, inactivity; B, gametogenesis; C, maturity; D, spawning; E, post-spawning.

spawning and in the muscle they showed low and virtually unchanged concentrations during the study period. Carbohydrates were found chiefly in the adductor muscle and digestive gland, whereas the gonad showed lower values. These same trends in energetic substrate concentration were found in *Pecten maximus* (Strohmeier et al. 2000). During gonad maturation of many marine bivalves, an increase of biochemical compounds is observed in the female gonad, followed by a drop during spawning (Ansell 1974, Taylor & Venn 1979, Barber & Blake 1981, Sundet & Vahl 1981, Pazos et al. 1996).

The slight variation of proteins during the summer, when the population is ripe, suggests a low demand of this energetic substrate during this stage. The trend in the lipid curve for the digestive gland suggests that lipids are used during the ripe stage; however the correlation obtained is low ($r_s = 0.55$) and nonsignificant. Lipid transfer from the digestive gland to the ovary has been demonstrated in *Chlamys hericia* (Vasallo 1973). Furthermore, digestive gland carbohydrates show a significant correlation with the ripe stage ($r_s = 0.83$, $P < 0.05$), indicating its use during this stage.

It is likely that during the last gamete growth stage, food is also used as an energy source. In this same population, ripe organisms have been found in July, coinciding with a peak in food availability, which may provide a portion of the nutrients required for the gonad's final ripening (Muñetón-Gómez et al. 2001). Similar behavior was reported for *Placopecten magellanicus*, which reside southeast of Terranova (Thompson 1977). In other bivalve species like *Donax vittatus* and *Tellina tenuis*, the accumulation of reserve glycogen seems to be related with the season when the highest phytoplankton biomass occurs (Ansell & Trevaillion 1967, Ansell 1972). American oysters (*Crassostrea virginica*) held at 14 to 19°C were able to exploit a large phytoplankton bloom and synthesize glycogen (Ruddy et al. 1975).

In *S. leucacanthus*, spawning is an event that demands a heavy energy expenditure, requiring the use of protein and carbohydrate from the adductor muscle, the gland, and the gonad. However, it is in this latter organ that a significant correlation is found ($r_s = -0.63$, $P < 0.05$). Lipids do not appear as an important source in this stage. Based on the analyses of morpho-physiologic indexes, gut content, and phytoplankton abundance in the environment, the required energy expenditure during spawning in this species had been suggested to be partially supported by reserves stored in the muscle, the digestive gland, and the gonad (Villalejo-Fuerte & García-Domínguez 1998, Muñetón-Gómez et al. 2001).

When spawning ends, lipids and proteins remain low whereas carbohydrates are stored in the digestive gland, providing the energy required for the resorption of residual gametes during the stage known as spent that occurs at the end of the autumn. In *Crassostra virginica*, the concentration of neutral lipids decreased considerably during the spawning (Trider & Castell 1980) in *C. gigas* was observed an increased in lipid during the gametogenesis phase (Deslous-Paoli & Héral 1988).

When the population enters reproductive inactivity (undifferentiated stage) in early winter, there is a reorganization in the gonad (Villalejo-Fuerte & García-Domínguez 1998); during this stage, carbohydrates from the digestive gland are used in the first place ($r_s = 0.59$, $P < 0.05$), followed by proteins stored in the digestive gland and the gonad.

Gametogenesis starts at the end of the winter and in early spring. Energy for the initial gamete development is provided by lipids from the digestive gland, an event occurring similarly in *Spondylus calcifer* (Villalejo-Fuerte et al. 2002). Proteins from the three organs analyzed are used afterwards, most probably during the gamete final development stages. In this respect, this species has been shown to undergo rapid gamete growth at the beginning of gametogenesis, followed by a slowdown at the end of this stage (Villalejo-Fuerte & García-Domínguez 1998).

Temperature has been reported to influence the gametogenic cycle through the regulation of nutrient transfer from reserve-storing organs (Sastry & Blake 1971, Barber & Blake 1981, MacDonald & Bourne 1987). Our findings suggest that nutrient transfer and use takes place when temperature varies as a result of the summer to autumn (spawning) and winter to spring (developing) seasonal changes. This had been previously suggested for this species by Villalejo-Fuerte and García-Domínguez (1998) and for *A. circularis* (Villalejo-Fuerte & Ceballos-Vázquez 1996). The reserve storage process takes place when the lowest temperatures occur.

The biochemical composition of marine bivalves is affected by exogenous factors, including food availability and temperature, as well as by endogenous factors such as reproduction (Gabbott 1975, Bayne 1976, Barber & Blake 1981, Boadas et al. 1997, Claereboudt & Himmelman 1997). "Opportunistic species" use the energy directly obtained from food for gonad development, and energetic substrate storage and gamete production cycles may overlap temporarily, whereas "conservative species" use previously stored energy reserves (Bayne 1976).

Based on the findings of this study, it is confirmed that *S. leucacanthus* has a markedly seasonal reproductive cycle. This species shows a reproductive inactivity period that is well delimited throughout the year, characterized by the accumulation of energy reserves in the adductor muscle, the digestive gland and the gonad. The reproductive cycle stages that demand a higher expenditure of reserves are developing and spawning. According to these features, it can be concluded that this species has a conservative reproductive strategy, which is a characteristic of iteroparous species that largely use energy reserves, supplemented by energy obtained from food for energetic expenditures.

Similar strategies have been identified in other bivalves. *Scapharca broughtonii* behaves as a typical conservative species, with gametogenesis taking place during the winter and spring at the expense of reserve glycogen stored during the autumn, and spawning occurring during the summer (Park et al. 2001); by contrast, *Crassostrea gigas* was shown to behave as an opportunistic species, given that gametogenesis starts simultaneously with reserve accumulation and proceeds until gonadic maturity is reached, coinciding with the highest level of biochemical components followed by a rapid drop of these substances during spawning (Kang et al. 2000). *Argopecten ventricosus* preferentially uses food available in the environment when it is abundant but makes use of reserves in the adductor muscle when food is scarce (Luna-González et al. 2000). In *Cerastoderma edule*, gametogenesis may occur at the expense of glycogen reserves or simultaneously with glycogen storage, depending on the amount of available food (Navarro et al. 1989).

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SEASONAL DISTRIBUTION OF THE OYSTER *OSTREA EDULIS* (LINNAEUS, 1758) LARVAE IN THE BAY OF MALI STON, ADRIATIC SEA

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ABSTRACT Study of spatial and temporal distribution of European flat oyster larvae in relation to temperature and salinity has conducted over a two-year period at four study sites in a Mali Ston Bay, largest bivalve production area in the Eastern Adriatic Sea. In 2000, significant number of larvae was noted in a period from May till September, with a peak in July at all study sites. Lower number of flat oyster larvae in 2001, than in 2000, were noted at three study sites, while larvae appeared to be similarly abundant in both years at site Bistrina. Maximal recorded number of larvae was 5029 ind/m^3 at site Bistrina in August 2001. Our results show between year and between site variations in distribution of oyster larvae.

KEY WORDS: bivalve, oyster, *Ostrea edulis*, larvae, Adriatic Sea

INTRODUCTION

The Bay of Mali Ston is the largest bivalve production area in the Eastern Adriatic Sea, with a tradition of collection and aquaculture that extends for few centuries, and according to some authors, even from the time of the Roman Empire (Basioli 1968). Today, production is still based on small family farms. Most important species cultured in this region is European flat oyster *Ostrea edulis* (Linnaeus, 1758), and according to Benović (1997), about 1.2 million oysters per year were produced in the1980s. In the early 1990s, due to the war situation in this region, aquaculture production was largely neglected and it is only in last few years that interest has been generated to improve oyster aquaculture in Mali Ston Bay as a result of market demands caused by developing tourism industry. According to Šimunović (2001), current annual production is only 300 thousand pieces of oysters.

European flat oyster is one of the few indigenous bivalve species in European commercial aquaculture. Since the mid-nineteenth century, in other European countries flat oyster beds have progressively shrunk making this species a scarce and expensive item (Cano et al. 1997). Naturally occurring flat oyster beds are of great interest as a natural source of seed for achieving a steady and viable culture of *O. edulis* (Cano et al. 1997). This gives Mali Ston Bay exceptional importance since it is one of the rare places in the Mediterranean where both an adequate collection of *O. edulis* spat and subsequent aquaculture are still possible.

A previous study conducted in Mali Ston Bay, described two oyster spawning periods, one in the spring and the other in the fall (Morović & Šimunović 1980). Between 1985 to 2000, local farmers noticed insufficient attachment of oyster spat. Change was especially evident in relation to fall-spat settlement, which according to farmers, did not occur in some years (Maškarić 2001, pers. com.). Therefore, a study of spatial and temporal distribution of oyster larvae in relation to environmental characteristics was undertaken with the objective of improving collection of oyster spat and its aquaculture in this region and to gain a better insight into possibilities of spat collection that could potentially be used for *O. edulis* aquaculture in other parts of Mediterranean.

Study Site

Mali Ston Bay is an extended and ramified bay situated between the mainland and the Pelješac peninsula (Fig. 1). It is relatively shallow (maximal depth = 26 m) and characterized by strong marine currents and underwater freshwater springs, which along with precipitation, lower the salinity of the bay and bring organic matter from the surrounding terrestrial area (Šimunović 1981). Abundant and constant sedimentation influences formation of soft-mud sediments. Owing to the high level of fresh water input, the concentration of nutrients is high, but there are no signs of eutrophication (Vukadin 1981, Carić et al. 1992). Analysis of zooplankton community has indicated that the bay is a naturally moderate eutrophic ecosystem (Lučić & Kršinić 1998). As a consequence, production in the entire bay area is high, providing a rich diet for filter feeders (Bahun 1981). To protect this unique ecosystem and its bivalve aquaculture production, Mali Ston Bay was declared a reserve in the sea.

MATERIALS AND METHODS

Investigation was conducted at four sites within the Bay of Mali Ston: Bistrina (9 m), Soca (8 m), Krstac (15 m), and Bjejevica (14 m) (Fig. 1), in a period from May 2000 until March 2002. Samples were collected by vertical tows of plankton net (1.5 m long, 56 cm in diameter, and 125 μm in mesh size) for 8 to 15 m, depending on a maximal depth of a study site. Sampling was conducted a few times a month, depending on the number of larvae in the water column and weather conditions, and was more intensive during the summer period. Collected samples were preserved in 4% formaldehyde solution and analyzed on a microscope in a laboratory within 24 h after the collection. Oyster larvae were determined according to Rees (1950) and Loosanoff et al. (1966), and their numbers were calculated per cubic meter of seawater. Temperature and salinity were measured with the WTW multiline hydrographical probe at three depths on each site: surface, mid water column and one meter above the bottom. Degree of association between larval number and environmental parameters was determined with Pearson's rank correlation analysis.

RESULTS

The surface temperature ranged between 7.0°C (Soca, January 2002) and 26.9°C (Bjejevica, August 2000). The rise in surface temperature above 21°C occurred in late May and temperature did not drop below 21°C before mid September, in both years and at all study sites. It is interesting to note that in 2000, surface temperature was higher in December than in November. In early fall

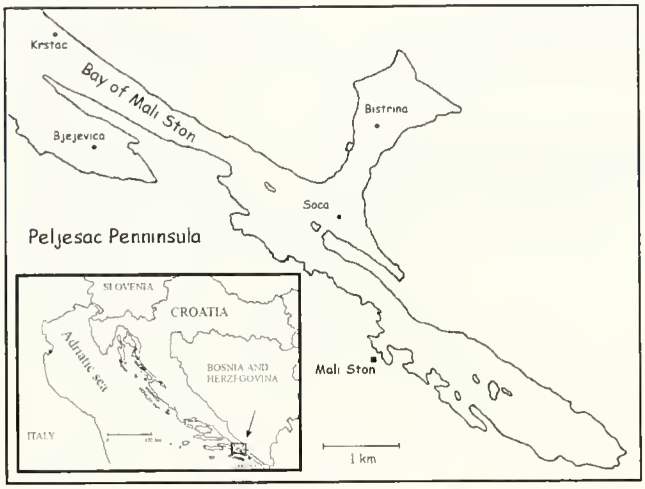

Figure 1. Location of Mali Ston Bay and four study sites: Bistrina, Soca, Krstac, and Bjejevica.

of 2001, surface temperature decreased slowly and was very similar in September and October at all study sites, that was followed by exceptionally low surface temperatures that dropped below 10°C in December and January.

In the late spring and early summer periods, due to stratification of a water column, bottom temperature is a few degrees lower than surface temperature. Toward the end of the summer, isothermia was observed in a water column, while during the fall and winter periods near bottom temperatures were higher than surface or mid water column temperatures (Table 1).

The surface salinity values ranged between 26.6 psu (Bistrina, May 2001) and 39.7 psu (Soca, March 2001). High variations in surface salinity were observed at Bistrina and Soca in a period from October till May and in Krstac and Bjejevica from October till February (Fig. 2). Near bottom layer was characterized with higher salinity values, than surface and mid water column layer, throughout the year.

European flat oyster larvae were present in the Bay of Mali Ston in varying numbers throughout the year. Maximal number of larvae recorded in a given month, and average surface temperature recorded in that month, are shown in Figure 3. High numbers of *Ostrea edulis* larvae were recorded in samples collected from May till September, while larvae were present in some samples, but not abundant, from November till April. Absence of larvae was primarily noted in samples collected between January and March 2002 at all study sites.

In year 2000, the highest larval numbers were recorded in July at all sites, with the highest value being recorded in the cove of Bjejevica (4875 ind./m^3) in late July. Minimal, maximal and mean values for number of *O. edulis* larvae recorded in study period are shown in Table 2. In 2001, maximal number of larvae collected in each month appeared to be lower than in 2000 at sites Soca, Krstac, and Bjejevica. At site Bistrina, the highest number of oyster larvae was recorded in August 2001 (5029 ind./m^3) while the number of larvae observed in other months of that year appears to be similar to values obtained for oyster larvae in year 2000.

There was a statistically significant positive correlation (P = 0.05) between number of *O. edulis* larvae and surface temperature and mid water column temperature (Table 3). Correlation between larvae number and near bottom temperature was significant only at

TABLE 1.

Water temperature at all four study sites from May 2001 to March 2002.

	Temperature (°C)											
	Bistrina			Soca			Krstac			Bjejevica		
Month	Sur.	Mid.	Bot.	Sur.	Mid.	Bot.	Sur.	Mid.	Bot.	Sur.	Mid.	Bot.
May	21.0	20.0	18.7	19.9	19.7	19.5	20.6	18.2	18.2	19.7	18.4	17.6
June	23.6	22.8	21.8	23.3	22.8	21.9	22.8	21.4	19.8	22.7	21.3	19.9
July	23.1	23.0	22.3	23.3	23.0	22.8	23.4	22.9	21.3	23.3	22.7	21.7
August	26.1	26.0	26.1	26.0	25.8	25.7	26.6	25.7	18.4	26.9	26.1	17.9
September	21.6	21.2	21.1	21.1	21.1	21.0	21.1	20.9	20.7	20.6	20.4	20.0
October	18.8	19.8	20.7	18.7	18.7	20.2	18.7	20.1	20.5			
November	15.4	17.9	17.8	12.2	17.3	17.5	13.8	17.5	18.0	12.8	18.3	17.9
December	18.4	17.4	17.4	13.8	17.0	17.2	16.8	17.7	17.6	15.3	15.6	16.0
January	12.0	13.7	14.7	11.4	13.1	15.8	12.4	14.7	15.8	12.8	15.4	15.5
February	12.0	12.3	13.8	11.4	12.5	14.3	11.5	14.1	14.3	11.8	13.3	14.5
March	14.4	14.6	14.5	14.5	14.2	14.2	14.1	14.3	14.0	14.0	14.0	14.0
April	14.4	14.9	15.2	14.5	14.7	14.9	14.9	14.7	14.7	14.5	14.4	14.7
May	21.1	17.7	17.5	20.2	18.4	17.8	18.2	17.0	16.8	18.3	17.1	16.8
June	21.0	20.5	19.8	21.2	21.1	20.5	20.6	20.4	20.1	20.8	20.4	19.9
July	24.4	24.2	22.9	24.3	24.2	22.6	24.5	23.8	19.9	24.6	23.8	18.8
August	25.5	23.9	21.8	25.4	23.8	21.7	25.0	24.7	20.0	25.3	23.7	20.0
September	20.0	20.0	19.9	20.1	19.8	19.7	20.3	20.1	19.6	20.2	19.7	19.3
October	20.0	20.2	20.5	19.9	20.1	20.2	20.0	20.6	20.4	20.0	20.5	20.4
November	13.8	15.6	16.3	13.8	15.2	16.1	15.2	15.6	16.2	14.1	15.3	16.0
December	9.5	9.8	10.6	8.9	9.0	10.6	10.0	10.7	10.6	10.1	10.1	10.3
January	9.3	10.6	11.1	9.1	9.5	11.1	9.3	10.6	11.1	9.0	10.6	11.0
February	11.5	12.6	13.1	11.4	11.8	12.7	11.8	12.0	12.5	11.6	11.6	12.4
March	13.5	13.4	13.3	13.4	13.2	13.3	12.8	13.1	13.2	13.0	13.0	13.4

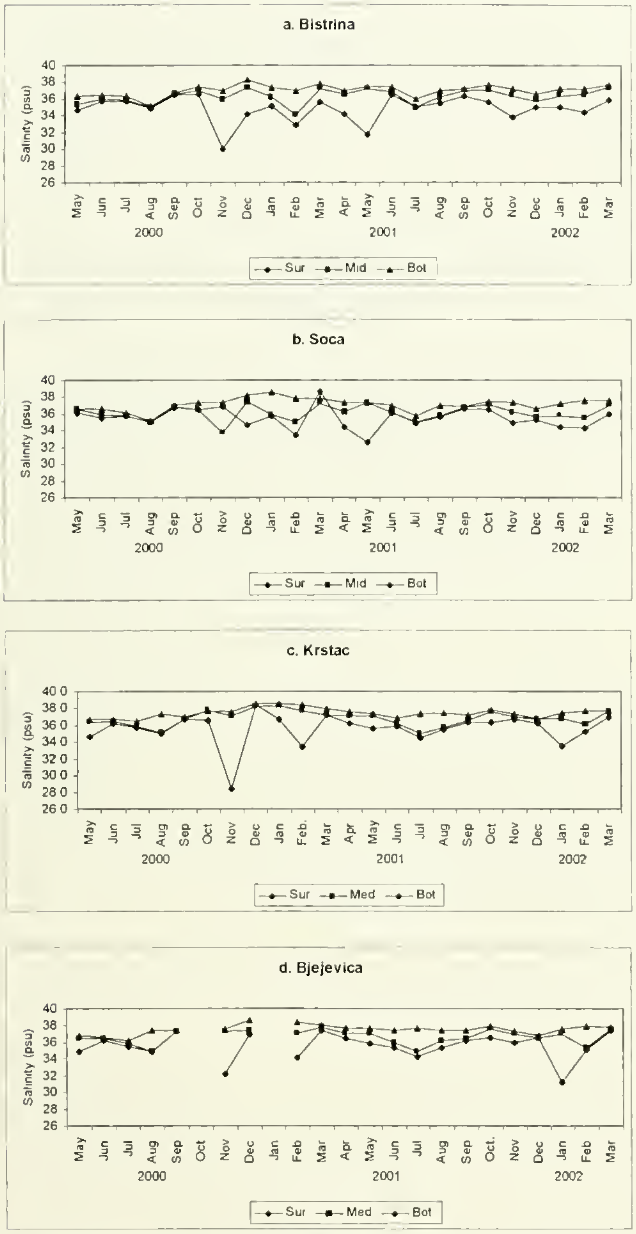

Figure 2. Time trend over a two-year study period for surface (Sur.), mid water column (Mid.) and near bottom (Bot.) salinity (psu) at four study sites: (a) Bistrina, (b) Soca, (c) Krstac, and (d) Bjejevica.

two shallower sites—Bistrina and Soca. Salinity values were not correlated with number of oyster larvae observed in samples, except at site Soca for near bottom salinity and at site Krstac for mid water column salinity.

DISCUSSION

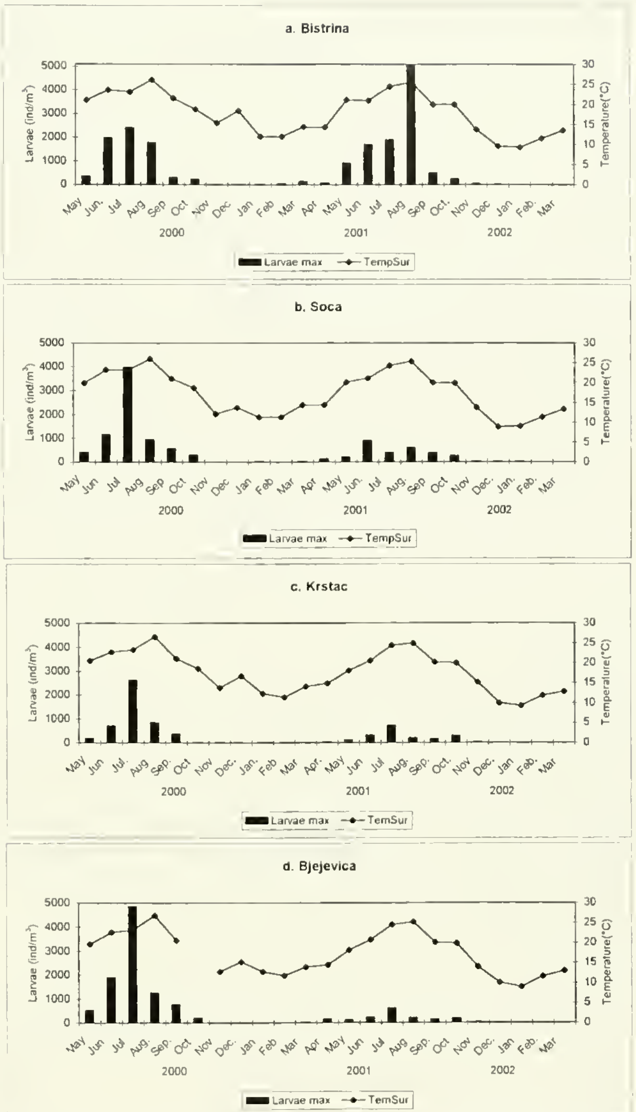

Figure 3. Time trend over a two-year study period for number of flat oyster larvae (ind/m^3) and temperature (°C) at for study sites: (a) Bistrina, (b) Soca, (c) Krstac, and (d) Bjejevica.

Traditionally, the Bay of Mali Ston was well known as an area where the European flat oyster has two spawning peaks per year, one in the late spring (May and June) and other in the autumn (September and October) (Morović & Šimunović, 1980). However, during the last 15 years, change in spawning season, was observed by local farmers. Results of this research confirm their observations. Although larvae were present throughout the year, the quantities suitable for commercial collection occurred several times during the warmest season, between May to September. Therefore, our results point out that in the summer period of 2000 and 2001, two previously known spawning peaks were combined to form a single, longer spawning period. Cano et al. (1997) also observed only one oyster spawning peak in Mar Menor (Spain) where significant oyster larvae numbers appeared at temperature

TABLE 2.

Number of *Ostrea edulis* larvae (ind./m^3) with respect to location.

Location	Records (n)	Maximum	Mean ± St.dev.
Bistrina	61	5029	473 ± 805
Soca	59	2651	247 ± 459
Krstac	60	3993	352 ± 592
Bjejevica	58	4875	366 ± 312

TABLE 3.

Pearson correlation matrix for *Ostrea edulis* larvae and environmental parameters considered at four study sites in Bay of Mali Ston.

Location	Temp. S	Temp. M	Temp. B	Sal. S	Sal. M	Sal. B
Bistrina	0.495***	0.501***	0.444**	N.S.	N.S.	N.S.
Soca	0.604***	0.575***	0.604***	N.S.	N.S.	−0.484***
Krstac	0.690***	0.654***	0.501***	N.S.	−0.451**	N.S.
Bjejevica	0.423**	0.369*	N.S.	N.S.	N.S.	N.S.

Temp. S. = surface temperature; Temp. M. = mid water column temperature; Temp. B. = temperature one meter above the bottom; Sal. S. = surface salinity; Sal. M. = mid water column salinity; Sal. B. = salinity one meter above the bottom. Level of significance: * $0.01 < p < 0.05$; ** $0.001 < p < 0.01$; *** $p < 0.001$; N.S. = non-significant.

from 15°C to 19°C, and maximum larvae number was noted at temperature between 24°C and 26°C.

The rise in mean temperature above 21°C in Mali Ston bay in early June correlates with the increased number of oyster larvae: over 500 individuals per m^3 at all sites from mid-June of 2000. The number of larvae reached the highest level in July, when the mean temperature of the water column was between 20°C and 24°C. When the mean water column temperature dropped to values between 12°C and 18°C, larval presence became negligible. The new spawning cycle and increase in larval numbers began again in April of 2001, correlating with the temperature increase above 14°C. This high dependence of initiation and duration of gametogenesis in *Ostrea edulis* on water temperature was already noted in many previous studies (e.g., Hrs–Brenko 1971, Sastry 1975, Mann 1979, Wilson & Simons 1985). In the summer of 2001 larval distribution was different than in summer of 2000. At sites Soca, Krstac, and Bjejevica lower number of larvae was collected in 2001 than in 2000. At site Bistrina, highest number of oyster larvae was observed in August 2001 while in other months larval distribution was very similar with respect to two observation years.

According to Morović and Šimunović (1980), maximum number of flat oyster larvae in Mali Ston bay in 1976 occurred in the first days of September and the minimum in August, while high numbers were also found in June and July, pointing out between year variation in distribution of oyster larvae.

Maximal number of larvae noted in this study is significantly different than values noted by Morović and Šimunović (1980). These authors noted over 75 thousand oyster larvae in 1 m^3 at site Krstac in May of 1974, while maximal number of larvae noted in this study was only 5029 ind/m^3. Observed differences might be attributed to reduced size of the flat oyster brood stock, water temperature, food availability, and water quality. Value obtained by Morović and Šimunović (1980) is also high when compared with the maximal number of oyster larvae recorded by Hrs–Brenko (1977) at several locations in Northern Adriatic where only up to 1000 ind/m^3 were recorded. Further on, Cano et al. (1997) also recorded a significantly smaller number of flat oyster larvae (6,000 ind/m^3) at one location in Spain.

In relation to environmental parameters recorded in this study, there was no apparent difference between year-temperature and salinity in the period between May and September that could explain the difference in numbers of flat oyster larvae observed in 2000 and 2001. In both years salinity in the bay was lower in comparison to open Adriatic waters (Vukadin 1981) and was within optimal values for larval survival. However, since flat oysters are filter feeders and phytoplankton is generally known to be the main source of nutrition for bivalve filter feeders (Dame 1996), possible explanation might be related to difference in distribution of phytoplankton biomass. According to Ninčević (unpublished data), phytoplankton biomass in Mali Ston Bay was lower in summer of 2001 than in summer of 2000. Another possible explanation for the reduced number of larvae in 2001 might be presence of unidentified species of ctenophore within the bay (Bolotin 2002, unpublished data). According to Dame (1996), ctenophores and jellyfish are the most commonly reported bivalve larval predators. However, at this point it is not possible to explain why the number of larvae decreased at three study sites in 2001 but did not decreased at site Bistrina.

To provide an explanation for observed differences in larvae distribution, further research should be conducted at all study sites, measure other environmental parameters, such as chlorophyl *a* and nutrients, as well as identification of ctenophore species. Observation of gonad maturation and settlement success should also be included to obtain a complete picture on reproductive effort and settlement of European flat oyster in this unique bay and to improve the aquaculture production.

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OBSERVATIONS ON THE EGG CAPSULES AND HATCHLINGS OF THE KNOBBED WHELK, *BUSYCON CARICA* (GMELIN, 1791) IN COASTAL GEORGIA

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ABSTRACT Despite the commercial importance and abundance of knobbed whelks on the east coast of the United States, very little life history information exists for juveniles in the egg capsule and the first few critical weeks post hatching. As a result, various intertidal sandy-mud flats around Wassaw Sound, Georgia were monitored from early April 2001 when copulating and egg-laying whelks were first observed to early June 2001 when most egg capsules had opened and the hatchlings had begun to disperse. During this spawning event, egg strings were collected, the length of the embryonated (mean = 339.86 mm) and unembryonated (mean = 199.67 mm) portions were measured, and the number of embryonated capsules per string (mean = 89.76) was determined. The middle section of the embryonated portion comprised the largest capsules (mean height = 22.12 mm, mean width = 31.11 mm, mean thickness = 6.03 mm) and had the highest number of encapsulated embryos with an average of 46 (up to 99). Egg strings were also hatched in the laboratory with the intention of examining the effects of diet (meat, macroalgae, and microalgae) and temperature (20, 25, and 30°C) on the growth and survival rates of newly hatched juveniles. Optimum growth and survival conditions resulted in those reared on a meat diet in aquaria maintained at 20°C.

KEY WORDS: knobbed whelk, *Busycon carica*, egg capsules, hatchlings

INTRODUCTION

The knobbed whelk, *Busycon carica* (Gmelin 1791), is a common prosobranch gastropod (Family Melongenidae) along the east coast of the United States from Cape Cod, Massachusetts to Cape Canaveral, Florida on sandy mud, in shallow water to 4.6 m (Rehder 1981). The reproductive cycle of *B. carica* has not been determined in Georgia, however, Walker (1988) reported spawning in the spring and fall based on the appearance of egg strings and copulating whelks. In North Carolina (Magalhaes 1948) also found biannual spawning between May to June and September to November. In South Carolina, *B. carica* was reported to reproduce in the fall (October to November) and spring (April) (Stevens 1976). Stevens (1976) suggested that the fall reproductive period was more important than the spring based on a comparison of body weights, digestive gland weights, and ovarian indices. Weinheimer (1982) reported that *B. carica* from South Carolina are not restricted to one or two breeding seasons, but reproduce from September to May. In Virginia, spawning was reported between mid August and November (Castagna & Kraeuter 1994).

Fertilization occurs internally in *Busycon* (Weinheimer 1982). Males are equipped with a large muscular penis, and the female oviduct is composed of a series of chambers and glands. Fertilized eggs are surrounded by a transparent viscous mass of albumin and are laid in protective disciform egg capsules arranged on a helicoid string (D'Asaro 1997). Several whelk species are reported to migrate to favorable egg laying sites during the breeding season (Power & Keegan 2001). In coastal Georgia, egg strings are commonly encountered near the low water mark on intertidal sandy mud flats. Ram et al. (1982) determined an egg deposition rate of 1.9 ± 1.5 h/capsule in the laboratory. According to Magalhaes (1948) long strings are usually laid over several days.

Egg strings in Virginia were reported to contain between 42 and 121 capsules (Castagna & Kraeuter 1994). The average embryos per egg capsule in Chesapeake Bay ranged from 46 to 67 (Harasewych 1982). Castagna and Kraeuter (1994) determined the average number of embryos per capsule varied according to position on the egg string, averaging 52 in the middle section and decreasing in the beginning and terminal portions. The female always begins by anchoring one end of the egg string deep into the substrate by several structurally modified capsules to prevent the string from being washed ashore (Magalhaes 1948). Ram (1977) and Ram et al. (1982) induced spawning in the knobbed whelk by stimulation with nervous system extractions. Spawning always began with a number of unembryonated capsules, a choice dictated by genome. It has not been determined how the female adjusts the number of anchoring capsules. Studies have found that the egg capsules in the anchoring portion are typically thinner walled and closely spaced (Harasewych 1982). The number of unembryonated capsules in this anchoring portion has been reported to range from 8 to 22 (Castagna & Kraeuter 1994), from 13 to 17 (Ram et al. 1982), and to average 21 (D'Asaro 1997).

The young pass the veliger stage within the egg capsule and emerge as fully developed miniature "adults." In Virginia, Castagna and Kraeuter (1994) found that egg strings deposited in the fall hatched from mid-March through early-May in the following year. The average size for hatchling juveniles is ~4 mm in length (Magalhaes 1948, Castagna & Kraeuter 1994).

Most studies that have been conducted were on adult whelks (Morton 1987). Very little information exists about the ecology of post hatchling knobbed whelks. As an adult, the knobbed whelk is a predator of bivalve species. There has been no research published on the habitat and nutritional requirements of juveniles in the wild. In Virginia, juvenile whelks were hatched and raised inside polypropylene bags in the laboratory to estimate post hatching growth rates (Kraeuter et al. 1989). There was limited information provided on rearing conditions (i.e., the temperature and diet). Length measurements were taken five times during the first year and one to three times per year for the subsequent nine years. The juveniles were fed live clams when they reached ~20 mm in length. Growth

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rates averaged 13.2 mm/y over the ten-year period, however juveniles grew from 4 to 36.5 mm (32.5 mm/y) in the first year. The only other published growth study on juvenile knobbed whelks was by Magalhaes (1948). She maintained juveniles in the laboratory for one month only and observed one individual added 1.5 mm in length in 22 days. This experiment attempts to determine the optimum food source for juvenile knobbed whelks with respect to growth and survival rates. Temperature is also considered since warmer temperatures foster accelerated growth.

MATERIALS AND METHODS

Weekly field trips to intertidal sandy-mud flats around Wassaw Sound, Georgia were carried out from April 2001, when copulating and egg-laying whelks were first observed, to early June 2001 when most egg capsules had opened and the hatchlings had begun to disperse. On each occasion, notes were taken on the behavior (i.e., copulating, depositing eggs, and feeding). If a whelk was in the process of laying, an estimate was made of the number of capsules already produced, her position was taken with a hand held global positioning system, and the location was physically marked with a metal stake. Once the strings were completed, the number of egg capsules per string was determined (excluding the anchoring section). The condition of the egg capsules was also monitored regularly to determine the larval developmental period in the field.

At the end of May 2001, egg strings were collected and returned to the Shellfish Research & Aquaculture Laboratory on Skidaway Island. Ten strings were selected to examine the capsule morphometrics, length, width, and thickness, as defined by D'Asaro (1997). The length of unembryonated and embryonated sections of each string was determined with a fish measuring board. Each string was then divided into four portions: the unembryonated anchor, and an inner, middle, and outer portion of the embryonated section. Five capsules were randomly selected from each section, the dimensions were measured and the capsules were opened to count the developing embryos.

Egg strings were also maintained at the laboratory in an open system raceway until hatching began. Hatchling juveniles were collected, measured for length, and isolated for growth rate experiments using diet and temperature variables. Three dietary treatments were chosen for the growth study: macroalgae, microalgae, and meat. Sea lettuce, *Ulva* sp., was harvested from Wassaw Sound and the Skidaway River for the macroalgae series. Benthic microalgae (species unknown) was cultured on mats in a closed system saltwater tank using nylon mesh as a substrate. The third diet consisted of chopped meat: clams, *Mercenaria mercenaria* (L.), brittle stars, *Ophiophragmus wurdemani* (Lyman), bloodworms, *Glycera dibranchiata* (Ehlers), and mussels, *Geukensia demissa* (Dillwyn), depending on what was available. Temperature was included in the design. At the time of hatching, water temperatures averaged 25°C (taken from the nearby Skidaway River), therefore three glass aquaria were set up in temperature controlled rooms, at 20, 25, and 30°C. For the duration of the experiment, the salinities were maintained at 35‰. Plastic jars (120.6 × 69.8 mm) were used to contain the juveniles. To help water circulation, thirty 2-mm holes were drilled in the top and bottom of the jars and 15 on each side. There were three replicates for each combination of diet and temperature treatment resulting in 27 containers, each holding 50 haphazardly chosen hatchlings. Jars were labeled and temperatures and salinities in the aquaria were tested and maintained regularly. The water in the aquaria was aerated, and changed monthly. All the juveniles were provided with an abundance of food, which was cleaned out and replaced approximately every three days. Juvenile shell length (to the nearest 0.01 mm) and body weight (to the nearest 0.0001 g) were recorded every two weeks using an electronic calipers and balance. The animals were first blotted dry by placing on a paper towel. Dead juveniles were removed and counted to determine mortality rates. The experiment was conducted over an eight-week period.

Statistical analysis of growth and survival data was performed using SAS (SAS Institute, Inc., Cary, NC). The two main effects (temperature and diet) and a nested effect (containers) on growth (length and weight) were analyzed by performing a nested factorial using GLM (General Linear Model) SAS. Survival data were arcsine transformed prior to analysis [$X' = \text{arcsine} \sqrt{(x/100)}$]. Two main effects were again analyzed (temperature and diet), with repeated measures of both factors using a GLM (SAS). An α level of $P = 0.05$ was used to determine if significant difference in mean juvenile length, weight, and survival occurred between different diets and temperature treatments.

At the termination of the eight-week laboratory growth experiment, we returned to the sites of egg deposition in Wassaw Sound to search for juveniles that had presumably dispersed on hatching. A circular area with a diameter of approximately 4 m was searched around approximately twenty marked egg cases. The goal was to obtain juveniles to compare the growth obtained in the laboratory with growth in their natural habitat. Methods for searching consisted of scooping up handfuls of the surface layer of sediment at low tide and sieving on a 1.40 mm screen. Submerged sites at lower tidal levels were searched by gently feeling the surface of the sediment.

RESULTS

The abundance of copulating whelks peaked in early April 2001 and was observed with decreasing frequency throughout the rest of the month. This period coincided with a dramatic increase in local water temperatures (Fig. 1). Often copulation was observed even while the female was in the process of depositing egg capsules (Fig. 2). Typically the larger female lay partially buried in the substrate and was surrounded by several smaller sized males (average of 3, but up to 9 observed). A large variation in the rate of egg deposition was observed: 1 to 23 capsules per day (7.69 ± 2.12, mean ± SE). This is a coarse estimate since the egg strings were observed once per week. An approximate total area of 2,500 m^2 was searched for whelks and eggs. By the end of April, most egg strings were complete and whelks were less abundant on the sandy-mud flats. From a total of 51 complete egg strings (0.02 m^2), the number of embryonated egg capsules per egg string averaged 89.76 ± 3.61, and ranged from 40 to 157. Egg capsules began to soften and deteriorate quickly, the escape aperture was open on all capsules examined on June 4, 2001 (approximately six weeks later). While all capsules were open at this time, only one third of those examined were empty, the remainder still held the fully developed hatchlings.

The average length of the unembryonated section for the ten egg strings selected for morphometric analysis was 199.67 ± 15.21 mm (ranged from 130 to 275 mm). For the embryonated section the average length was 339.86 ± 26.17 mm (ranged from 245 to 450 mm). The number of unembryonated capsules per egg string ranged from 6 to 26 (mean = 13.67 ± 1.97). The number of developing embryos per egg capsule ranged from 0 to 99. The

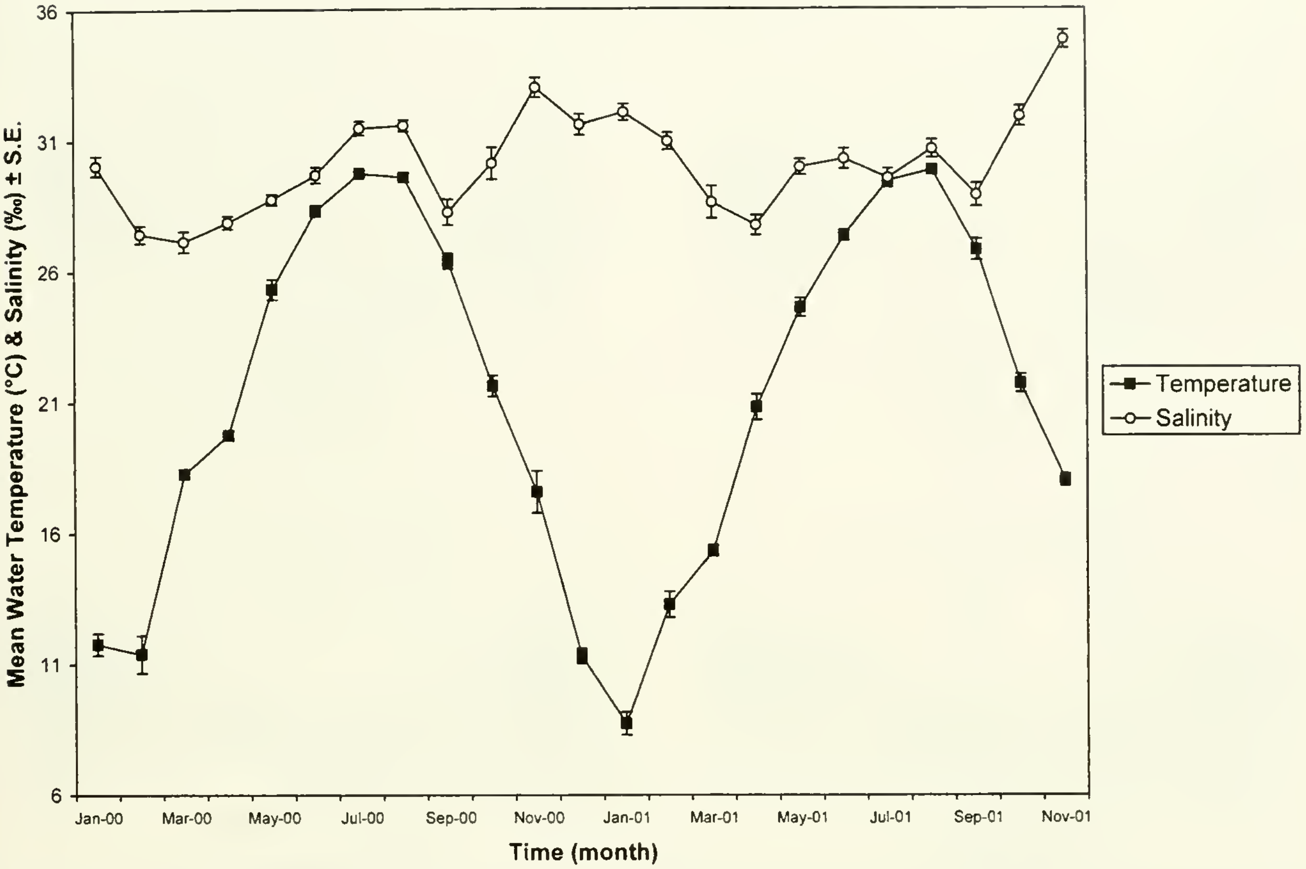

Figure 1. The mean monthly water temperature and salinity of the Skidaway River, Georgia from January 2000 to November 2001 (vertical bars indicate ± 1 standard error from the mean).

middle portion of the embryonated section of the egg strings had longer, thicker, and wider capsules with a higher number of embryos per egg capsule. The inner and outer portions averaged 23.41 ± 2.77 embryos per capsule, with capsule dimensions of 20.20 ± 0.47 mm in length, 27.26 ± 0.69 mm in width and 5.66 ± 0.09 mm in thickness. The middle portions had an average of 45.86 ± 3.95 embryos per capsule, with capsule dimensions of 22.12 ± 0.41 mm in length, 31.11 ± 0.56 mm in width, and 6.03 ± 0.14 mm in thickness.

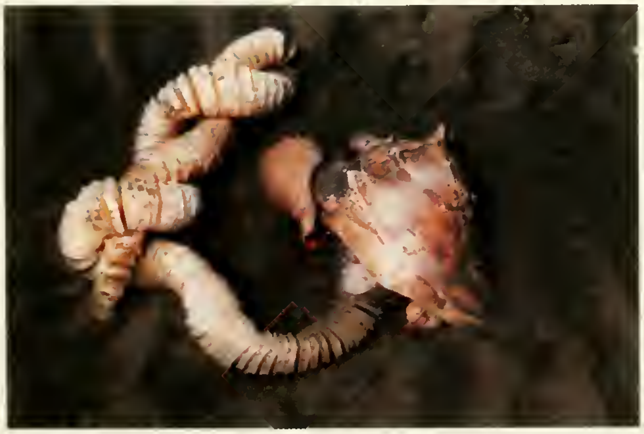

Figure 2. Small male knobbed whelk, *Busycon carica*, copulating with a larger female that is also in the process of depositing an egg string.

In the laboratory, the egg strings maintained in raceways also began to hatch in early June 2001. On hatching the juveniles averaged 5.60 ± 0.02 mm in shell length and 23.10 ± 0.24 mg in weight (n = 540). These were haphazardly assigned to the three different diet (meat, algae and diatoms) and temperature (20, 25, and 30°C) treatments. Figure 3 and Figure 4, present the growth rates of these juveniles for a period of eight weeks, in terms of shell length (mm) and weight (mg), respectively. Figure 5 presents the percentage survival rate at the termination of the experiment for each temperature and diet treatment. Growth in terms of length and weight was significantly affected by diet (P = 0.003 and 0.001, respectively). Duncan's Multiple Range Test revealed no difference in the growth of whelks fed on the macroalgae and microalgae diets (macroalgae: mean length 5.67 mm, mean weight 21.29 mg; microalgae: mean length 5.65 mm, mean weight 21.47 mg), however those fed on the meat diet were significantly larger (mean length 6.35 mm, mean weight 27.81 mg). Survival was not significantly affected by diet (P = 0.2084). There was a significant (P = 0.001) temperature effect on survival. Duncan's Multiple Range Test revealed no difference in survival at temperatures 25°C and 30°C (15.31% and 7.73%), but a much greater survival rate occurred at 20°C (69.50%). Percent survival rates decreased with temperature increase for all dietary treatments (Fig. 5).

It proved very difficult to locate juvenile knobbed whelks on the intertidal flats in August and September. The best approach proved to be feeling the surface layer of sediment when a shallow depth of water covered the flats. Even with this approach only nine

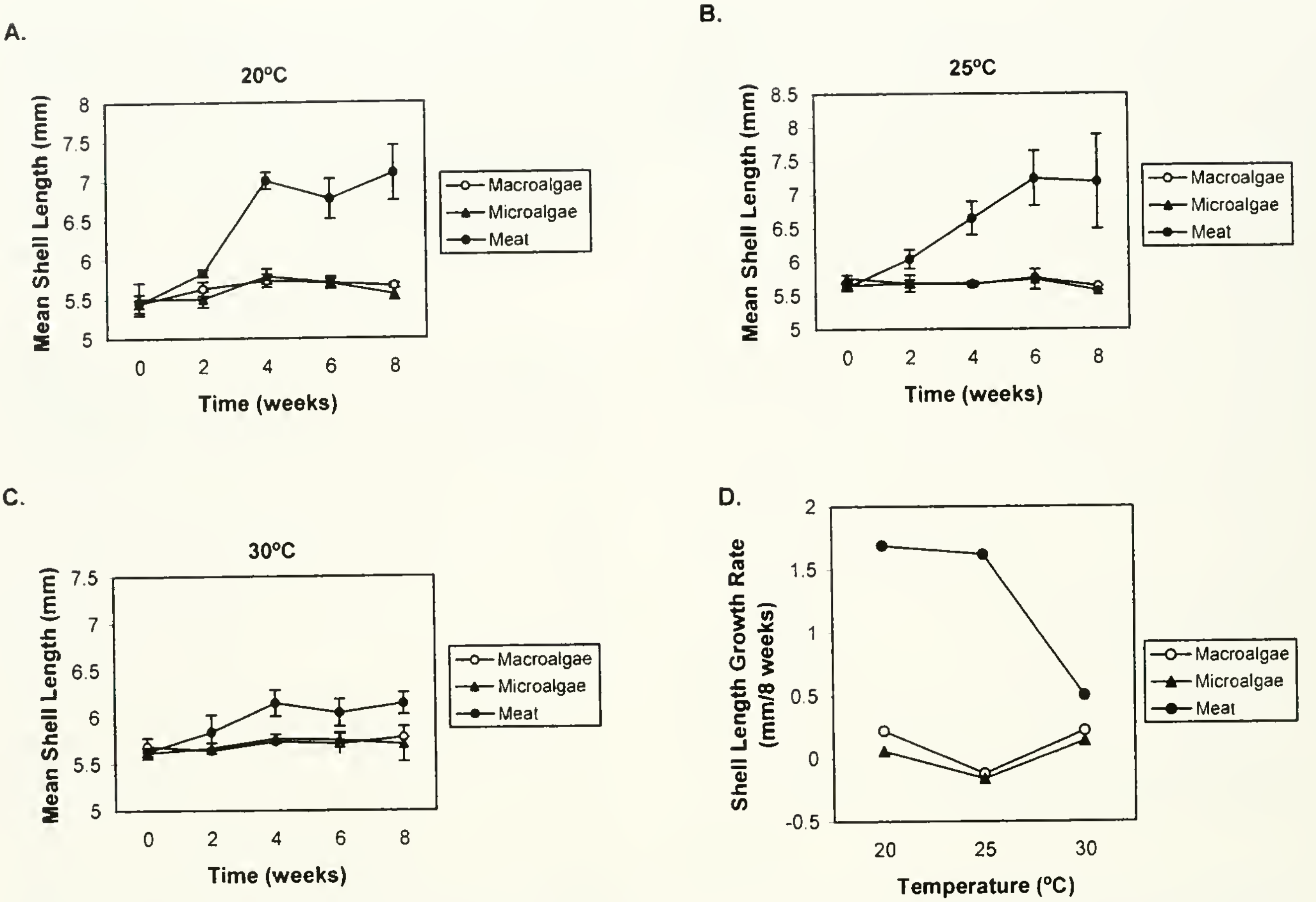

Figure 3. A–C. The mean shell length (mm ± SE) of knobbed whelks reared in the laboratory on macroalgae, microalgae and meat diets at temperatures of 20, 25, and 30°C, respectively, for eight weeks post hatching. D: The overall growth rate (shell length, mm) per diet and temperature treatment over eight weeks.

juveniles were recovered (Table 1), from a total area of approximately 250 m^2 (~0.04 m^{-2}). The wild juveniles located, averaged a much larger size (mean shell length = 21.2 mm) than those reared in our laboratory treatments.

DISCUSSION

Copulation occurred throughout April 2001 during which average seawater temperatures increased rapidly from a mean of 15.3°C in March 2001 to 20.8°C in April 2001. Walker (1988) observed six pairs of copulating whelks in Wassaw Sound in March 1980 (mean water temperature 14.3°C). We do not believe that whelks commenced spawning earlier than April in 2001, since intertidal flats were thoroughly searched throughout March 2001 as part of an ongoing whelk mark and recapture study. Another spawning event may take place in Georgia, when seawater temperatures drop to a similar range in the fall. Along the eastern coast of the United States, the reported spawning period of the knobbed whelk is progressively later as one moves northwards: March to April in Georgia (Walker 1988, present study) and South Carolina (Stevens 1976), May to June in North Carolina (Magalhaes 1948), and mid August to November in Virginia (Castagna & Kraeuter 1994).

In the natural environment, the timing of the reproductive period is usually synchronized with conditions that are most favorable for maximization of juvenile survival and continuity of the species (Sastry 1986). Gastropod egg capsules are preyed on by fish, crustaceans, other prosobranchs, polychaete worms, and even shorebirds (Penchaszadeh et al. 2000). Predator activity typically increases in the warm temperatures of the early summer months. However, these months also permit a more rapid embryonic development of encapsulated larvae that may reduce overall losses. A rapid developmental period of six weeks was observed in the present study, which concurs with findings for the pear whelk in Florida (6 wk: Kent 1983). This is however, significantly lower than the six-month period (mid summer to following spring) reported for the same species in the cooler northern waters of Virginia (Castagna & Kraeuter 1994). Embryonic development of encapsulated knobbed whelks has been noted to temporarily arrest and resume after egg capsules were refrigerated for four days at 0°C (Dr. DeEtte Walker, pers. comm., UGA Dept. of Genetics). According to Hain and Arnaud (1992) embryonic development for related species can be up to thirty times longer in Antarctic than in tropical waters.

Although most egg capsules in the field were open in early June, two-thirds of all capsules examined still held hatchlings. A gradual dispersal has also been noted for juveniles of the lightning whelk, with many found within and between the opened capsules (D'Asaro 1997). Juveniles may use the structure as a refugium. Egg strings also provide a substratum for algae, diatoms and even other invertebrates in their juvenile stages and could therefore also serve as a food source for newly hatched whelks.

In this study the hatchlings (n = 540) averaged 5.60 mm in

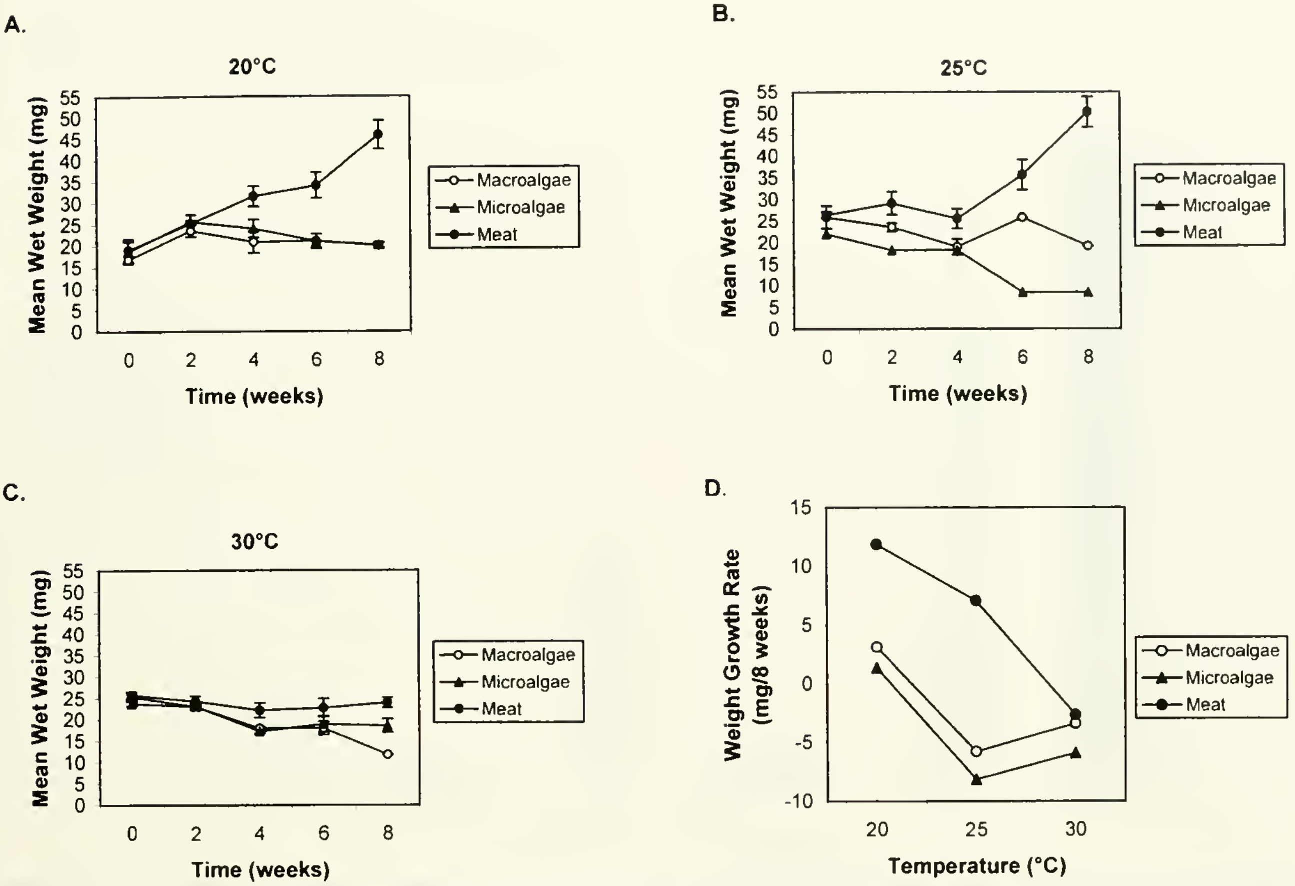

Figure 4. A–C. The mean wet weight (mg ± SE) of knobbed whelks reared in the laboratory on macroalgae, microalgae and meat diets at temperatures of 20, 25, and 30°C, respectively, for eight weeks post hatching. D: The overall growth rate (wet weight, mg) per diet and temperature treatment over eight weeks.

shell length (ranged from 3.31 to 6.96 mm). This is larger than the 4 mm previously recorded for whelks in North Carolina (Magalhaes 1948) and Virginia (Castagna & Kraeuter 1994). Similarly to residence time within the capsules, the size at hatching may also be influenced by environmental factors including water temperature.

In this study, there was a large range in the number of encapsulated embryos per egg capsule (ranging from 0–99). The middle section of the embryonated portion comprised the largest sized capsules (mean length 22.12 mm, mean width 31.11 mm, mean thickness 6.03 mm) and contained the highest number of embryos (mean = 46). The inner and outer portion capsules contained about half the number of embryos (mean = 23) and were smaller (averaging 21.45 mm and 18.96 mm in length, 29.27 mm and 25.26 mm in width, and 5.60 mm and 5.71 mm in thickness, respectively). The middle section of egg strings in Virginia was reported to contain a slightly higher average of 52 embryos, but the number of embryos in the outer capsules was not provided (Castagna & Kraeuter 1994). In shape, egg capsules were taken to roughly approximate a prism on an elliptic base. The following formula was used to obtain the mean number of embryos per unit of capsule space for the middle, inner and outer portions of egg strings = (# embryos)(4) / (π) (length) (width) (thickness). Embryos were packed closer in the middle capsules (14.07 cm^{-3}) than in either the inner (8.45 cm^{-3}) and outer (10.95 cm^{-3}) portions.

There is typically a wide variation in the number of embryos occurring in egg capsules within the family Melongenidae, e.g., the lightning whelk is reported to have between 25 and 200 (Perry & Schwengel 1955), the channeled whelk between 73 and 130 (Conklin 1907), and the pear whelk between 9 and 50 (Odhner 1927, D'Asaro 1997). The number of knobbed whelk egg capsules per egg string averaged 90 and ranged from 40 to 157, which is similar to the range 42–121 reported from Virginia (Castagna & Kraeuter 1994). This species appears to have more egg capsules per egg string than the channeled whelk (up to 70: Conklin 1907), but less than the lightning whelk (up to 175: Perry & Schwengel 1955), which could be expected in relation to size (shell length) differences between the species (channeled: <19 cm; knobbed: <22.9 cm; lightning: <40.6 cm, Rehder 1981). Miloslavich and Dufresne (1994) have directly related the size (shell length) of female *Buccinum undatum* whelks depositing egg capsules to the number of capsules, the size of the capsules, and to the number of developing embryos within. According to Spight and Emlen (1976), the fecundity of an organism increases with an increase in size and age.

From the growth and survival data presented for juveniles at the laboratory, it is clear that a carnivorous diet is optimal post hatching. Cannibalism at this stage was also observed in the treatments provided with macroalgae and microalgae diets. Morton (1987) suggested that juveniles of *Hemifusus tuba* might consume one another outside of the capsules to enhance the survival of an individual in the transition time needed to discover natural prey. Survival rates were much reduced at 25°C and 30°C indicating that the temperature was approaching the upper physiologic tolerance. Most tropical marine organisms cannot survive at temperatures

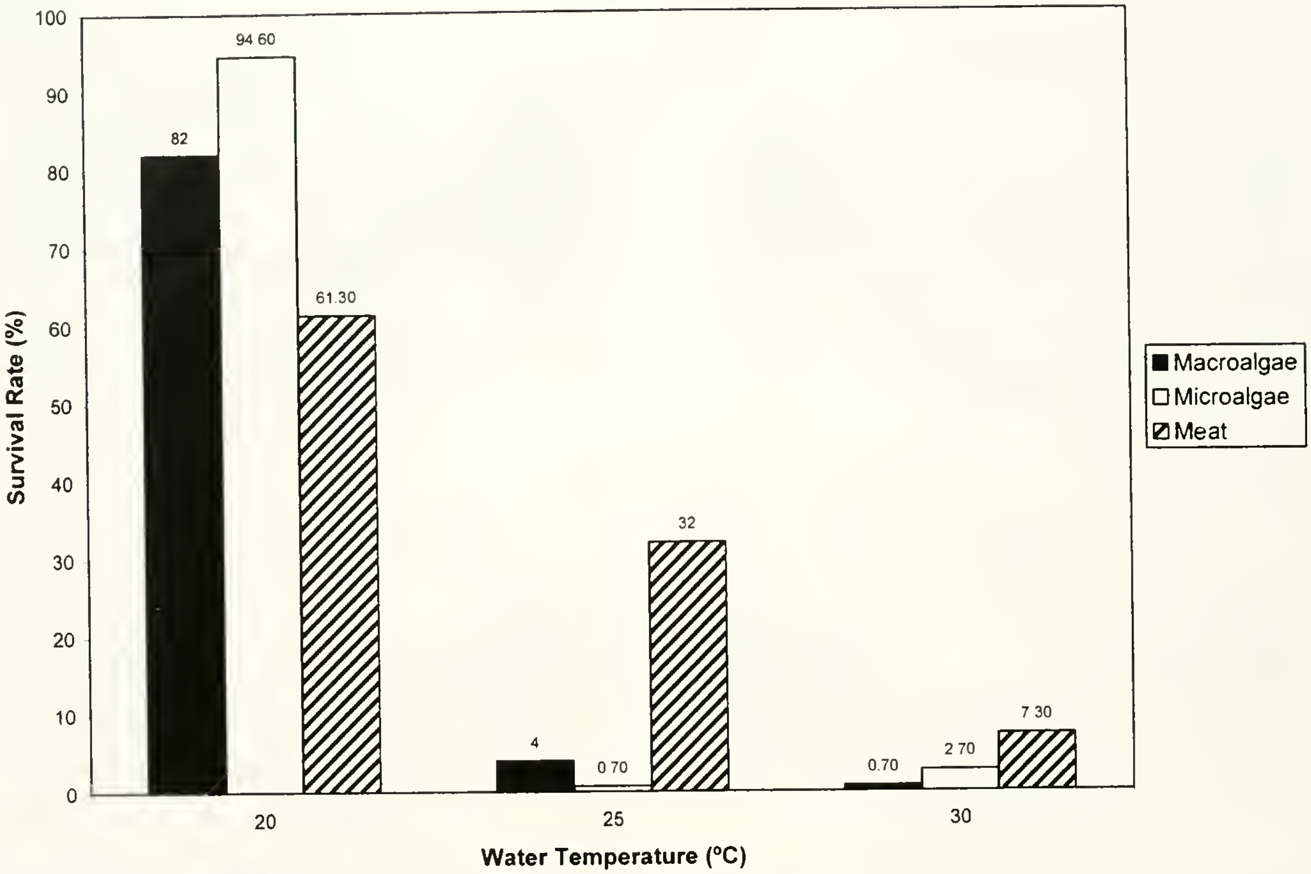

Figure 5. The percentage survival rate of knobbed whelks reared in the laboratory on macroalgae, microalgae and meat diets at temperatures of 20, 25, and 30°C, respectively, for eight weeks post hatching.

higher than 35°C (Kinne 1963). Juveniles that hatched in June 2001 would have experienced temperatures up to 30°C on the intertidal flats, but would also have had the ability to bury themselves in the substrate. Chaitanawisuti and Kritsanapuntu (1998, 1997) found that the highest growth rates of hatchery reared juvenile spotted Babylon, *Babylonia areolata* (Link 1807) whelks occurred in treatments provided with sand substrate and flow through seawater, while the lowest occurred in those with no sand substrate and static seawater. In our experimental treatments, both water flow and a sand substrate were not provided and may have led to sub-optimal growth and survival rates. The lack of substrate has been shown to induce interactive energy expenditure by juvenile queen conchs (Siddal 1984).

TABLE 1.

Juvenile *Busycon carica* whelks captured on intertidal flats (previously marked sites of egg string deposition) at Wassaw Island (0.04 individuals m^2) in August/September, 2001.

Length (mm)	Width (mm)	Weight (mg)
17.6	7.5	0.2
21.1	9.4	0.4
18.8	8.3	0.3
19.7	8.8	0.4
22.7	9.7	0.6
22.5	9.7	0.6
21.8	9.8	0.5
20.2	8.8	0.4
26.3	11.5	1
Means: 21.2	9.3	0.5

Catterall and Poiner (1983) describe small juvenile colonies (area of 2–15 m in diameter) of the gastropod *Strombus luhuanus* with between 5 to 100 individuals per meter squared. These colonies may persist for up to two years and become larger in area and less dense as members grow and mature. This type of spatial segregation may also occur for juvenile knobbed whelks in the wild. It is likely that juveniles spend a large proportion of time completely buried in the sand, particularly when the tide retreats, which would explain our difficulty in locating them. We only found juveniles at the surface of the sediment after the tide had advanced over the intertidal flats. In the laboratory the largest sized juvenile at the termination of our rearing experiment (8 weeks later) was approximately 12.5 mm. The average size of the juveniles found in the wild was much larger at 21.2 mm. These wild juveniles may have resulted from the previous fall spawning event and would therefore be approximately eight months old, which resembles the annual year one growth rate of 34.5 mm reported by Kraeuter et al. (1989) in Virginia.

ACKNOWLEDGMENTS

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RAPA WHELK *RAPANA VENOSA* (VALENCIENNES, 1846) PREDATION RATES ON HARD CLAMS *MERCENARIA MERCENARIA* (LINNAEUS, 1758)

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ABSTRACT The recent discovery of adult veined rapa whelks *Rapana venosa* (Valenciennes, 1846) in the Lower Chesapeake Bay, U.S.A. offers cause for both ecological and economic concern. Adult rapa whelks are large predatory gastropods that consume bivalves including commercially valuable species such as hard clams, *Mercenaria mercenaria* (Linnaeus, 1758). Laboratory feeding experiments were used to estimate daily consumption rates of two sizes of whelks feeding on two size classes of hard clams. Large rapa whelks (shell length, SL >101 mm) are capable of consuming up to 2.7 g wet weight of clam tissue daily, equivalent to 0.8% of their body weight. Small whelks (60–100 mm SL) ingest an average of 3.6% of their body weight per day.

KEY WORDS: rapa whelk, *Rapana venosa*, hard clam, *Mercenaria mercenaria*, predation, Chesapeake Bay

INTRODUCTION

The veined rapa whelk, *Rapana venosa*, (Valenciennes 1846; Gastropoda: Muricidae) was discovered in the Hampton Roads region of the Chesapeake Bay, USA, in the summer of 1998 (Harding & Mann 1999). The species is native to the Sea of Japan, Yellow Sea, East China Sea and the Bohai Sea (Tsi et al. 1983) but was introduced to the Black Sea in the 1940's (Drapkin 1953) and has since spread to the Aegean Sea (Koutsoubas & Voultsiadou-Koukoura 1990) and the Adriatic Sea (Ghisotti 1974). Recently a female specimen together with egg masses was found in the Rio del Plata, an estuary between Argentina and Uruguay in South America (Pastorino et al. 2000).

The predatory activity of rapa whelks in the Black Sea is considered by Zolotarev (1996) to be the prime reason for the decimation of native Black Sea oyster, scallop and mussel populations. Given this history, there is both ecological and economic concern for the future of shellfish stocks in the Hampton Roads region of the Chesapeake Bay. Hard clam, *Mercenaria mercenaria*, populations are of particular concern in that the Hampton Roads region supports a substantial local commercial hard clam fishery. Laboratory feeding experiments were used to quantify daily feeding rates for two size classes of adult rapa whelks offered hard clams.

MATERIALS AND METHODS

Twelve adult rapa whelks, collected from the lower Chesapeake Bay, USA, between March and May 2000, were separated into two different size classes: small (60–100 mm shell length (SL), the maximum dimension from the apex of the spire to the end of the siphonal canal) and large (101–160 mm SL). Rapa whelks were maintained individually in 60 × 40 × 30 cm plastic net cages submerged in a shallow flume (250 × 70 × 30 cm) with a constant flow of unfiltered York River water as described in Savini (2001). The bottom of each cage was covered with 15 cm of clean hard sand substrate. Rapa whelks were starved for 48 hours prior to the addition of hard clams (prey) to each enclosure. Each whelk was given five small (50–70 mm maximum dimension, hereafter shell height, SH) and five large (71–100 mm SH) hard clams as potential prey. Clams were arranged in the experimental cages so that whelks initially had the same probability of encountering each size of prey (i.e., whelks at the center of a circle with clams of alternating size classes spaced evenly around the circumference).

The experimental flume was covered with a fixed plastic net to prevent escape of the whelks and maintained on a 14/10 h natural light/dark schedule. Water temperature and salinity data were collected daily from the flume for the 38 day duration of the experiment (June 11 to July 18, 2000). Experimental cages were examined daily and the empty shells of all prey were removed and measured. Clams that were consumed were replaced daily with clams of similar dimensions thus maintaining constant prey availability.

A size range (30–100 mm SH) of fifty hard clams was selected from the pool of potential prey items and used to create size-weight relationships for the prey. Individual hard clams were measured (SH, mm) and weighed (g) prior to the removal of soft tissue. Clam soft tissue was weighed (wet weight, g) to the nearest 0.1 g.

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 analyses. When appropriate, Fisher's test was used for post-hoc multiple comparisons.

Feeding Rates

The numbers of clams consumed by each size class of whelks during the entire experimental period were compared using a one-way ANOVA with individual whelk as a factor. The number of clams consumed satisfied the assumptions of homogeneity of variance and normality without transformation. Daily feeding rates were calculated for each whelk by dividing the total number of clams consumed during the experimental period by the duration of the experiment (38 days).

Consumption on a Weight-Weight Basis

Clam wet and dry tissue equivalents consumed by whelks were compared using a two-way ANOVA with whelk size class and individual whelk as factors. Tissue equivalent data satisfied the assumptions of homogeneity of variance and normality without transformation.

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RESULTS

Average water temperature during the experimental period was 26°C (±1°C). Average salinity was 21 ppt (±1 ppt). During the 38 day experiment, the six small whelks ate a total of 19 clams while the six large whelks consumed a total of 15 clams. There was no significant difference in the total number of clams eaten by small and large whelks (ANOVA, $F = 0.67$; $P > 0.05$). Small rapa whelks did not show any clear size preference when offered hard clams as prey (Fig. 1) although small whelks consumed a total of 11 small clams and 8 large clams. It should be noted that 5 of the 11 small clams were consumed by one individual. Large rapa whelks consumed large clams more frequently than small clams (4 small clams vs. 11 large clams; see Fig. 1).

Clam Size-Weight Relationships

Clam tissue wet and dry weights were plotted in relation to shell height and used to calculate shell height-wet tissue weight relationships for hard clam prey. These relationships were used to calculate wet tissue equivalents for each clam consumed by an individual whelk and were described with the following equations:

$$\mathrm{Log}\ (\mathrm{CWWgt}) = -3.93 + 2.77 * (\mathrm{Log\ SH}); \qquad R^2 = 0.96$$

where CWWgt is clam tissue wet weight (g) and SH is clam shell height.

Rapa Whelk Size-Weight Relationships

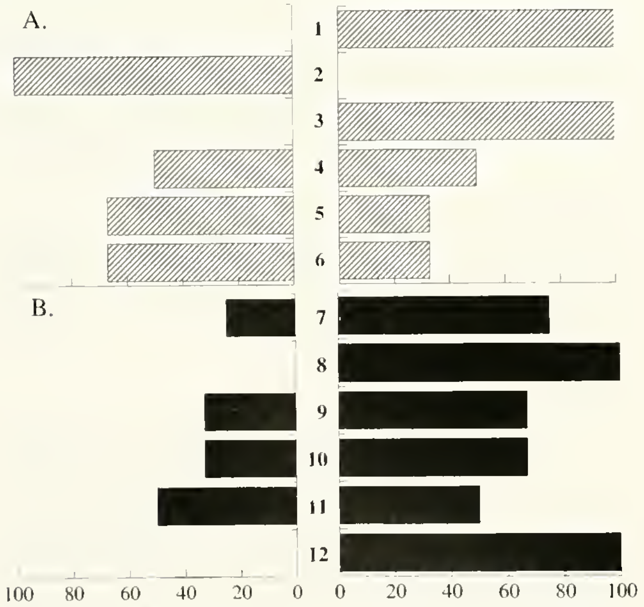

Figure 1. Percentage of small (50–70 mm SH) and large (71–100 mm SH) hard clams eaten in each experimental cage by each rapa whelk during the whole experimental period (June 11 to July 18, 2000). A) N. 1–6 = small whelks (60–100 mm SL), B) N. 7–12 = large whelks (101–160 mm SL).

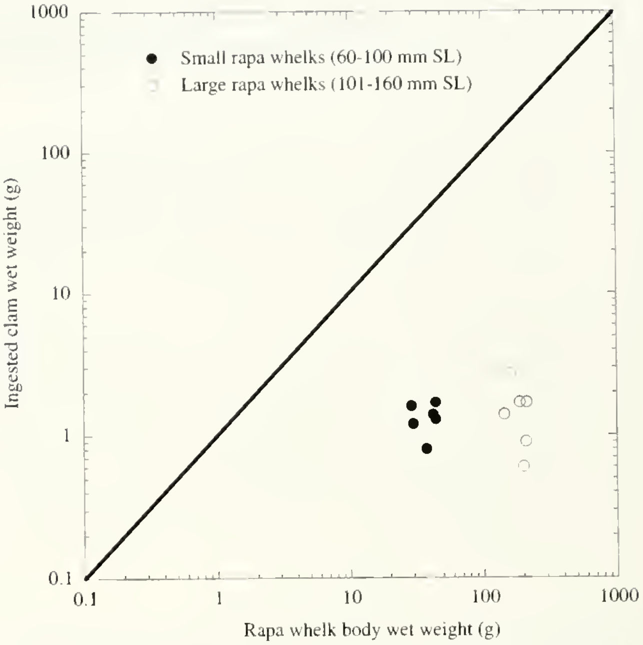

Figure 2. Daily ingested clam wet weight (g) in relation to rapa wet weight (g) observed in laboratory feeding experiments during June and July, 2000. The solid diagonal line represents clam consumption equal to the body weight of the predator (whelk) or a 1:1 consumption relationship on a prey wet weight: predator wet weight basis. Points above the line indicate prey consumption at a rate greater than one while points below the line indicate daily consumption rates less than the body weight of the predator.

Rapa whelk tissue wet weight was plotted in relation to shell length and used to calculate a shell length–wet tissue weight relationship for whelk predators. This relationship is based on 150 animals (80–165 mm SL) collected from lower Chesapeake Bay, USA between October 1999 and July 2000 (Harding and Mann, unpublished data):

$$\mathrm{WWWgt} = 6.4908 * e(0.0229 * \mathrm{SL}), \qquad R^2 = 0.69$$

where WWWgt is whelk tissue wet weight (g) and SL is whelk shell length (mm).

On the basis of tissue wet weight, large whelks consumed significantly more prey flesh tissue than small whelks (ANOVA, $F = 4.45$, $P < 0.05$). Individual small whelks ate proportionately more hard clam tissue on a clam wet weight: whelk wet weight basis than large whelks (Fig. 2). Maximum daily clam consumption rates of 5.6% of body wet weight were recorded for small whelks as compared to 1.6% of body wet weight for large whelks.

DISCUSSION

Large rapa whelks (101–160 mm SL) are able to consume up to 2.7 grams of clam tissue (wet weight) per day or 0.8% of their body weight per day at water temperatures of approximately 26°C. In contrast, small rapa whelks (60–100 mm SL) ingested an average 3.6% of their body weight every day, which is more than four times that observed for larger rapa whelks at similar water temperatures on a weight-specific basis. Edwards and Huebner (1977) suggest that temperature affects feeding rate in the moon snail *Polinices* by increasing predators' metabolic rate, and thus the requirement for a larger amount of food. The present investigation was conducted during warmer months and is probably indicative of the maximum feeding activity of rapa whelks. There is considerable variation in reported ingestion rates for predatory gastropods with values up to 25% of its body weight per day reported for the moon snail, *Polinices duplicatus* (Thorson 1971).

The hard clam fishery in the lower Chesapeake Bay is already in decline. Hard clam landings during 1999 were less than 10% of landings during 1973 (Virginia Marine Resources Commission, Newport News, VA). The observed decline in hard clam stocks may be related to increased anthropogenic impacts on the Chesapeake Bay ecosystem in the past 20 years including overfishing, water pollution and disease. Habitat changes are considered the major threats to estuarine ecosystem (Smith et al. 1999). The superimposition of a novel invading predator on this already stressed population has clear ecological and economic implications.

Virnstein (1977), found that particularly in Chesapeake Bay, densities of infaunal species are not controlled by competitive interactions for food or space but mainly by the action of predators. If the introduction of *Rapana venosa* into the lower Chesapeake Bay results in a large scale successful invasion, rapa whelks could have a serious negative impact on the density and distribution of the native hard clam population in the lower Chesapeake Bay. At this time we do not have a good estimate of the resident population of rapa whelks in the Chesapeake Bay but is possible to use our data for a hypothetical calculation to estimate potential impact of the whelk on the clam population. The rapa whelk distribution in the Chesapeake Bay, which extends from the mouth of the Rappahannock River in the North, to the Chesapeake Bay Bridge tunnel in the southeast and to the Lafayette River in the south (Harding & Mann 1999, Mann & Harding 2000), is within the historic distribution of *M. mercenaria* (Roegner & Mann 1991). The 1999 summer fishing season for hard clams in the lower Chesapeake Bay produced a harvest of 27388 kg or approximately 3,040,000 individual clams. Based on the predation rates observed in this study, a population of 1000 rapa whelks in the lower Bay could reduce this yield by between 0.3 to 0.9%.

ACKNOWLEDGMENTS

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ECONOMIC ANALYSIS OF A PILOT COMMERCIAL HATCHERY-BASED OPERATION FOR SPOTTED BABYLON, *BABYLONIA AREOLATA* LINK 1807, JUVENILES IN THAILAND

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ABSTRACT An economic analysis of constructing and operating a pilot hatchery and nursery for the commercial production of spotted babylon, *Babylonia areolota*, juveniles in Thailand was presented on the basis of proven practical techniques and published data from larval culture trials. The total initial investment requirement for the construction of a spotted babylon hatchery producing 1.2 million juveniles per year was 9,310.5 US$. Annual ownership costs were estimated to be 2,497.5 US$, with annual operating costs of 5,311.8 US$. Total annual cost for the juvenile production (Hatchery) phase of spotted babylon was 7,809.3 US$, and the cost of producing 1.2 million juveniles in this hatchery design was estimated at 6.09 US$ per 1,000 juveniles. Under the basic assumptions in this study (juvenile production of 1.2 million/year), a selling price of 13.8 US$ per 1,000 juveniles results in a positive cash flow by year 2. Price and survival sensitivity resulted in substantial variability in net returns. This pilot hatchery operation is marginally feasible under these conditions if costs can be lowered considerably by improving growth and survival.

KEY WORDS: Spotted babylon, *Babylonia areolata*, economic analysis, hatchery-based operation

INTRODUCTION

Spotted babylon, *Babylonia areolata* (Link), are promising a new aquacultural marine gastropod in Thailand. Fast growth, high survival, and low feed conversion ratio (FCR) in grow-out culture provide this species with the biologic production and market characteristics necessary for a profitable aquaculture venture (Chaitanawisuti & Kritsanapuntu 1999a, Chaitanawisuti & Kritsanapuntu 1999b). Considerable interest has developed recently regarding the commercial culture of spotted babylon in Thailand because of a growing demand and an expanding domestic market for seafood, as well as a catastrophic decline in the natural spotted babylon population in the Gulf of Thailand. An accurate economic assessment of culturing spotted babylon in Thailand is required before producers can make informed decisions regarding the potential of this enterprise. A lack of economic data can be an important constraint to the successful development of spotted babylon aquaculture in Thailand. A financial investment analysis describes the relationships between yield (growth and survival), market price, fixed and variable costs, and profitability indicators. From 1998 to 2000, Chulalongkorn University conducted a collaborative research and development project with the National Research Council of Thailand (NRCT) to develop the land-based aquaculture system for large-scale hatchery and grow-out operations for spotted babylon in Thailand. Thereafter, the methods and techniques are intended to transfer for the economically hatchery-based operations in Thailand. This study was the first attempt to present estimates of the cost of producing juvenile spotted babylon, *B. areolata*, under a successful management scheme. The specific objectives were performed to identify the investment requirements, annual ownership and annual operating costs associated with the hatchery, and the net returns, returns on investment and cash flow according to selected survival and prices of juvenile.

MATERIALS AND METHODS

Hatchery Operation

A pilot commercial-scale hatchery in this study is designed to produce a total of 100,000 juveniles in 30-day production cycle for transfer to growout phase of culture. The design and operation of the hatchery was based upon the techniques of Chaitanawisuti and Kritsanapuntu (1997). The data necessary to estimate investment requirements for the hatchery were mainly gathered from a pilot research. The broodstock spawned and reared the larvae up through the post-set stage to 1.0 cm juvenile. The process involves manipulating adult snails until natural spawning occurs. The egg capsules are collected and placed in rearing tanks, where they develop into veliger larvae. The set are then placed in rearing units in which algae-enriched water is offered once daily. The algae are cultured from unicellular algal flask cultures of preferred species (*Chaetoceros calcitrans* and *Tetraselmis* sp.) in a controlled system. The average monthly productions of egg capsule and veliger larvae are approximately 8,180 and 7,116,600, respectively (Chaitanawisuti & Kritsanapuntu 1999b). The initial stocking density rate was specified at 500 larvae L^{-1} of water. The entire hatchery process require 14–18 days to go from spawning to newly settled juveniles ready for placement in the nursery system. This method requires a substantial level of capital investment and technical expertise to produce a consistently high quality of algae supply. The nursery is a critical link in the spotted babylon culture process. Placing hatchery juveniles directly into the grow-out system may induce unacceptably high mortality levels. The nursery provides an intermediate step that nurtures hatchery-reared juveniles to a size less vulnerable to the stress of grow-out operation. In addition, growing juveniles to the size required for the grow-out stage within the intensive hatchery environment would likely not be cost effective. The length of time (30–45 days) required by a nursery to produce 1.0-cm juveniles from newly settled juveniles. Based on this pilot hatchery, an average survival of veliger larvae to 1.0 cm juveniles is approximately 1.5%.

Financial Analysis

Financial analysis was based on investment cost, production, and marketing data from the pilot-scale trials. The components of the economic analysis include the following.

For the initial investment, the building used for hatchery operations included a 300-m² space made of concrete floor and sheet zinc roof with no window and wall for reducing construction cost and entrance of natural light. The hatchery design consists of three

TABLE 1.

Initial investment requirements for hatchery production of spotted babylon, *B. areolata*, juveniles.

Item	Number, (unit)	Investment, (US$)	Percent of Total Cost
Land		1,149.4	12.35
Building (300 m^2)	1	3,448.3	37.04
Broodstock tanks (3 × 3 × 0.7 m)	3	229.9	2.47
Larval rearing tanks (500 L)	30	689.7	7.40
Nursery tanks (500 L)	15	344.8	3.70
Algal rearing tanks (500 L)	10	229.9	2.47
Mass Algal rearing tanks (3 ton)	5	689.7	7.40
Aeration system	1	229.9	2.47
Water supply and drainage	1	574.7	6.17
Storage tanks (20 m^3)	2	459.8	4.94
Algal laboratory	1	804.6	8.65
Hatchery equipment	1	459.8	4.94
Total		9,310.5	100

TABLE 3.

Estimated annual operating costs (Baht) for hatchery production of the spotted babylon, *B. areolata*, juveniles.

Item	Cost (US$)	Percent of Total Cost
Repairs and maintenance	408.0	7.68
Hired labors (2 full time)	2,758.6	51.94
Feed	724.1	13.63
Broodstock purchase	413.8	7.79
Electricity	827.5	15.58
Interest on operating cost	179.6	3.38
Total operating cost	5,311.6	100

rectangular broodstock rearing tanks (3.0 × 3.0 × 0.7 m canvas tanks), 30 cylindrical larvae rearing tanks (500-L plastic tanks), 20 cylindrical (nursery tanks (500-L plastic tanks), 15 conical algae culture tanks (500-L plastic tanks), and 4 mass algae culture tanks (4-ton concrete tanks). A small 3.0 × 3.0 m room is also included for algae culture and stock maintenance. Sand filter rated at 150 L per minute are specified for filtering water before its initial use in the hatchery. Two sets of 2-horsepower pumps rated seawater used are necessary to transport water through the system. Two 10-ton canvas tanks were used as seawater stocking tanks. The two-horsepower blower is designed to provide a high volume of dissolved oxygen, low pressure, and uncontaminated air necessary for larval survival and to keep food suspended in the water column after it has been introduced into the larval culture tank. Operating a hatchery requires the regular monitoring of water-quality variables such as pH, salinity, water temperature, ammonia, nitrite, and nitrate levels, and these parameters are maintained at satisfactory levels for optimum growth and survival. A refractometer is specified for salinity measurements and a seawater test kit is specified for the determination of important water-quality parameters. Other designated equipment includes a refrigerator for the storage of supplemental feed ingredients and algae stocks. A binocular microscope is needed for the daily inspection of the quantity of food and growth of the larvae. PVC pipes and fittings are used to transport seawater and air from their sources to the culture system. Additional miscellaneous equipment also is specified for the daily operation of the hatchery. Interest rates for capital cost is based on 2000 bank loan rate of 3.5% for business enterprise.

Annual ownership costs mainly consisted of annual depreciation and interest on investment. These costs are fixed and incurred in the long run regardless of whether the facilities are operated. Annual depreciation was based on the expected useful life of each equipment item. A zero salvage value was assumed on all items constituting the facilities. The hatchery building, canvas tanks, and plastic tanks were assumed to have a useful life of 10 years because of the seawater environment. The life expectancies of equipment ranged from 3–5 years. Interest rates are based on 2000 bank loan rates of 3.5% of the original prices for all investment items.

Annual operating costs are incurred upon actual operation of the hatchery and include repairs and maintenance, labor, feed, utilities, and interest on operating capital. The annual cost of repairs and maintenance for the hatchery was computed as 5.0% of purchase price. Two labor requirements were estimated based on the particular needs for full-time operation of the hatchery. Labor cost for each individual was calculated at a rate of 114.9 US$ per month, without fringe benefits. Feed cost is based upon the assumption that larvae are fed microalgae for 14 days of the cycle.

TABLE 2.

Estimated depreciation, interest charges, and repairs and maintenance of hatchery production for *B. areolata* juveniles.

Item	Cost (US$)	Economic Life (yr)	Annual Depreciation (US$)	Annual Interest Charges (US$)	Annual Repair and Maintenance (US$)
Land	1,149.4	1	1,149.4	40.2	–
Buildings	3,448.3	10	344.8	120.7	172.4
Broodstock tanks	229.9	10	22.9	8.0	11.5
Larval-rearing tanks	689.7	10	68.9	24.1	34.5
Nursery tanks	344.8	10	34.5	11.9	17.2
Algal tanks	229.9	10	22.9	8.0	11.5
Mass algal tanks	689.7	10	68.9	24.1	34.5
Aeration system	229.9	3	76.6	26.6	11.5
Water supply and drainage	574.7	3	191.5	20.1	28.7
Storage tanks	459.8	10	2,000	16.1	22.9
Algal culture laboratory	804.6	10	80.5	28.2	40.2
Hatchery equipment	459.8	5	45.9	1.6	22.9
Total cost/year	**9,310.3**		**2,153.1**	**344.4**	**408.0**

TABLE 4.

Estimated annual costs for hatchery production of spotted babylon, *B. areolata*, juveniles.

Item	Cost (US$)	Percent of Total
Ownership costs		
Depreciation	2,153.1	27.57
Interest on investment	344.4	4.41
Total ownership cost	2,497.5	31.98
Operating costs		
Repairs and maintenance	408.0	5.23
Hired labor	2,758.6	35.33
Feed	724.1	9.28
Broodstock purchase	413.8	5.29
Electricity	827.6	10.59
Interest on operating capital	179.6	2.30
Total operating cost	5,311.8	68.02
Total annual cost	7,809.3	100

Thereafter, feed is principally composed of the fresh meat of fish. The cost of purchasing and caring for broodstock was estimated to be 4.6 US$/kg of spotted babylon. Based on the production scenario, the hatchery designed in the present study requires 40 kg of broodstock. This estimate was based on the assumption that approximately 52,200 larvae are produced from one female broodstock and an average of 1.5% survival of juveniles was expected. Electricity is used for operating the various pumps and lighting units in the hatchery. The average charge of electricity was assumed to be 68.9 US$ per month. Interest rates are based on 2000 bank loan rates of 3.5% per year for all depreciable items that compose the hatchery.

Return Analysis

Net returns and returns on investment for hatchery production were computed at final survivals ranging from 1.0–3.5% and the selling price of juvenile ranging from 9.2–22.9 US$ per 1,000 juveniles. Gross return was computed for each level of survival and each selling price. Net return was calculated from the gross return minus the total annual cost. Return to capital and management was computed for each level of survival and each selling price by subtracting annual operating cost from gross returns. Subsequently, return on investment was estimated by dividing returns to capital and management by initial capital investment (Fuller et al. 1992). An annual production budget was developed from the variable and fixed costs, and cash flow budgets were developed to examine profitability in relation to the timing of expenditures and earning. Net cash flow was determined by projecting estimated revenues and costs over a 10-year period because an aquaculture enterprise would be an attractive investment opportunity if it were profitable within this period. The initial investment was charged in the first year, and costs in subsequent year included annual variable and fixed costs. (Rubino 1992, Head et al. 1996).

TABLE 5.

Estimated total annual cost for production of spotted babylon, *B. areolata*, juveniles at selected survival rates.

Survival Rate (%)	Annual Production (Juvenile)	Annual Costs (US$)	Cost per 1,000 Juveniles (US$)
0.5	426,996	7,809.3	18.29
1.0	853,992	7,809.3	9.14
1.5	1,280,988	7,809.3	6.09
2.0	1,707,984	7,809.3	4.57
2.5	2,134,980	7,809.3	3.66
3.0	2,561,976	7,809.3	3.05
4.0	3,415,968	7,809.3	2.29

Survival rate is calculated from veliger larvae to juveniles of 1.0-cm shell length with an average monthly egg capsule and veliger production of 8,180 and 7,116,600, respectively.

TABLE 6.

Gross return for hatchery production of spotted babylon, *B. areolata*, juveniles at selected survival rates and selling prices.

Survival (%)	Selling Price (US$ per 1,000 Juveniles)				
	9.2	13.8	16.1	18.4	22.9
0.5	3,928.3	5,892.5	6,874.6	7,856.7	9,778.2
1.0	7,856.7	11,785.0	13,749.3	15,713.4	19,556.4
1.5	11,785.1	17,677.6	20,623.9	23,570.1	29,334.6
2.0	15,713.4	23,570.2	27,498.5	31,426.9	39,112.8
2.5	19,641.8	29,462.7	34,373.2	39,283.6	48,891.0
3.0	23,570.2	35,355.3	41,247.8	47,140.3	58,669.3
4.0	31,426.9	47,140.4	54,997.1	62,853.8	78,225.7

Gross return was calculated for each level of survival and selling price.

RESULTS

Total investment requirements for construction of the hatchery were 9,310.5 US$ (Table 1). The building was the largest cost component (37.04%) of the hatchery. The rearing tank, land, water supply and storage tanks, and algal culture tanks are the second most expensive items in equipping the hatchery, representing 13.57%, 12.35%, 11.11%, and 9.87% of total investment, respectively. These five components of the hatchery represent 83.94% of total investment requirements for production of spotted babylon juveniles. Annual ownership costs were estimated to be 2,497.5 US$ with annual depreciation and interest of 2,153.1 and 344.4 US$, respectively (Table 2). The annual operating cost is estimated to be 5,311.8 US$. Hired labor was the largest cost component (51.94%) of the operating cost, followed by electricity, feed, and repairs and maintenance of which comprised 15.58%, 13.63%, and 7.68%, respectively (Table 3). Total annual cost for the juvenile

TABLE 7.

Net return for hatchery production of spotted babylon, *B. areolata*, juveniles at selected survival rates and selling prices.

Survival (%)	Selling Price (US$ per 1,000 Juveniles)				
	9.2	13.8	16.1	18.4	22.9
0.5	−7,556.3	−19,168	−934.7	47.4	1,968.9
1.0	47.4	3,975.7	5,940.0	7,904.1	11,747.1
1.5	3,975.8	9,868.3	12,814.6	15,760.8	21,525.3
2.0	7,904.1	15,760.9	19,689.2	23,617.6	31,303.5
2.5	11,832.5	21,833.4	26,563.9	31,474.3	41,081.7
3.0	15,760.9	27,546.0	33,438.5	39,331.0	50,860.0
4.0	23,617.6	39,331.1	47,187.8	55,044.5	70,416.4

Net return was calculated from the gross return minus total annual cost (7,809.3 US$).

TABLE 8.

Return to capital and management for hatchery production of spotted babylon, *B. areolata*, juveniles at selected survival rates and selling prices.

Survival (%)	Selling Price (US$ per 1,000 Juveniles)				
	9.2	13.8	16.1	18.4	22.9
0.5	−1,383.5	580.7	1,562.8	2,544.9	4,466.4
1.0	2,544.9	6,473.2	8,437.5	10,401.6	14,244.6
1.5	6,473.3	12,365.8	15,312.1	18,258.3	24,022.8
2.0	10,401.6	18,258.4	22,186.7	26,115.1	33,801.0
2.5	14,330.0	24,150.9	29,061.4	33,971.8	43,579.2
3.0	18,258.4	30,043.5	35,936.0	41,828.5	53,357.5
4.0	26,115.1	41,828.6	49,685.3	57,542.0	72,913.9

Return to capital and management was calculated for each level of survival and selling price by subtracting annual operating cost (5,311.8 US$) from gross returns.

production (hatchery) phase of spotted babylon culture was 7,809.3 US$ (Table 4). Annual ownership and operating costs accounted for 31.98% and 68.02% of the total annual cost, respectively. The major ownership cost item was depreciation on investment representing 27.57% of total annual cost. Hired labors was the highest operating cost item, representing 35.33% of total annual cost. The cost associated with producing juvenile spotted babylon is expressed as US$ per 1,000 juveniles (43.5 Thai Baht is approximately 1US$). The cost of producing 1,200,000 juveniles in this hatchery design was estimated at 6.09 US$ per 1,000 juveniles. However, as the total number of juveniles produced per year decreases, then cost increases. For example, if 426,996 juveniles (approximately 0.5% survival) are produced, utilizing the same level of inputs, the estimated cost of production increases to 18.29 USS per 1,000 juveniles. Estimated total annual cost for production of juveniles at selected survivals is presented in Table 5. At 1.5% survival in this study reveals the 13.8 US$ Baht breakeven price. Thereafter, gross return and net return at these levels are 17,677.6 US$ and 9,868.3 US$, respectively (Tables 6 and 7). Return to capital and management and return on investment at these levels are 12,365.8 US$ and 1.33, respectively (Tables 8 and 9). Under the basic assumptions in this study (juvenile production of 1.2 million/year), a selling price of 13.8 US$ per 1,000 juveniles results in a positive cash flow by year 2 (Table 10).

TABLE 9.

Return on investment for hatchery production of spotted babylon, *B. areolata*, juveniles at selected survival rates and selling prices.

Survival (%)	Selling Price (US$ per 1,000 Juveniles)				
	9.2	13.8	16.1	18.4	22.9
0.5	−0.15	0.06	0.17	0.27	0.48
1.0	0.27	0.69	0.91	1.12	1.53
1.5	0.69	1.33	1.64	1.96	2.58
2.0	1.11	1.96	2.38	2.80	3.63
2.5	1.54	2.59	3.12	3.65	4.68
3.0	1.96	3.22	3.85	4.49	5.73
4.0	2.80	4.49	5.34	6.18	7.83

Return on investment was calculated by dividing return to capital and management by initial capital investment (9,310.5 US$).

DISCUSSION

Based on juvenile production of 1.5% survival and selling price of 13.8 US$ per 1,000 juveniles, the culture system is economically feasible under the assumptions used. The cost of producing *B. areolata* postlarvae according to the procedures and assumptions outlined in this study is considerably higher when the survival is very poor. The stand-alone hatchery operation becomes profitable at output level of 1.2 million juveniles. Additional research needs to refine the lower limit of profitability and the nature of marginal costs (i.e., the change in variable costs with changes in output) within this range of facility sizes. An underlying assumption in this analysis shows that survival rate and market price are sensitive to farm output. However, potential investors must assess the impact to local market prices resulting from large production levels. The analysis assumes a constant market price, which may not be valid as the production volumes from large-scale operations are released onto the market. In general, prices are sensitive to changes in supplies. Prices decrease (increase) as spotted babylon landing increase (decrease). This relationship is not incorporated into the preceding analysis, which focuses primarily on cost changes as output levels vary. Investors in spotted babylon aquaculture should be aware of the potential negative effects on market prices as output levels increase. Costs presented in this study are based on limited available data. An economic analysis of a pilot hatchery production for spotted babylon would be commercially

TABLE 10.

Ten-year cash flow of a pilot hatchery production for spotted babylon, *B. areolata* juveniles.

Year	Variable Cost (US$)	Fixed Cost (US$)	Investment (US$)	Total Annual Cost (US$)	Receipt (US$)	Net Return (US$)	Cumulative (US$)
Year 1	5,311.8	2,497.5	9,310.5	17,119.8	17,677.6	9,868.3	−7,251.5
Year 2	5,311.8	2,497.5	0	7,809.3	17,677.6	9,868.3	2,616.8
Year 3	5,311.8	2,497.5	0	7,809.3	17,677.6	9,868.3	12,485.1
Year 4	5,311.8	2,497.5	0	7,809.3	17,677.6	9,868.3	22,353.4
Year 5	5,311.8	2,497.5	0	7,809.3	17,677.6	9,868.3	32,221.7
Year 6	5,311.8	2,497.5	0	7,809.3	17,677.6	9,868.3	42,090.0
Year 7	5,311.8	2,497.5	0	7,809.3	17,677.6	9,868.3	51,958.3
Year 8	5,311.8	2,497.5	0	7,809.3	17,677.6	9,868.3	61,826.6
Year 9	5,311.8	2,497.5	0	7,809.3	17,677.6	9,868.3	71,694.9
Year 10	5,311.8	2,497.5	0	7,809.3	17,677.6	9,868.3	81,563.2

Based on an annual production of 1.2 million juveniles per year and selling price of 13.8 US$ per 1,000 juveniles.

feasible at current selling price at survival rate of 1.5%, marginally feasible. This study serves as a guideline for understanding the economics of commercial juvenile production. Deviation from the hatchery specifications and management techniques of this study will likely result in altered costs. Costs can be lowered considerably by improving growth and survival rate. This economic analysis is intended as a guide and must be modified to reflect individual situations.

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INCIDENTAL DAMAGE OF BLACKLIP ABALONE (*HALIOTIS RUBRA*) BY COMMERCIAL DIVERS IN NEW SOUTH WALES, AUSTRALIA

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ABSTRACT Rates of incidental damage to blacklip abalone (*Haliotis rubra*) were investigated in a retained, commercial catch in New South Wales, Australia. On average, 10.2% (8.9–11.6%, 95% CI) of retained abalone were damaged, but there was no relationship between the level of experience of divers and the degree or frequency of damage. Most damage was relatively minor, such as small abrasions to the foot or cuts to the mantle, and wounds healed rapidly in aquaria. Major damage, such as large cuts and abrasions to the foot, occurred in 4.2% (3.6–4.9%) of retained abalone. For individuals with major damage, any mortality was rapid and significantly greater than for undamaged abalone in aquaria. Rates of growth were also lower for individuals with major damage. An average of 19.1% (17.2–21.1%) of abalone removed from the reef were found to be under the minimum legal size and replaced. Experienced divers removed and replaced significantly fewer abalone under the minimum legal size (11% vs. 23.8% of those handled) than inexperienced divers. Using the estimated rates described here, approximately 40 tons of abalone are estimated to be damaged by the fishery each year. Most damaged abalone are retained, heal rapidly in aquaria, and can be exported live, but approximately 3 tons of damaged abalone (both retained and discarded) may die.

KEY WORDS: abalone, commercial fishing, damage, incidental, mortality

INTRODUCTION

Incidental damage to species not targeted by a fishery can be a substantial source of mortality (e.g., Broadhurst 2000). Damage to individuals of species that are targeted but subsequently found to be inappropriate for market also can be substantial (e.g., Blount & Worthington 2001). These types of mortality are most common in fisheries with nonselective collection methods (e.g., prawn trawls), or those with uncertainty about the quality or size of the product (e.g., sea urchin roe). Despite this, incidental mortality may be an issue in selective fisheries when a minimum size limit requires the handling of individuals (to identify their size) before they are retained. Damage and any consequent mortality to individuals 1) above the size limit and retained and 2) below the size limit and returned can be problematic in fisheries where most of the product is sent live to markets.

Compared with many other fisheries, commercial fishing for abalone in New South Wales (NSW) is relatively benign, with minimal direct impact on other species or on abalone not being targeted for collection. In NSW, divers use a blunt, chisel-shaped iron to remove individual abalone from the reef. Abalone are then quickly measured and, if larger than the size limit (115 mm in shell length, hereafter referred to as legal size), placed in bags that are sent to the surface for more controlled measuring. Any individuals that are removed from the rock, measured, and found to be below the size limit (hereafter referred to as undersized), either by the diver in the water or on the boat, are returned to the reef by hand. Although this process is relatively simple, the action of levering individuals off the bottom can result in cuts and abrasions to the foot of the abalone. Similarly, damage can occur during handling and storage of the catch on the boat before landing at processing factories.

There has recently been a major change in management of the NSW abalone fishery. Before 1995, each of the 37 shareholders was required to harvest the quota they owned, except in exceptional circumstances such as illness. This resulted in a group of shareholders that entered the fishery in the early 1970s and had collected abalone commercially for over 20 y. The change during 1995 enabled shareholders to nominate a diver to collect abalone on their behalf and, as a consequence, there was an influx of inexperienced divers. This change generated concern within the industry about the impact of increased rates of damage by inexperienced divers on populations of abalone. In addition, over the same time period, the fishery began to export live almost all abalone that were landed. This has also led to concern within the industry over the handling of abalone by divers and the rates of recovery of damaged abalone before export.

Different types of damage are likely to have different effects on abalone, ranging from decreased growth and reproduction to death. Even the slightest disturbance, such as tagging, has been known to reduce growth rates and, in some cases, increase mortality (McShane et al. 1986). There is also some evidence from aquaculture that suggests small disturbances may disrupt reproduction (our unpublished data). Abalone have no clotting agent in their blood, so that damage to the foot that severs any of the major arteries is likely to lead to death (Armstrong et al. 1971). Any type of injury may also inhibit their ability to feed or attach to the reef, leaving them more susceptible to predation (Pirker 1992). When disturbed, abalone also clamp down on the reef, making them considerably harder to remove, which may further increase the likelihood of damage.

In this article, we describe the frequency of different types of damage to abalone by commercial divers in NSW. We estimate rates of damage in the commercial catch and the number of abalone that are removed from the rock, measured, found to be undersized, and replaced. Finally, to investigate the possible effects of damage to abalone that are not retained, we estimated the rates of recovery, growth, and mortality of individuals with different types of damage when maintained in aquaria. This has implications for marketing because retained abalone are held in tanks at processing plants before export.

MATERIALS AND METHODS

Frequency of Damage by Commercial Divers

To estimate the frequency of different types of damage by commercial divers, a two-phase sampling design was used. First, divers' daily catches were haphazardly selected for sampling in commercial processing factories from August 1996–December 1997. An individual bin (holding 50–100 abalone) from the diver's catch was then randomly selected, and the length of all abalone within measured to the nearest mm and weighed to the nearest 5 g.

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Damage was categorized into several types and magnitudes based on injuries observed in the commercial catch. Categories (in order of increasing severity) were: no damage (ND), abrasions to the edge of the foot or mantle (MA), cuts to the edge of the foot or mantle (MC), small (<10 mm) abrasions to the foot (FA1), small (<10 mm) cuts to the foot (FC1), large (>10 mm) abrasions to the foot (FA2), large (>10 mm) cuts to the foot (FC2), and major breaks to the shell (BS). These categories were further grouped into minor damage (MA, MC, FA1) and major damage (FC1, FA2, FC2, BS), based on their likely effects on abalone. For the analyses presented here, individuals with more than one type of damage were categorized to the most severe grouping. Confidence limits were calculated using the binomial distribution, and a generalized logit model was used to relate the size of abalone to rates of minor and major damage (see Richards et al. 1994).

Although the sampling design described above provided detailed information on the frequency of damage, a second sampling design was used to provide less detailed but more comprehensive information. In this design, all abalone from all divers' daily catch supplied to one commercial processing factory were assessed for damage over a four month period (July to August 1999). Abalone were assessed as either damaged, not damaged, or dead. In total 68,900 abalone from 15 divers on 207 diver-days were assessed in this design, compared with only 9,732 in the first design. Although the second sampling design is more comprehensive than the first in terms of the absolute number of abalone sampled, it covers only a proportion of the year and only one major processing factory.

To estimate the rates of damage to abalone in the nonretained catch, information was collected on the frequency of removing, measuring, and replacing undersized abalone. Commercial divers were supplied with a logbook to record the number of undersized abalone removed and replaced and the number of legal-sized abalone retained in their first bag (divers retain harvested abalone in rope catch bags) of the day. Six commercial divers completed this logbook for a period of 15–30 days each between April–December 1999.

Recovery, Growth, and Mortality of Damaged Abalone

To investigate the effects of damage on individual abalone, two experiments were conducted in aquaria at a commercial processing factory. Experiment 1 began on 31 October 1996 and included 242 damaged abalone selected from the commercial catch. Replicate individuals from each damage category were placed in one of three randomly chosen aquaria. The number of replicate individuals within a damage category was related to the frequency of the category in the commercial catch supplied to the factory on that day (Table 1). Every two weeks, all individuals were briefly removed from the aquaria and any damage reassessed. A BS treatment was not included because individuals with this type of major damage were often dead before landing or were known to die rapidly thereafter.

In experiment 2, the effects of damage on growth were examined and the damage categories with the highest mortality in experiment 1 were further investigated. On 9 April 1997, at least 50 abalone from each of four damage categories (ND, FC1, FA2, and FC2) were selected at the factory from the commercial catch and placed in one of three randomly chosen aquaria (Table 1). Individuals were not disturbed throughout the six weeks of the experiment.

TABLE 1.

Number of abalone within each treatment of different types of damage, and within each tank for both the first and second experiments.

	Damage Category							
Tank	**ND**	**MA**	**MC**	**FA1**	**FC1**	**FA2**	**FC2**	**Total**
Experiment 1								
1	16	5	9	12	10	21	7	80
2	4	6	14	15	9	25	7	80
3	21	8	14	12	10	10	7	82
Total	41	19	37	39	29	56	21	242
Experiment 2								
1	17	—	—	—	17	17	19	70
2	17	—	—	—	16	16	19	68
3	17	—	—	—	17	17	18	69
Total	51	—	—	—	50	50	56	207

Damage categories in order of increasing severity, are no damage (ND), abrasions to the edge of the foot or mantle (MA), cuts to the edge of the foot or mantle (MC), small abrasions to the foot <10 mm (FA1), small cuts to the foot <10 mm (FC1), large abrasions to the foot >10 mm (FA2) and large cuts to the foot >10 mm (FC2).

All abalone used in both experiments were initially measured (i.e., maximum shell length, maximum shell width, and wet weight), and a small plastic tag was attached to the shell with glue. Each experiment lasted for six weeks, and the animals were fed fresh drift algae as required. The tanks were checked daily for dead abalone, which were removed immediately. In experiment 1, abalone were disturbed every two weeks to investigate recovery of their wounds. This disturbance may have affected growth, so in experiment 2, abalone were not disturbed and weighed at the end of the experiment to investigate growth. Results from both experiments were combined to investigate mortality.

RESULTS

Frequency of Damage by Commercial Divers

In total, 9,732 abalone from 29 divers on 123 days were examined for damage. On average, 10.2% (8.9–11.6%, 95% CI) of abalone retained by commercial divers were damaged. There was significant variation among divers in the rate of damage to abalone (t test of highest vs. lowest, $P < 0.05$). Total rates of damage varied among divers from 1–17% of abalone retained, with little relationship to the experience of divers ($R^2 < 0.1$, $P > 0.05$, Fig. 1). To some extent, this was caused by large variation in rates of damage among days for individual divers, with daily rates of total damage ranging from 1–30% of abalone retained, and an average coefficient of variation among days of 67%.

Most damage was relatively minor, occurring in 6.0% of abalone retained (5.2–6.9%, 95% CI), but some divers had significantly greater rates of major damage than others (t test of highest vs. lowest, $P < 0.05$, Fig. 1). For example, rates of minor damage varied among divers from 0–13%, and rates of major damage ranged from 0–12%. Major damage occurred in 4.2% (3.6–4.9%, 95% CI) of abalone retained. Again, there was no relationship between the rates of different types of damage and the experience

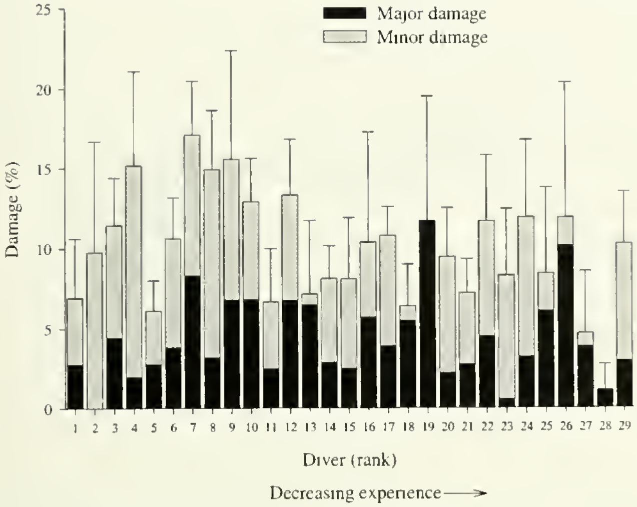

Figure 1. Number of abalone with major and minor damage as a percentage of all abalone sampled in the commercial catch of 29 divers. Error is 95% CI on total damage.

of divers (both $R^2 < 0.1$, $P > 0.05$). MA was the most common type of damage (3.7%) whereas FA2 (1.8%) was the most common type of major damage (Fig. 2). Rates of different types of damage varied greatly among divers and days. For example, rates of FC2 ranged among days from 0–8% and FA2 ranged from 0–16%. There was little relationship between the rates of different types of damage among days (all $R^2 < 0.07$, $P > 0.05$), but rates of damage were related to the size of abalone. Large abalone were more likely to be damaged than small ones (Fig. 3). For example, individuals of 115 mm were predicted to have rates of minor and major damage of 6.9% and 2.4%, whereas individuals of 140 mm were predicted to have rates of 9.1% and 4.7%, respectively.

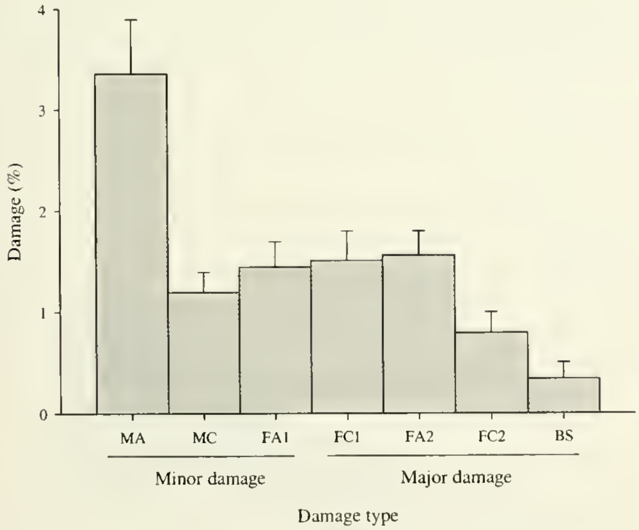

Figure 2. Number of abalone with different types of damage as a percentage (+95% CI) of all abalone sampled in the commercial catch. Damage categories were abrasions to the edge of the foot or mantle (MA), cuts to the edge of the foot or mantle (MC), small (<10 mm) abrasions to the foot (FA1), small (<10 mm) cuts to the foot (FC1), large (>10 mm) abrasions to the foot (FA2), large (>10 mm) cuts to the foot (FC2), and major breaks to the shell (BS).

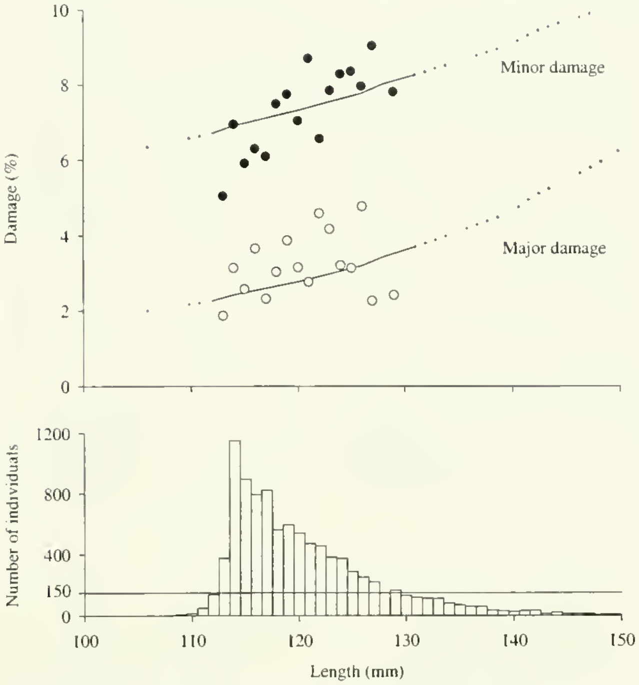

Figure 3. Top, number of abalone with major and minor damage as a percentage of all abalone sampled. Bottom, number of abalone sampled in each size class from the commercial catch in NSW. Percentage damage is shown for those size classes with more than 150 individuals. A generalized logit model was used to fit the solid line for size classes with more than 150 individuals and a dotted line for those size classes with less.

The second sampling design provided a significantly greater estimate (t test, $P < 0.05$) of the total rate of damage at 14.1% (12.6–15.8%, 95% CI). This included 0.7% (0.6–0.8%, 95% CI) of abalone that were dead at landing. Rates of total damage by individual divers ranged from 7–34% and were related to the size of the daily catch ($R^2 = 0.03$, $P < 0.05$). For example, at an average catch of 140 kg per day, predicted rates of damage were 12%, which was 4.5% lower than at a catch of 40 kg per day. Despite these differences, there was large variation in rates of damage for all catches, with a standard error of 9.4%.

On average, 19.1% (17.2–21.1%, 95% CI) of abalone removed from the reef were replaced. Rates of replacement varied among divers from 10–31%, with the two most experienced divers having significantly lower rates than others (i.e., 11.0% vs. 23.8%, t test, $P < 0.05$). There was large variation in rates of removal and replacement for individual divers, with daily rates ranging from 0–34% for the experienced divers and from 3–55% for the inexperienced, with an average coefficient of variation among days of 51%.

Recovery, Growth, and Mortality of Damaged Abalone

Recovery of wounds of abalone occurred rapidly, and at a similar rate among treatments. After 6 wk, there was no visible wound in 35.8% (30–42.0%, 95% CI) of previously damaged abalone, varying among treatments from 32–54% (Fig. 4). In addition, most remaining damaged abalone were recovering, so that after 6 wk only 21.3% (15.8–27.2%, 95% CI) of all abalone had failed to show any visible recovery. Abalone within the ND treatment remained undamaged throughout the experiment.

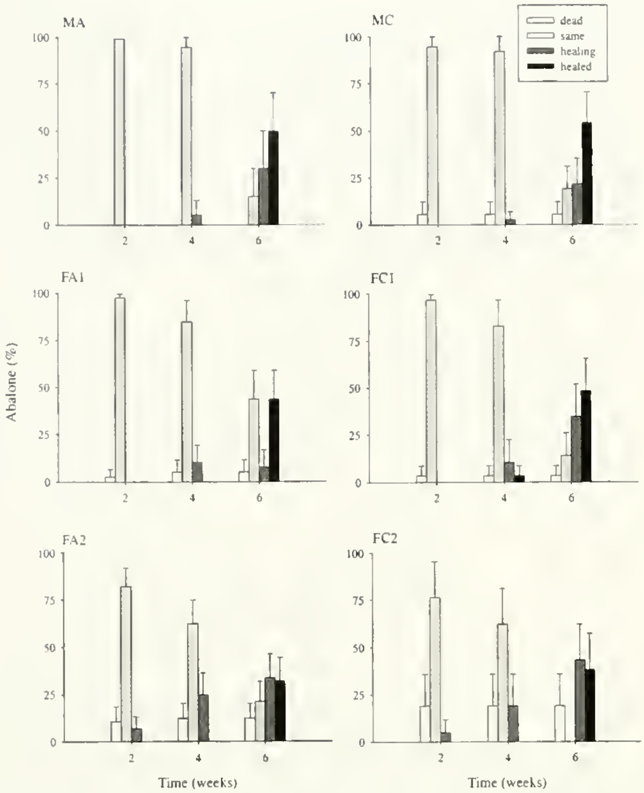

Figure 4. Number of abalone with different types of damage in four categories of wound progression over a 6-wk period, as a percentage (+95% CI) of the original number damaged. Damage categories were abrasions to the edge of the foot or mantle (MA), cuts to the edge of the foot or mantle (MC), small (<10 mm) abrasions to the foot (FA1), small (<10 mm) cuts to the foot (FC1), large (>10 mm) abrasions to the foot (FA2), and large (>10 mm) cuts to the foot (FC2).

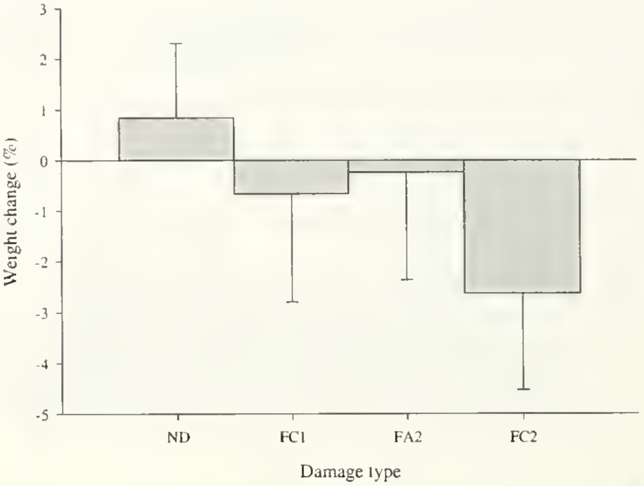

Figure 5. Change in weight as a percentage of original weight (+95% CI) for abalone with different types of damage after 6 weeks in experimental aquaria. Damage categories are no damage (ND), small (<10 mm) cuts to the foot (FC1), large (>10 mm) abrasions to the foot (FA2), and large (>10 mm) cuts to the foot (FC2).

Rates of growth of damaged abalone were significantly different among treatments (*t* test, FC2 vs. ND, $P < 0.05$). Individuals within the ND treatments increased their weight by 0.8% over the 6 wk of the experiment (Fig. 5). Within all other treatments, the average rate of growth was negative. Growth of individuals within the FC2 treatment was significantly less than ND (Fig. 5). The remaining treatments were not significantly different from ND (*t* tests, FA2 vs. ND, FC1 vs. ND, $P > 0.05$), but followed a consistent gradient of greater weight loss with increasing injury.

In experiment 1, rates of mortality of damaged abalone were significantly different among treatments after 6 wk in aquaria (Fig. 4). FC2 and FA2 caused the highest mortality of 19% (4.8–38.1%, 95% CI) and 12.5% (5.4–21.4%, 95% CI), respectively. The remaining treatments had much lower rates of mortality ranging from 0–5.1%. One individual within the ND treatment died (i.e., 2.4%), and 88% of all the mortality occurred in the first two weeks of the experiment. In experiment 2, results were similar, with generally lower rates of mortality. The highest mortality again occurred for FC2 with 14.3% (5.4–23.2%, 95% CI), whereas mortality in the other treatments ranged from 2–6%. One individual within the ND treatment died (i.e., 2%), and 79% of the mortality occurred in the first 2 wk of the experiment. When rates from both experiments were combined (Fig. 6), FC2 treatments caused the highest mortality averaging 15.6% (7.8–24.7%, 95% CI) followed by 8.5% for FA2 treatments (3.8–14.2%, 95% CI) and FC1 with 5.1% (1.3–10.1%, 95% CI). There was significantly greater mortality in the major damage treatments than ND ($\chi^2 = 5.25$, 1 df, $P < 0.05$). Most of this mortality was attributable to FC2 treatments, which were significantly greater than ND treatments ($\chi^2 = 9.84$, 1 df, $P < 0.01$) with all other comparisons not significant.

DISCUSSION

Frequency of Damage by Commercial Divers

Commercial divers damage a significant proportion of the abalone they retain in NSW. Most of the wounds are minor and heal quickly, but over 4% have major injuries that may affect their growth and survival (Fig. 2). Although rates of major damage were variable among divers and days, there was little apparent relationship to the experience of divers. Abalone size and size of the daily catch did explain some of the variation in rates of damage. The

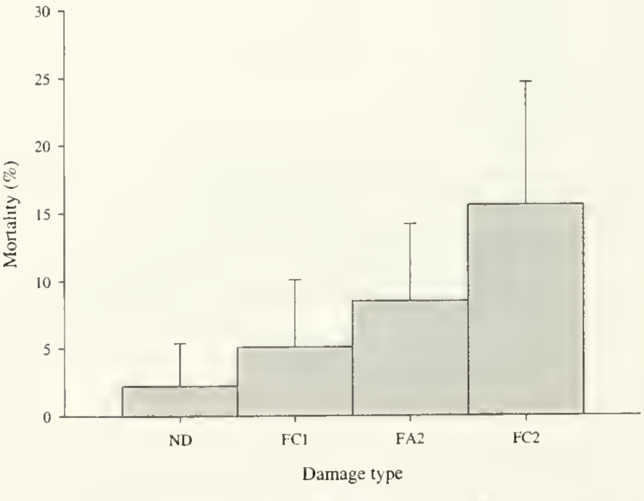

Figure 6. Mortality of abalone with different types of damage as a percentage (+95% CI) of the original number damaged after 6 wk in an experimental aquaria. Damage categories are no damage (ND), small (<10 mm) cuts to the foot (FC1), large (>10 mm) abrasions to the foot (FA2), and large (>10 mm) cuts to the foot (FC2).

rates of damage were greater for larger abalone and small daily catches, but this effect was small compared with variation among divers and among days for individual divers. Local wind and swell conditions are known to affect catch rates (Worthington et al. 1998) and are likely to influence rates of damage to abalone due to the difficulty of handling during harvesting and transport in strong winds and large swells.

The rate of damage in the commercial catch varied among divers from 1–17%, and averaged 10.2% (Fig. 1). Although the second sampling design provided a greater rate of damage (14.1%), it represented only one major processing factory over a small portion of the year. Rates of damage in NSW were considerably lower than those found for other abalone fisheries. Pirker (1992) found rates of damage between 44–78% in the commercial fishery for *Haliotis iris* in New Zealand, whereas Bruge et al. (1975) found rates above 90% in the recreational fishery for *H. rufescens* in California. Several differences among the fisheries may explain these differences. For example, in NSW a limited number of commercial divers use surface-supplied air and blunt irons to remove abalone from the reef. Perhaps most importantly, the NSW fishery exports most of the catch live and divers are encouraged by beach price to minimize the damage to their catch. Since this study was completed, further improvements in the handling of abalone by commercial divers have been made so that current rates of damage may be even lower. These include recirculating seawater-holding tanks on board fishing vessels, divers being encouraged by beach price to handle their catch appropriately, and an improved knowledge of handling techniques throughout the industry.

In contrast to the frequency of damage to retained abalone, the rate of removal and replacement of undersized abalone was related to the experience of divers. Experienced divers removed and replaced fewer undersized abalone. For a given catch of abalone, this would reduce the rate of damage caused by their removal. The lower rate of removal and replacement by experienced divers could be caused by a variety of factors. For example, more experienced divers may be able to identify undersized individuals or areas where there is little need to remove and measure individuals. Alternatively, less-experienced divers generally have lower catch rates and may be forced to work in times and places more experienced divers can avoid. Some populations are dominated by individuals who are close to legal size (Andrew et al. 1997) and fishing in these areas would require more measuring. Indeed, in some areas few, if any, individuals reach legal size (Worthington et al. 1995), and this can lead to inexperienced divers measuring large numbers of abalone to identify any individuals above the size limit. Damage to undersized individuals at these sites could be a substantial source of mortality (see also Tegner et al. 1989).

Recovery, Growth, and Mortality of Damaged Abalone

Rates of mortality and growth largely depended on the type of injury sustained. Damage to the foot that results in severing of any of the major arteries is likely to lead to death (Armstrong et al. 1971). Minor injuries can be controlled and repaired with the aid of muscle contractions (Armstrong et al. 1971). Within the aquaria, major damage resulted in mortality rates of up to 19% and reduced body weight (Figs. 5 and 6). Mortality rates and growth reduction may be much greater in the wild because of increased vulnerability to infection and predators (Bruge et al. 1975, Pirker 1992). For similar reasons, reductions in reproductive ability, such as reabsorption of eggs (observed when abalone are disturbed in aquaculture), may also be more intense in the wild.

Within aquaria, most mortality occurred within the first two weeks. Recovery of damaged abalone was rapid in aquaria, with most individuals completely recovered or recovering after six weeks (Fig. 4). These results confirm the usefulness of retaining damaged abalone within aquaria until their wounds have recovered and they are appropriate for live export. Most importantly, the rapid time for mortality or recovery is consistent with potential tanking times in the industry within NSW.

The estimated rates of damage and mortality can be used to estimate the total weight of abalone being damaged and killed each year by the fishery. During 2000, approximately 1 million abalones were retained and landed by the commercial fishery. With the estimated rate of removal and replacement of undersized abalone from this study (i.e., 19.1%), this suggests almost 1.2 million abalone were exposed to potential damage by being removed from the rock. At the observed rate of damage in the commercial catch (i.e., 10.2%), almost 120,000 individuals or approximately 40 tons may be damaged by the commercial fishery each year. Of these damaged abalone, approximately 8 tons are undersized and replaced on the reef, and the fishery retains 32 tons. Most of the damaged individuals probably recover rapidly but, even at the low rates of mortality likely in aquaria, the fishery may kill approximately 3 tons of abalone each year (i.e., 0.81% of those removed from the reef, estimated from rates of damage and mortality). Of this, approximately 500 kg are undersized abalone that are replaced, suggesting such mortality has a relatively minor impact on the productivity of the population. Furthermore, although many-damaged abalones that are replaced may survive, they are still likely to suffer complications, including reduced growth and lower reproduction.

Damage in the recreational and illegal abalone fisheries in NSW may also have important effects. For example, illegal fishers do not observe the minimum length limit and kill many undersized abalone. Many recreational fishers also handle and retain undersized abalone (unpublished data) with the potential for high rates of damage because they are not permitted to use scuba and generally have little experience removing abalone. Recreational divers often use unconventional tools to remove abalone, which may cause high rates of damage. During 1997, the recreational catch was estimated at 52 tons (unpublished data), when the commercial catch was over 330 tons. As a result, total rates of damage may be similar between the two sectors.

Early in the history of the commercial fishery for abalone in NSW there was no minimum legal size. In 1972, a minimum legal size was introduced at 100 mm, but most abalone were well above this size and almost all individuals removed from the rock were retained. With the progression of size limit upwards to the current 115 mm minimum legal size, and the decline in the size-structure of the population, more removal, measurement, and replacement of under-size abalone has occurred. Current management measures, such as temporal closures, attempt to minimize the disturbance and damage to abalone, particularly during reproductive season. Beach price incentives have also been developed by the industry to encourage the landing of undamaged individuals and to create an awareness of the damage to the resource caused by injuring abalone that are not retained. Incidental damage of undersized individuals in the NSW abalone fishery is small when compared with some other abalone fisheries, but could still be a significant source of mortality. This is obviously undesirable in an already heavily exploited fishery.

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THE EFFECT OF NONNUTRITIVE FILLERS ON THE DIGESTIBILITY OF A MANUFACTURED ABALONE DIET

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ABSTRACT In this study the effects on nutrient digestibility of adding nonnutritive fillers (kaolin, bentonite, sand and diatomaceous earth) to a research diet for greenlip abalone, *Haliotis laevigata* Donovan, were investigated. The addition of kaolin significantly improved the gross energy digestibility of the diet with the greatest improvement in gross energy digestibility occurring at the highest level of inclusion investigated (20%). Possible explanations for the positive effect of kaolin on nutrient digestion in abalone include assisting with the breakdown of diets through an abrasive/grinding effect, increasing gut transit time, decreasing digesta viscosity or through supplementation of minerals that may be deficient.

KEY WORDS: abalone, digestibility, kaolin, bentonite, sand, diatomaceous earth

INTRODUCTION

Silicates, more commonly known as bentonite, kaolin, zeolites and other clays, have been used in poultry and swine nutrition for many years (Dias et al. 1998). The reason for their inclusion is due to their binding and lubricating property in the production of pelleted feeds. There is also suggestion that some clay products may have direct beneficial effects on animal performance, however, results from studies aimed at establishing this have produced mixed results. Experiments with pigs, chickens and fish have shown responses ranging from increases in growth, feed efficiency and nutrient utilization (Kurnick & Reid 1960, Onagi 1966, Kondo & Wagai 1968, Quinsberry 1968, Han et al. 1975, Han et al. 1976, Smith et al. 1980) to negative or no responses (Reinitz 1983, Reinitz 1984, Dias et al. 1998).

Because kaolin contributes no protein or energy, it is regularly used as a filler in research diets for abalone at the South Australian Research and Development Institute's Aquatic Sciences Center. Since the addition of silicates, including kaolin, have been reported to improve nutrient utilization in other animals, it is important that its effects on nutrient digestion in abalone be investigated. The aim of this study is to assess whether four, nonnutritive, fillers (beach sand, diatomaceous earth, kaolin and sodium bentonite) affect the digestibility of a research diet for greenlip abalone, *Haliotis laevigata*.

MATERIALS AND METHODS

Diets

The basal diet used in all experiments was developed by the South Australian Research and Development Institute's Aquatic Sciences Center. The exact diet formulation is confidential; however, semolina constitutes the largest percentage of the diet followed by soy flour, which is the main source of protein. Chromic oxide was included in the diets at 0.5% by dry weight at the expense of semolina, as an indigestible marker for use in calculating the apparent protein and energy digestibility. All diets were initially hand mixed and then mixed in a spiral action dough mixer ('Impastrice', Hill Equipment and Refrigeration, Adelaide, South Australia). The mixture was then fed through a commercial pasta machine (La Prestigiosa medium, IPA, Vicenza, Italy) where it was made into 300 mm long strips using a die with slots 18 × 1.5 mm. The strips were dried on mesh trays overnight in a forced draft oven at 55°C.

Experiment 1—The Effect of Different Nonnutritive Fillers on the Protein and Energy Digestibility of a Manufactured Abalone Diet

Six diets were formulated in total, including the basal diet (Table 1). Four of the diets consisted of the basal diet with 5% of the semolina substituted for one of the following nonnutritive fillers: kaolin, bentonite, diatomaceous earth, and beach sand. The fifth diet contained 5% of pre-gelatinized waxy maize starch (Wades BO11C, Goodman Fielder Mills, NSW, Australia). The beach sand was obtained from West Beach, South Australia and was autoclaved at 121°C for 20 min before inclusion in the diet. The size of the sand grains ranged from around 192–346 μm. Each dietary treatment was randomly allocated to one of 18 digestibility tanks to provide three replicate fecal samples per diet.

Experiment 2—The Effect of Kaolin Inclusion Level on the Protein and Energy Digestibility of a Manufactured Abalone Diet

Six diets were formulated in total including the basal diet and the basal diet with 5% pre-gelatinized waxy maize as in experiment 1 (Table 2). The other four diets contained increasing levels of kaolin (5%, 10%, 15%, and 20%) again at the expense of semolina. The six diets were randomly allocated to one of 18 digestibility tanks to provide three replicate fecal samples per diet.

Abalone and Feeding

Juvenile greenlip abalone (shell length 40–60 mm) were used in the experiments. The abalone had been obtained from a commercial hatchery and raised on manufactured abalone feed. The abalone were preconditioned for one week on the test diet assigned to their tank. During both the pre-conditioning and experiment periods the animals were fed to excess every day at approximately 1700 h.

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TABLE 1.

Composition, proximate analysis and gross energy content (MJ/kg) of experimental diets from experiment 1 (g/kg, air dry basis).

	Basal Diet	5% Starch	5% Bentonite	5% D. Earth*	5% Sand	5% Kaolin
Basal diet	995.0	945.0	945.0	945.0	945.0	945.0
Starch	0	50.0	0	0	0	0
Bentonite	0	0	50.0	0	0	0
Diatomaceous earth	0	0	0	50.0	0	0
Sand	0	0	0	0	50.0	0
Kaolin	0	0	0	0	0	50.0
Chromic oxide	5.0	5.0	5.0	5.0	5.0	5.0
Gross energy (MJ/kg)	17.79	17.77	17.03	16.93	16.98	16.90
Crude protein (N × 6.25)	311.9	311.9	312.5	312.8	311.9	311.3
Dry matter	919.2	916.4	923.4	927.8	926.3	922.4

* diatomaceous earth.

Tanks and Collection System

Conical shaped digestibility tanks were used. Abalone were housed in 20-L buckets (approximately 80–100 per bucket) that fitted inside the tanks. All the buckets were fitted with plastic mesh bottoms (1.3 cm × 1.5 cm mesh) allowing containment of the abalone while permitting feces to drop into the collection tube at the base of the tank. Three 25 cm lengths of PVC pipe (8 cm in diameter) were placed in the buckets as shelters for the abalone. Attached to the bottom of each digestibility tank was a screw-on collection tube (11 cm long, 15 mm diameter). Tanks were on a flow-through water system at a rate of about 2 L/min. The seawater was filtered to 30 μm by primary sand filters, then to 10 μm by secondary composite sand filters before entering the tanks. Aeration was supplied at 0.5 L/min to each tank at all times by an air stone. Water temperature and lighting were controlled during the experiment with temperature maintained at 18.0°C ± 1.0 and a light regimen of 12 h light: 12 h dark. Salinity was 35–36‰ throughout the experiment.

Fecal Collection

Feces were collected by settlement every day until 5–6 g of feces (dry weight) was collected for each replicate sample. This took approximately 2 wk for each replicate. On each day of fecal collection the buckets containing the abalone were removed and the digestibility tanks were drained of water and all fittings were cleaned of feces and uneaten feed. Following cleaning, the tanks were refilled and the buckets replaced. Collection tubes were fitted by 0900 h. A small foam container was placed underneath each tube and filled with ice to keep the collecting feces cold and thus reduce its degradation by microbes. The feces were collected from the tubes at about 1630 h by gently pouring the contents onto a 1 mm diameter mesh. The mesh was then placed into a petri dish and frozen at −30°C. The following day the frozen fecal sample was scraped off the mesh, pooled into a composite sample, and stored in the freezer until required for analysis. Prior to analysis the samples were freeze-dried and ground with a mortar and pestle.

Chemical Analyses

Dry matter was determined by drying samples at 100°C overnight until a constant weight was achieved. Gross energy was determined using a Parr 1281 bomb calorimeter. Crude protein was determined by the combustion method using a LECO® CN-2000 Carbon and Nitrogen Analyser (Royal Australian Chemical Institute 1999). Chromic oxide was determined using atomic absorption spectroscopy based on a modification of the methods described by Hillebrand et al. (1953). The modified methodology involved preliminary ignition of the sample at 500°C to remove organic material and the dissolution of the sample in hydrochloric acid instead of sulphuric acid (M. Frith, personal communication, University of Tasmania, Launceston, Australia).

Digestibility Determination

The apparent digestibilities of nutrients in the diets were calculated using the following formula (Hardy 1997):

$$\text{Apparent digestibility} = 1 - \left(\frac{Cr_{diet} \times Nutrient_{feces}}{Cr_{feces} \times Nutrient_{diet}}\right)$$

where *Cr* is chromium content and *Nutrient* is nutrient or energy content of the diet.

Statistical Analysis

The data were analyzed by use of general linear model and the treatment means were compared by least significant difference. Prior to analysis, data were analyzed using a univariate procedure and normal plots to establish that the data were in fact normally distributed, which was the case. The presence of outliers was assessed using the RANK procedure in SAS (SAS Institute Inc., 1988) and normal scores computed from the ranks following Blom (1958). As no outliers were detected using the RANK procedure, all data were used in the calculation of digestibility estimates for all parameters, respectively.

RESULTS

Experiment 1—The Effect of Different Nonnutritive Fillers on the Protein and Energy Digestibility of a Manufactured Abalone Diet

Although very close, no significant difference was found among the six diets in their apparent protein digestibility for abalone at the 0.05 significance level ($F_{5,10}$ = 2.76; P = 0.0804) (Table 3). However, the diets were significantly different at the 0.10 significances level. This was entirely due to the 5% bentonite diet having significantly lower apparent protein digestibility than all the other diets. A significant difference was observed in the apparent dry matter digestibility ($F_{5,10}$ = 6.93; P = 0.0048) and

TABLE 2.

Composition, proximate analysis and gross energy content (MJ/kg) of experimental diets from experiment 2 (g/kg, air dry basis)

	Basal Diet	5% Starch	5% Kaolin	10% Kaolin	15% Kaolin	20% Kaolin
Basal diet	995.0	945.0	945.0	895.0	845.0	695.0
Starch	0	50.0	0	0	0	0
Kaolin	0	0	50.0	100.0	150.0	200.0
Chromic oxide	5.0	5.0	5.0	5.0	5.0	5.0
Gross energy (MJ/kg)	17.65	17.74	16.87	15.97	15.11	14.21
Crude protein (N × 6.25)	315.3	308.1	310.3	302.2	297.2	291.3
Dry matter	914.7	920.6	924.9	928.0	936.7	940.7

apparent gross energy digestibility of the diets ($F_{5,10} = 3.29$, $P = 0.0516$) (Table 3). The diet containing 5% starch had significantly higher apparent dry matter digestibility than all the other diets (Table 3). No significant differences in dry matter digestibility occurred among the diets with fillers and the control diet (Table 3). The diet with 5% starch had significantly higher apparent gross energy digestibility than all the other diets, excluding the kaolin diet (Table 3). There was no significant difference in apparent gross energy digestibility among the control, bentonite, diatomaceous earth and sand diets. Similarly, there was no significant difference in apparent gross energy digestibility among the kaolin, bentonite, diatomaceous earth and sand diets, but the kaolin diet did have significantly higher apparent gross energy digestibility than the control diet (Table 3).

Experiment 2—The Effect of Kaolin Inclusion Level on the Protein and Energy Digestibility of a Manufactured Abalone Diet

The digestibility coefficients for the control diet and that with 5% starch were comparable with those from experiment 1 (Table 3 and Table 4). No significant difference in protein digestibility occurred among the six diets in experiment 2 ($F_{5,10} = 1.41$, $P = 0.3017$) but there were significant differences among the diets in apparent gross energy ($F_{5,10} = 18.99$, $P = 0.0001$) and dry matter digestibility ($F_{5,10} = 8.56$, $P = 0.0022$) (Table 4). The diet with 5% starch had significantly higher apparent dry matter digestibility than all the other diets (Table 4). The diet with 10% kaolin had significantly higher dry matter digestibility than the control diet, but not the other kaolin diets. Apparent gross energy digestibility significantly increased with increasing level of kaolin in the diet (Table 4). The diet with 20% kaolin had significantly higher gross energy digestibility than all other diets (Table 4).

DISCUSSION

Substitution of semolina with kaolin in a manufactured diet for juvenile greenlip abalone increased the apparent digestibility of its gross energy. The gross energy digestibility of the manufactured diet significantly increased with increasing inclusion level of kaolin (Table 4). Unlike kaolin, the addition of other inert fillers (sand, bentonite and diatomaceous earth) did not significantly improve abalone's apparent gross energy digestibility of the diet when included at 5% (Table 3). It is possible that these fillers may improve the apparent gross energy digestibility if included in diets at higher levels.

The improved digestibility/growth rates observed in other animals fed diets supplemented with inert fillers such as kaolin, may have resulted, singly or in combination, from their assisting with the breakdown of diets through an abrasive/grinding effect, increasing gut transit time and through supplementation of minerals that may be deficient. Any of these mechanisms could also explain why kaolin significantly improved the gross energy digestibility of a commercial diet for juvenile abalone. Two other possible mecha-

TABLE 3.

Apparent fecal digestibility coefficients of protein, gross energy and dry matter of the 6 diets from experiment 1.

	Basal Diet	5% Starch	5% Bentonite	5% D. Earth†	5% Sand	5% Kaolin	Statistics		
							P	SEM	LSD
Protein	0.717 ± 0.01303	0.708 ± 0.01069	0.665 ± 0.01287	0.699 ± 0.00543	0.703 ± 0.00742	0.713 ± 0.01144	NS	0.01137	
Gross energy	0.490^{c} ± 0.00589	0.560^{a} ± 0.00841	0.509^{bc} ± 0.02258	0.501^{bc} ± 0.00945	0.515^{bc} ± 0.00665	0.540^{ab} ± 0.00901	*	0.01435	0.0452
DMD	0.362^{a} ± 0.00840	0.434^{b} ± 0.03165	0.331^{a} ± 0.00935	0.327^{a} ± 0.01303	0.348^{a} ± 0.01041	0.370^{a} ± 0.00614	**	0.01479	0.0466

* $P = 0.05$.

** $P < 0.001$.

† diatomaceous earth.

NS, not significant ($P > 0.05$).

SEM, standard error of the mean.

DMD, dry matter digestibility.

LSD, least significant difference.

Diets in a row with different superscripts differ significantly ($t_{0.05(10)} = 2.23$).

Data are mean ± SE, $n = 3$.

TABLE 4.

Apparent fecal digestibility coefficients of protein, gross energy and dry matter of the 6 diets from experiment 2.

	Basal Diet	5% Starch	5% Kaolin	10% Kaolin	15% Kaolin	20% Kaolin	Statistics		
							P	SEM	LSD
Protein	0.687 ± 0.00542	0.687 ± 0.00846	0.685 ± 0.00612	0.696 ± 0.00495	0.688 ± 0.00713	0.707 ± 0.00532	NS	0.00692	
Gross energy	0.455^{d} ± 0.00897	0.549^{b} ± 0.007484	0.505^{c} ± 0.02084	0.553^{b} ± 0.01155	0.551^{b} ± 0.00788	0.605^{a} ± 0.01112	***	0.01164	0.0367
DMD	0.327^{c} ± 0.01320	0.426^{a} ± 0.00515	0.362^{bc} ± 0.01828	0.376^{b} ± 0.01148	0.341^{bc} ± 0.01225	0.358^{bc} ± 0.01002	**	0.01169	0.0369

** $P < 0.01$.
*** $p < 0.001$
NS, not significant ($P > 0.05$).
SEM, standard error of the mean.
DMD, dry matter digestibility
LSD, least significant difference.
Diets in a row with different superscripts differ significantly ($t_{0.05(10)} = 2.23$).
Data are mean ± SE.

nisms are its reducing digesta viscosity and osmotic pressure in the lumen.

Reduction in Osmotic Pressure in the Lumen

The increase in apparent gross energy digestibility of diets with the increased level of kaolin could be related to the unusual oligosaccharides in soyflour, the second largest component of the diet in this study. Pedersen (1989) suggested that the post-weaning diarrhea found in piglets fed soy-based diets might not be due solely to an allergic reaction elicited by soy antigens, but to the soy's oligosaccharides. The accumulation of these components in the intestine not only provides a substrate for the growth of undesirable bacteria, but also could lead to an "osmotic" diarrhea that occurs when body fluid is drawn into the lumen of the gut to counter a raised osmotic pressure (Pedersen 1989). Soyflour may possibly produce the same response in the gut of abalone. The positive effect kaolin had might be related to its ability to counteract this osmotic diarrhea caused by soyflour and thus increase the digestibility of dietary nutrients. Evidence for this is that the addition of zeolites (alumino-silicates) to piglet diets has been found to reduce diarrhea. Inclusion of zeolite to the diet of piglets severely affected with scours (prolonged diarrhea in livestock) markedly reversed the progress of this disease within a few days (Morita 1967). Similarly, the incidence of scours in young swine fed a diet supplemented with 5% clinoptilolite was significantly reduced (England 1975). Zeolites are characterized by their ability to lose and gain water reversibly and by a very effective ion exchange capacity (Mumpton & Fishman 1977, White & Ohlrogge 1974). It is these properties of zeolites (ion exchange and reversible binding of water) that could have reduced diarrhea in piglets when added to their diet. Kaolin, also an alumino-silicate, could have had the same effect in the gut of abalone resulting in the increased gross energy digestibility of the diet.

Abrasive or Grinding Effect

Kaolin may perform a similar function to sand and grit that abalone ingest while feeding on their natural diet, assisting in grinding up and breakdown of food. Insoluble grit given to chickens remains in their gizzard and assists in grinding, crushing and breaking up food particles (Bruce 1976). Silt is believed to have a similar effect in the style sac of mussels (Murken 1976). Sand and grit, which may have a grinding effect in the gut, is taken in by wild abalone when they feed on their natural diet algae. In the wild, abalone consume algae by either catching it as it drifts past or by grazing it off rocks. When they graze rocks, they also take in sand and other sediments. The fine sand and grit can be seen in their intestine when dissected. A study on the composition of food in the stomachs of wild *H. laevigata*, *H. rubra* and *H. roei* (Shepherd 1973) revealed algae, and other browsed matter, including sand grains, small gastropods, bryozoa and detrital matter. The stomachs of *H. laevigata* and *H. scalaris* also contained quartz sand (Shepherd & Cannon 1988). Kaolin contains approximately 46.3% silica (Reis 1908). Like sand, silica may have an abrasive/grinding action in the gut of abalone. However, the mechanism that kaolin improves digestion in abalone through its grinding effect in the abalone's gizzard implies that the addition of sand, bentonite and diatomaceous earth would be expected to increase their digestibility. Therefore, it does not seem likely that a grinding effect of kaolin caused the increase in digestibility observed in this present study.

Supplementation of Minerals

It is possible kaolin is not nonnutritive but is actually supplying essential minerals that abalone require but that are deficient in manufactured diets. Deshimaru and Kuroki (1974), using a semi-purified diet, found that mineral rich diets (as high as 19.5% ash) produced the best growth of *Penaeus japonicus*. Similarly, Wheeler and Oltjen (1977) reported faster daily weight gains, feed conversion efficiency and carcass quality in finishing steers fed a corn and hay ration containing 8% protein and 3.5% cement kiln dust to replace trace minerals, than in control animals receiving an otherwise similar diet containing 12% protein plus trace minerals. They speculated that the cement kiln dust may have provided hitherto unknown but necessary trace minerals. Also, increasing levels of bentonite in a high roughage ration for steers reduced the retention of dietary calcium but increased the retention of phosphorous (Martin et al. 1969). Thus kaolin may have provided minerals essential for certain enzyme systems and biochemical functions involved in digestion in abalone but lacking in the diet used in this study.

Alteration of Gut Transport Time

It is possible that kaolin increases digestion in abalone by altering gut transport time. Increased gut transport time can increase digestibility, because the food is exposed for a longer time to digestive enzymes. The improved caloric efficiency by the addition of kaolin to poultry diets as observed by Osterhaut (1967), was due possibly to a slowing down of the rate of feed passage through the intestinal tract (Quinsberry 1968). Kurnick and Reid (1960), in their study of the effect of the addition of bentonite to 3 diets differing in energy levels for Leghorn cockerels, observed a significant increase in growth rate with the feeding of 2.5% bentonite to the low energy level diet. For each diet, the feeding of bentonite in the diet slightly delayed the passage of feed through the digestive tract. Similarly, by testing the effect of inclusion of silica, cellulose and chabamin at 10% and 20% in diets for European seabass juveniles Dias et al. (1998) observed that in fish fed the control diet, the totality of the feces was expelled after 31 h, whereas in those fed with 20% bulk incorporated diets, fecal egestion continued over 35 h. By contrast, however, Grove et al. (1978) reported that the gastric evacuation time in rainbow trout was reduced from 15 h to 10 h when the energy content of the feed pellet was reduced by 50% by dilution with kaolin.

In the wild, herbivores, such as abalone, eat macrophytes, which are low in digestibility and possess high levels of indigestible materials that pass rapidly through the gut (Wee 1992). Their relatively long guts increase retention time and allow enzymes to work on the ingested material to optimize nutrient extraction and absorption. Wild abalone take approximately 24 h to digest preferred species of algae and species less digestible may remain identifiable in abalone guts for more than 48 h (Foale & Day 1992, Day & Cook 1995). The addition of indigestible kaolin to the diet may have affected transit time in a manner similar to that of poorly digestible algal species that remain in the gut for a longer period of time, thereby increasing exposure to digestive enzymes. The digesta transit times of the diets in this study were not measured and thus cannot be compared, however, an increase in transit time caused by the addition of kaolin can not be ruled out as a possible mechanism for the increase in energy digestibility of the diet.

Reduction in Diet Viscosity

Kaolin may have increased the gross energy digestibility of the commercial diet by reducing its viscosity and hence increasing access to it by digestive enzymes. The basal diet used in this study consisted mainly of semolina and soyflour. It is well known, particularly for poultry, that soluble polysaccharide (fiber), such as arabinoxylans in wheat (semolina) and β-glucans in barley and oats elicit negative effects on digestion through increasing intestinal viscosity (Annison 1990, Bedford et al. 1991, Choct & Annison 1992, Annison 1993, Choct et al. 1996, Dusel et al. 1997). High gut viscosity decreases the rate of diffusion of substrates and digestive enzymes and hinders their effective interaction at the mucosal surface (Choct 1997). The viscous polysaccharides probably complex directly with digestive enzymes and reduce their activity (Ikeda & Kusano 1983). As with poultry, non-starch polysaccharides in wheat may also elicit negative effects on digestion in abalone through increasing intestinal viscosity. Therefore, the significant improvement in digestion by the addition of kaolin may be because it decreases intestinal viscosity.

If kaolin did increase the digestibility in abalone by decreasing the diet's viscosity, the fact that other fillers used (bentonite, diatomaceous earth and sand) had no effect on its digestibility may be due to their physical/chemical properties. The contradictory results of the effects of nonnutritive fillers incorporation on feed evacuation time in fish are, in some cases, related to the different physico-chemical properties of the various bulk agents tested (Dias et al. 1998). The properties, such as ion binding or water holding capacity, have a strong influence on solubility, gelling and viscosity of food during its passage through the intestinal tract. The phenomenon of gelation caused by polysaccharides is because they are hydrophylic molecules and consequently have the ability to hold water. Kaolin is hydrophilic, disperses in water readily, is non-expanding, has low viscosity, low sorptivity, and a low surface charge (Murray 1993). Thus, it may reduce viscosity caused by soluble polysaccharides in diets by binding with water and reducing the polysaccharide's gelation ability. The same did not occur with the other fillers and this may be due to their different chemical properties. For example, bentonite, albeit hydrophilic, has high viscosity, high sorptivity, is expanding, and has a high surface charge (Murray 1993). The effect of nonnutritive fillers seems related to their physico-chemical properties and properties of other ingredients in the diet. Thus, the positive effect of kaolin on digestibility may have been related to the basal ingredients in the diets and it may not have a positive effect on diets with different basal ingredients.

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THE DIGESTIBILITY OF WHOLE AND DEHULLED LUPINS *(LUPINUS ANGUSTIFOLIUS)* FED TO JUVENILE GREENLIP ABALONE, *HALIOTIS LAEVIGATA*

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ABSTRACT The apparent digestibility of protein, amino acids, and gross energy from whole and dehulled lupins (*Lupinus angustifolius*) and defatted soyflour was investigated for greenlip abalone, *Haliotis laevigata* Donovan. Protein digestibility of all three feedstuffs was high with a coefficient of 0.91 obtained for soyflour and whole lupins and a coefficient of 0.92 obtained for dehulled lupins. Gross energy digestibility was much more variable with the energy from soyflour being significantly more digestible (coefficient of 0.87) than that from dehulled lupins (coefficient of 0.83), which was significantly more digestible than the energy from whole lupins (coefficient of 0.50). The significantly lower energy digestibility of the whole lupins compared with the dehulled lupins could possibly be due to abalone's poor ability to digest cellulose, which is the major component of the hull of *L. angustifolius*.

KEY WORDS: abalone, digestibility, lupins, *Lupinus angustifolius, Haliotis laevigata*

INTRODUCTION

In Australia, lupins, in particular the species *Lupinus angustifolius*, are used as a source of dietary protein and energy for both pigs and poultry. *L. angustifolius* has protein levels ranging from 271.9 to 372.3 g/kg and a gross energy content of 17.9–18.6 MJ/kg (Petterson et al. 1997). Lupins are an attractive feedstuff for use in animal diets, as selective breeding programs have produced varieties of lupins with low concentrations of anti-nutritive factors such as alkaloids (Annison et al. 1996).

Successful results have been obtained on incorporation of *L. angustifolius* in manufactured diets for a range of aquaculture species including juvenile prawns, silver perch, snapper, carp, gilthead seabream and rainbow trout (Jenkins et al. 1994, Viola 1988, Robaina et al. 1995, Gomes et al. 1995, Smith et al. 1998, Allan et al. 1998). Jenkins et al. (1994) found no significant differences in growth rates of snapper when fed balanced diets containing either 20% soybean meal, 15% *L. angustifolius* seed meal plus 13% soybean or 28% *L. angustifolius* seed meal. Similarly, excellent results *with L. angustifolius* have been found for carp. Growth of carp fed a diet containing 30% lupins exceeded that of a control diet by 25% while carp fed a diet containing 45% lupins grew at the same rate as those on the control diet (Viola et al. 1988). Robaina et al. (1995) found no significant difference in weight gain, protein efficiency ratio and feed efficiency between gilthead seabream (*Sparus aurata*) fed a fishmeal based control diet and a control diet with 30% of the fishmeal protein replaced by *L. angustifolius* as a source of protein. Positive results have also been reported for prawns with no significant differences in daily growth rate, feed intake, feed conversion rate, protein conversion efficiency or apparent protein utilization of *P. monodon* when fed diets containing either dehulled *L. angustifolius* or deffated soybean meal as the main source of protein (Sudaryono et al. 1999).

This study was undertaken to determine the apparent digestibility of protein, amino acids and gross energy from the lupin, *Lupinus angustifolius,* for greenlip abalone, *Haliotis laevigata.* A potential problem that could affect abalone's ability to digest energy from *L. angustifolius* is the large proportion of cellulose in its hull. To investigate this the digestibility of both whole and dehulled *L. angustifolius* for abalone was determined.

MATERIALS AND METHODS

Diets

Two lupin diets, one containing whole, and the other containing dehulled *L. angustifolius,* were formulated (Table 1). A diet containing defatted soyflour was formulated as a control diet for comparative purposes ("Bakers Nutrisoy" brand; Archer Daniels Midland Company, Decatur, Illinois). Each feedstuff was the sole source of protein in the diet and each diet was formulated to have a crude protein content of 160 g/kg. The lupins were ground in a hammer mill and then in a ball mill before inclusion in the diets. Equal amounts of vitamins and minerals, as described by Uki et al. (1985), Jack Mackerel oil (Triabunna Fish Oils, Triabunna, Tasmania), and pre-gelatinized maize starch were added to each diet. Kaolin was included as filler and chromic oxide was included as an indigestible marker (0.5%) for use in digestibility calculations. Shipton and Britz (2001) found that chromic oxide was a suitable marker in protein digestibility studies on *Haliotis midae* as it was inert, was not absorbed, did not interfere with the digestive process, and moved through the intestine at a similar rate to the protein. Proximate analyses of the experimental diets and of each feedstuff were determined (Table 2 and Table 3).

Diet Allocations

The three diets were randomly allocated to 4 of 12 digestibility tanks to provide four replicate fecal samples per diet based on a completely randomized design.

Abalone Feeding and Fecal Collection

Abalone and Feeding

Juvenile greenlip abalone (shell length 40–60 mm, 70 g wet weight) were used in the experiment. The abalone had been obtained from a commercial hatchery and raised on manufactured abalone feed. The abalone were preconditioned for one week on

TABLE 1.

Composition of experimental diets (g/kg, air dry basis).

	Soyflour	Whole Lupin	Dehulled Lupin
Soyflour[1]	335.4		
Lupin (whole)[2]		500.0	
Lupin (dehulled)[2]			421.1
Pre-gelatinised starch[3]	100.0	100.0	100.0
Kaolin	534.0	369.4	448.4
Jack Mackerel oil	20.0	20.0	20.0
Mineral premix[4]	2.0	2.0	2.0
Vitamin premix[4]	3.0	3.0	3.0
Ascorbic acid	0.5	0.5	0.5
DL- -tocopheryl acetate	0.1	0.1	0.1
Chromic oxide	5.0	5.0	5.0

[1] Defatted soyflour (Baker's Nutrisoy; ADM).
[2] *Lupinus angustifolius* cv. Gungurru.
[3] Pre-gelatinized waxy maize starch (Wades BO11C, Inpak Foods, South Australia).
[4] Vitamin and mineral premixes as described by Uki et al. (1985).

TABLE 2.

Proximate analysis, total amino acid content (g/kg, air dry basis), and gross energy (MJ/kg, air dry basis) of experimental diets.

Diet	Soyflour Diet	Whole Lupin Diet	Dehulled Lupin Diet
Crude protein (N × 6.25)	167.5	169.8	155.0
Gross energy (MJ/kg)	8.28	9.88	10.45
Dry matter	968.1	950.0	974.6
Ash	539.4	373.8	462.2
Fiber extract			
Crude	25.0	34.3	55.3
Acid detergent	427.6	270.0	387.8
Neutral detergent	719.3	469.0	618.6
Ether extract (bp. 40–60°C)	12.4	38.1	38.6
Amino acids			
Aspartic acid	11.60	11.50	9.30
Glutamic acid	26.10	32.50	23.40
Serine	7.70	8.50	6.60
Glycine	6.75	7.00	6.05
Histidine	3.95	4.00	4.15
Arginine	9.60	16.00	13.00
Threonine	6.50	6.00	4.95
Alanine	7.05	5.50	5.10
Proline	14.0	7.50	10.55
Tyrosine	5.70	6.50	5.70
Valine	8.30	5.50	5.65
Methionine	1.25	2.50	1.05
Cystine	3.00	2.00	1.65
Isoleucine	7.75	6.50	5.85
Leucine	13.45	12.00	9.80
Phenylanaline	8.85	6.50	6.05
Lysine	8.20	7.50	4.80

the test diet assigned to their tank. During both the preconditioning and experimental periods the animals were fed to excess every day at approximately 1700 h.

Tanks and Collection System

Conical shaped digestibility tanks were used. Abalone were housed in 20-L buckets (approximately 80–100 per bucket) that fitted inside the tanks. All the buckets were fitted with plastic mesh bottoms (1.3 × 1.5 cm mesh) allowing containment of the abalone while permitting feces to drop into the collection tube at the base of the tank. Three 25-cm lengths of PVC pipe (8 cm in diameter) were placed in the buckets as shelters for the abalone. Attached to the bottom of each digestibility tank was a screw-on collection tube (11 cm long, 15 mm diameter). Tanks were on a flow-through water system at a rate of about 2 L/min. The seawater was filtered to 30 μm by primary sand filters, then to 10 μm by secondary composite sand filters before entering the tanks. Aeration was supplied at 0.5 L/min to each tank at all times by an air stone. Water temperature and lighting were controlled during the experiment with temperature maintained at 18.0°C ± 1.0 and a light regimen of 12 h light: 12 h dark regimen. Salinity was 35–36‰ throughout the experiment

Fecal Collection

Fecal collection occurred every day until 6–10 g (dry weight) of feces was collected for each replicate sample. This took 22 days in total. On each day of fecal collection the buckets containing the abalone were removed and the digestibility tanks were drained of water and all fittings were cleaned of feces and uneaten feed. Abalone were out of water for about 1 to 2 min. Following cleaning, the tanks were refilled and the buckets replaced. Collection tubes were fitted by 0900 h A small foam container was placed underneath each tube and filled with ice to keep the collecting feces cold and thus reduce degradation by microbial action. The feces were collected from the tubes at about 1630 h by gently pouring the contents onto a 1 mm mesh. The mesh was then placed into a petri dish and frozen at −30°C. The following day the frozen fecal sample was scraped off the mesh, pooled into a composite sample, and replaced into the freezer until required for analysis. Prior to analysis the samples were freeze-dried and ground with a mortar and pestle.

Chemical Analyses

Chemical analysis for proximates (i.e., dry matter, ash, ether extract, neutral-detergent fiber, acid-detergent fiber, and crude fiber) were undertaken using the methods of the Association of Official Analytical Chemists (1984). Gross energy was determined using a Parr 1281 bomb calorimeter. Proteins were analyzed by the combustion method using a LECO® CN-2000 Carbon and Nitrogen Analyser (Royal Australian Chemical Institute 1999).

All amino acids were determined by the Water's PICOTAG amino acid analysis method (Sarwar et al. 1988) using pre-column phenylisothiocyanate (PITC) derivitization and liquid chromatography was used for the determination of all amino acids. Protein sources were hydrolyzed for 22 h in duplicate with 6 N HCL at 110°C for the determination of all amino acids, except for methionine and cystine. Hydrolysates for the determination of methionine as methionine sulfone and cystine as cysteic acid were prepared by performic acid oxidation of the protein prior to hydrolysis using 6 N HCL. Alpha-aminobutyric acid was used as an internal standard.

Chromic oxide was determined using atomic absorption spectroscopy based on a modification of Hillebrand et al.'s (1953) method. The modified method involved preliminary ignition of the sample at 500°C to remove organic material and the dissolution of the sample in hydrochloric acid instead of sulphuric acid (M. Frith,

TABLE 3.

Proximate analysis and total amino acid content (g/kg, air dry basis), and gross energy content (MJ/kg, air-dry basis) of soyflour, whole and dehulled lupins used in experimental diets.

Feedstuff	Soyflour	Whole Lupin	Dehulled Lupin
Crude protein (N × 6.25)	454.0	338.6	380.0
Gross energy (MJ/kg)	17.33	17.74	18.28
Dry matter	876.5	894.5	891.9
Ash	58.8	25.0	25.3
Fibre extract			
Crude	30.5	76.9	93.9
Acid detergent	79.9	132.8	108.6
Neutral detergent	94.6	186.3	157.3
Ether extract (bp. 40–60°C)	7.5	63.5	64.4
Amino acids			
Aspartic acid	61.40	32.85	41.00
Glutamic acid	88.10	75.85	82.15
Serine	26.15	18.00	18.45
Glycine	20.80	14.85	14.95
Histidine	12.70	7.60	9.85
Arginine	35.65	37.65	37.60
Threonine	18.05	12.15	13.05
Alanine	19.10	11.60	11.65
Proline	20.65	21.15	23.95
Tyrosine	17.95	13.55	12.35
Valine	24.45	12.50	13.45
Methionine	6.10	2.35	2.00
Cystine	6.70	3.50	2.00
Isoleucine	23.10	13.20	14.30
Leucine	36.90	21.40	22.75
Phenylanaline	24.95	13.45	14.05
Lysine	30.85	14.60	15.35

personal communication, University of Tasmania, Launceston, Australia).

Digestibility Determination

The apparent digestibilities of nutrients in the diets were calculated using the following formula (Hardy 1997):

$$\text{Apparent digestibility} = 1 - \left(\frac{Cr_{diet} \times Nutrient_{feces}}{Cr_{feces} \times Nutrient_{diet}}\right)$$

where Cr is chromium content and *Nutrient* is nutrient or energy content of the diet.

The digestibility of gross energy for each ingredient was calculated by subtracting the amount of digestible energy contributed from the oil, sodium alginate and pre-gelatinized starch in each diet.

Statistical Analysis

The data were analyzed by a general linear model and the treatment means were compared by least significant difference. Prior to analysis, data were analyzed using a univariate procedure and normal plots to establish that the data were in fact normally distributed, as was the case. The presence of outliers was assessed using the RANK procedure in SAS (SAS Institute Inc., 1988) and normal scores computed from the ranks following Blom (1958). As no outliers were detected using the RANK procedure, all data were used in the calculation of digestibility estimates for all parameters, respectively.

RESULTS

A significant difference was found among the lupins and soyflour in abalone's digestibility of protein ($F_{2,6} = 17.22$, $P = 0.0033$), gross energy ($F_{2,6} = 1340.96$, $P = 0.0001$), arginine ($F_{2,6} = 7.24$, $P = 0.0251$), proline ($F_{2,6} = 10.87$, $P = 0.0101$), methionine ($F_{2,6} = 9.16$, $P = 0.0150$) and isoleucine ($F_{2,6} = 18.94$, $P = 0.0026$) (Table 4).

Dehulled lupins had significantly higher apparent gross energy digestibility for abalone than whole lupins, but had significantly lower gross energy digestibility for abalone than soyflour (Table 4). The protein from dehulled lupins was significantly more digestible than that from either whole lupins or soyflour (Table 4).

DISCUSSION

Dehulling significantly improves abalone's digestibility of energy from *L. angustifolius*. The low gross energy digestibility of whole *L. angustifolius* seeds and the significant increase in gross energy digestibility of dehulled compared with whole *L. angustifolius* has also been observed for other aquacultured species. For example, the gross energy digestibility increased from 45–74% for juvenile *P. monodon* (Smith et al. 1998) and from 59.4–74% for silver perch (*Bidyanus bidyanus*) (Allan et al. 1998) by use of dehulled as against whole *L. angustifolius* lupins.

The abalone's significantly lower digestibility of energy from whole compared with dehulled *L. angustifolius* indicates it has a poor capacity to digest the cellulose in lupins. Cellulose constitutes approximately 57.3–58.4% of the hull of *L. angustifolius* (Evans & Cheung, 1993) and thus is likely to be cause of abalone's poor gross energy digestibility of whole *L. angustifolius*. Indeed, Uki et al. (1985) demonstrated that the growth rate of *Haliotis discus hannai* decreased as the cellulose content of the diet increased from 0–20% and concluded that abalone must have a poor capacity for digesting it.

Some may expect that abalone should be able to digest cellulose, given that it forms the structural basis of many algae, their natural diet (McCandless 1985) and cellulase activity has been documented for a number of *Haliotis* spp. (Gianfreda et al. 1979, Elyakova et al. 1981, Boyen et al. 1990, Gomez–Pinchetti & Garcia–Reina 1993). A complication in interpretation of those results is that despite many studies, the origin of the cellulose activity in abalone is inconclusive. This is because most studies have not identified whether the cellulase was of microbial origin. Erasmus (1997) was the first to demonstrate that abalone possess a cellulase by detecting the presence of carboxymethylcellulase in gnotobiotic *H. midae*. Erasmus (1997) suggested that this cellulase was most likely to have been poly-β-glucanase, which hydrolyzes carboxymethylcellulose. Whether abalone possess the other two cellulases required to hydrolyze cellulose remains to be confirmed. The substrate carboxymethylcellulose does not require a true cellulase for hydrolysis. Erasmus et al. (1997) hypothesized that bacteria possibly secrete a true cellulase to completely degrade cellulose and abalone only partially hydrolyze the substrate. This hypothesis was supported by Enriquez et al. (2000) who examined the *in vitro* digestion of pure cellulose (alphacel) using stomach extracts from *Haliotis fulgens* and reported that bacteria play an important role in cellulose digestion in abalone, as stomach ex-

TABLE 4.

Apparent faecal digestibility coefficients of protein, gross energy and amino acids in soyflour, whole and dehulled *L. angustifolius* lupins fed to juvenile greenlip abalone (*H. laevigata*). Data are mean ± SE, $n = 4$.

Feedstuff	Soyflour	Whole Lupin	Dehulled Lupin	Statistics		
				P	SEM	LSD
Protein	$0.904^{a} \pm 0.00151$	$0.909^{a} \pm 0.00267$	$0.919^{b} \pm 0.00222$	**	0.00199	0.0069
Gross energy	$0.870^{a} \pm 0.00293$	$0.499^{b} \pm 0.00502$	$0.831^{c} \pm 0.00838$	***	0.00556	0.0192
Amino acids						
Aspartic acid	0.980 ± 0.00153	0.949 ± 0.03647	0.978 ± 0.00130	NS	0.02078	
Glutamic acid	0.981 ± 0.00146	0.982 ± 0.00168	0.979 ± 0.00124	NS	0.00157	
Serine	0.938 ± 0.00233	0.935 ± 0.00229	0.938 ± 0.00335	NS	0.00228	
Glycine	0.919 ± 0.00573	0.924 ± 0.00306	0.923 ± 0.00924	NS	0.00591	
Histidine	0.914 ± 0.00702	0.918 ± 0.00448	0.929 ± 0.00607	NS	0.00655	
Arginine	$0.947^{a} \pm 0.00425$	$0.962^{b} \pm 0.00142$	$0.962^{b} \pm 0.00230$	*	0.00306	0.0106
Threonine	0.880 ± 0.00716	0.895 ± 0.00360	0.884 ± 0.00666	NS	0.00472	
Alanine	0.902 ± 0.00561	0.889 ± 0.00250	0.893 ± 0.00363	NS	0.00361	
Proline	$0.947^{a} \pm 0.00549$	$0.916^{b} \pm 0.00927$	$0.945^{a} \pm 0.00450$	*	0.00525	0.0182
Tyrosine	0.885 ± 0.00397	0.908 ± 0.00153	0.907 ± 0.01080	NS	0.00723	
Valine	0.873 ± 0.00170	0.857 ± 0.00367	0.869 ± 0.00808	NS	0.00447	
Methionine	$0.815^{a} \pm 0.01077$	$0.907^{b} \pm 0.00474$	$0.828^{a} \pm 0.02372$	*	0.01647	0.0150
Cystine	0.972 ± 0.01379	0.975 ± 0.00688	0.969 ± 0.01379	NS	0.00344	
Isoleucine	$0.693^{a} \pm 0.00664$	$0.660^{a} \pm 0.00225$	$0.596^{b} \pm 0.00664$	**	0.01130	0.0391
Leucine	0.881 ± 0.00716	0.888 ± 0.00174	0.887 ± 0.00716	NS	0.00455	
Phenylanaline	0.870 ± 0.01009	0.875 ± 0.00206	0.870 ± 0.01010	NS	0.00577	
Lysine	0.927 ± 0.00895	0.920 ± 0.00634	0.913 ± 0.00895	NS	0.00624	

NS, not significant.
* $P < 0.05$
** $P < 0.01$
*** $P < 0.001$
SEM, standard error of the mean
LSD, least significant difference
Values in a row with different superscripts differ significantly ($t_{0.05(6)} = 2.45$).

tracts showed significantly decreased cellulolytic activity in the presence of antibiotics.

A factor to consider when evaluating abalone's ability to digest cellulose based on previous studies done to determine cellulase activity, is that many have estimated cellulase activity by examining the production of glucose from abalone stomach homogenates incubated with α-cellulose. This type of cellulose is a powder prepared by treating dewaxed cotton (ethanol extracted) for 8 h with 1% boiling sodium hydroxide solution (Whistler & Smart 1953). Thus, it is probably much easier for the abalone's own endogenous enzymes and gut bacterial enzymes to digest then cellulose present naturally in algae or in land based plants. Evidence for this is that cellulose that has been treated with alkali to increase the proportion of amorphous cellulose is more rapidly decomposed than untreated cellulose (Whistler & Smart 1953). In addition bacterial attack of cellulose is inhibited by the presence of lignin (also a component of lupin hulls). Thus bacteria that readily hydrolyze isolated cellulose may have little, if any, effect on wood (Whistler & Smart 1953). In addition, algae contain only low levels of cellulose. In divisions Phaeophyta, Rhodophyta and much of the Chlorophyta, cellulose is a minor component of the structural polysaccharides. In fact, in Phaeophytes and Rhodophytes, the amount of á-cellulose ranges from 1 to 8% of thallus dry weight whereas the proportion is often 30% in terrestrial plants (Kloareg & Quatrano 1988). Thus it is possible that although abalone possess cellulase it does not necessarily mean that they are able to effectively hydrolyze the cellulose present in terrestrial plants, thereby resulting in the lower gross energy digestibility of the whole compared with dehulled lupins in this study.

Abalone must be able to digest the major energy reserves in the cotyledon of lupins (lipids and cell wall non-starch polysaccharides (Evans 1994) relatively well, as indicated by the significant increase in gross energy digestibility of the lupins after dehulling. The lipid content of the cotyledon is relatively low and comprises approximately 85 g/kg by dry weight (Evans & Cheung 1993). The predominant monosaccharides in the cotyledon non-starch polysaccharides are galactose (67%), arabinose (12%) and uronic acid (10%) residues (Evans & Cheung 1993), and are the constituent sugars of the reserve pectic substances. Pectin is also one of the principal polysaccharides in cell walls of algae (Chapman & Chapman 1973), and so it would not be surprising if abalone were able to digest it.

The finding of comparable digestibility of lupins and soyflour indicates that there is potential for use of lupins in manufactured feeds for abalone. Since lupins are considerably cheaper than defatted soyflour, the replacement of soyflour with lupins in diets would result in a reduction in the cost of manufactured feeds.

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LARVAL ESCAPE THROUGH ABALONE CULTURE EFFLUENT SYSTEMS: AN ANALYSIS OF THE RISK

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ABSTRACT Expansion of abalone culture in Western Australia has resulted in the acquisition of juvenile stock (*Haliotis laevigata*) from interstate, particularly South Australia. This movement has raised concerns about the possibility of compromising the genetic integrity of domestic wild stocks of abalone with exotic genetic material. Such a compromise could arise from the escape of larvae from a production system in the event of a spawning event during the growing period. The use of a fine mesh filter may reduce this risk but is considered impractical because of frequent blockage. A risk model was constructed, which interfaces with an abalone growth model, to predict spawning events and the likelihood of viable larvae surviving in the open sea until they mature and spawn again. The model predicts that the probability of a spawning event leading to larvae escaping, maturing and spawning again in the wild is in the order of 3.7×10^{-6} per production cycle (about 3 y), and if such an event occurred, around 10 abalone would reach maturity, or about 17 in the absence of a settling pond for effluent water before discharge. Sensitivity of the model to input variables and implications for further research are discussed.

KEY WORDS: abalone culture, larval escape, risk analysis

INTRODUCTION

Abalone (*Haliotis* spp.) are highly prized edible marine gastropod mollusks. Their culture is increasing around the coastline of Australia with national production anticipated in the order of 485 tons in 2000–2001 (Hone & Fleming 1997). Increasing world prices for abalone have increased the viability of abalone aquaculture, with the majority of Australian aquaculture production being in South Australia, Victoria, and Tasmania. The industry is comparable to most intensive animal production systems, with a degree of specialization occurring. In particular, the potential exists for the production of large quantities of juvenile abalone for sale to grower units enabling growers to focus on a single aspect of production by purchasing stock from a specialist producer.

Production occurs in both sea- and land-based systems, with a trend toward land-based systems. Land-based systems tend to be operated close to the ocean, with sea-water supplied from the ocean, and effluent returned some distance from the inlet system. Australian abalone farms vary in size and layout. The profitability of such farms with either a 100 or 200 ton annual production was modeled by Weston et al. (2001).

Development of the industry in Western Australia is expanding, although only a small number of hatcheries have been established to date. To facilitate industry development, there has been pressure on Government to permit the introduction of juvenile stock from other states in Australia. Movement of any aquaculture stock into Western Australia is subject to an environmental assessment process. When assessing the movement of juvenile green-lip abalone (*Haliotis laevigata*) from South Australia to Western Australia, a concern was expressed that the genetic integrity of the Western Australian stocks could be compromised. During the growing period of some three years, it was hypothesized that a spawning event could occur in the growing tanks, resulting in discharge of viable juvenile abalone into the wild. Although the species of abalone in Western Australia is the same as that around other parts of the coastline, biotypes may vary (Brown 1991, Elliot et al. 2001) and an exotic or hybrid biotype may establish in the wild following a spawning event (Gaffney et al. 1996).

This proposed risk to the genetic integrity of wild abalone has been the subject of unpublished consultancies, and there is evidence that in greenlip abalone, stocks are genetically differentiated, that differences are detectable over small distances, and that interaction between different stocks is limited. More recent data published by Hancock (2000) show only small differences among *Haliotis roei* populations in Western Australia and, on this basis, genetic zones should no longer be applied for broodstock collection with this species. For black lip abalone (*H. rubra*), genetic differences seem to be even smaller than for green lip, with data on other species being inadequate for reliable conclusions to be made (Westaway & Norriss 1997). However, the potential for introducing new genetic material into existing stocks, and the possibility of reducing genetic diversity remain of concern to environmentalists and the abalone aquaculture industry, and management of this perceived risk is appropriate. Recommended risk management strategies include the use of progeny derived from a sufficiently large number of parent stock to ensure adequate genetic diversity; obtaining broodstock from local populations; and locating the sea culture facilities at a prescribed distance from wild populations of abalone. In addition, circumstances may require that effluent tank water be discharged over sand or filtered to minimize the ability of escaped larvae to settle on a suitable substrate. Current policy in this state dictates the use of a settlement pond or sediment filter for land-based farms.

Other risk reduction measures have been suggested, including the culture of polyploid (specifically triploid) organisms and harvesting before sexual maturity is reached. However, these options are not viable using present technology, because polyploidy is difficult to guarantee, and sexual maturity may occur some considerable time before harvestable size is reached in some species.

One management strategy to minimize the risk of larval escape from abalone culture ventures is the use of an effluent filter to trap larvae should a spawning event occur. In practice, given the volume of water involved, the use of a fine-mesh (100 μm) filter on the discharge outlet from abalone farms poses significant difficulties because of rapid clogging with particulate matter and restriction of water flow.

It is the intent of this risk assessment to quantify the probability of escape of larvae from a land-based abalone culture system using flow-through seawater in the absence of an effluent filter. The assessment relates to the risks of a spawning event, which is affected by the volume of water used, but independent of the number of abalone in the farm and their density.

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METHOD

A quantitative risk model (Vose 2000) was constructed using Microsoft® Excel, with the Palisade® @Risk add-in. However, because of the changing risks associated with the growth and development of abalone during the growing period, relevant parts of the growth and production system were also modeled. The model tracks abalone growth and associated aquaculture events daily.

Abalone production is based on a grow-out raceway fed by seawater, the flow rate of which increases as the abalone grow. Initially, approximately 0.2 megaliters (ML) of water per day is used, increasing to 40 ML per day for the 2 mo before harvest. A linear relationship between size and water flow is assumed: Flow Rate = Age (days) * 0.04 ML/day to day 1010, then 40 ML thereafter.

Indications from Purdom (1980) are that growth in fish is linear under optimal conditions, restrained by food, temperature, and sexual maturity. Social interaction has an effect in free swimming fish, but it is anticipated that such interaction is minimal in abalone. Hahn (1989) also showed that growth in length is relatively linear for abalone over a wide size range. Sexual maturity in greenlip abalone is generally after reaching 90 mm, so the constraints of feed and temperature remain the major ones, although precocious maturation occurs in abalone farms. Under farmed conditions, feed should be supplied *ad libitum* and temperatures maintained at or around optimum. Thus, a linear growth rate between 0.2 and 70 mm, over about 1,000 days is used. The growth expression is:

$$\text{Size (mm)} = \text{Age (days)} * 0.07.$$

Considerable attrition is noted in farmed abalone, with survival to maturity being very low. Large losses before settlement have been noted. Shepherd et al. (2000) observed settlement rates between 0.02–1.2% for *H. rubra*, and 0.1–6.5% for *H. laevigata* in experimental stock enhancement trials in South Australia. At the request of the Department of Fisheries, two linear death rates were used. In this situation, mortalities reach 95% by the time abalone are 5 mm (approximately day 127 of the model) and a further 50% mortality by the time abalone reach 88 mm. Simple linear regression lines were used for this purpose.

Abalone are harvested at approximately 70 mm in length, some 20 mm short of the anticipated size for sexual maturity. However, abalone have been reported to spawn when only 25 mm in length. Synchronous spawning may occur in abalone, although Hahn (1989) reports that spawning is poorly synchronized in some species. Egg production is from 0.5×10^6 to 3×10^6 per abalone, with larger shellfish producing more eggs. The relationship between size and spawn production is considered linear. Throughout this model, assumptions are made that favor the production of larvae and their escape, so as to avoid underestimating the risk. Thus, abalone as small as 15 mm are permitted to spawn in the model. The quantity of spawn (eggs) produced is calculated as:

$$\text{Spawn (millions)} = \text{length in mm} * 0.023809524 + 0.142857143.$$

Discharge water from the grow-out tanks is fed into a settling pond, which is believed to remove up to 80% of solid material. Although more accurate figures would be desirable, this percentage is an upper limit, with the minimum and means probably being 30 and 65%. There is a negative correlation between the flow rate of seawater through the farm and the efficiency of the settling pond: Proportion settling in the pond is: flow (ML) * (-0.0125628) + 0.8. The effectiveness of the settling pond was evaluated by running the model with zero settling.

From the settling pond, effluent is discharged through a pipeline to a subsurface outlet in the sea. This outlet is located at the maximum practical distance from the seawater inlet for the facility. Discharge is required to be over sand, to minimize the chance of any abalone larvae that escape of finding a suitable (rocky) habitat on which to settle. However, the effect of discharge over sand is not directly modeled, rather a probability that such larvae will survive and settle is used. Water from the subsea discharge pipe is expected to plume rather than disperse evenly into the sea, thereby maintaining a higher level of suspended particles for some distance from the outlet.

The model uses a random number generator to determine if a spawning event occurs during a growing cycle. It is assumed that spawning is an unexpected event that may occur once before harvest. The likelihood of spawning is set as a probability, using a beta-pert distribution. This type of distribution has been chosen, because it offers the capacity to specify minimum, maximum, and most likely values, and the curve is such that the extremes are not favored, and the bulk of the values generated cluster around the most likely value. The shape of the beta-pert curve is one that can be grasped intuitively, appearing as a bell-shaped curve, with or without a skew. The values for the distribution are: Minimum: 0.001; Most Likely 0.005; Maximum 0.0095. That is, there is a 1 in 200 chance of a spawning event occurring, but this may be as low as 1 in 1,000, or as high as (approximately) 1 in 100. These data have been provided from production experience. Although spawning is most likely to occur with larger abalone in the third year of the growing period, the model allows spawning on any day once abalone reach 15 mm. (This may seem very low, but gonad maturation occurs very early for some species; e.g., *Haliotis roei*) The day on which the spawning event occurs is randomly set between the time abalone reach 15 mm, to the end of the growing period.

When a spawning event occurs, a proportion of abalone present in the grow-out tank is able to spawn as females. This is also represented as a beta-pert distribution, with the following values: Minimum 0.05; Most Likely 0.1; Maximum 0.3. Proportions calculated in this manner were subsequently used to determine the number of individuals reaching the next stage, as a binomial probability.

Although a number of environmental variables interact at the time of spawning to influence fertilization, and the window of opportunity for fertilization may be short, is assumed that all spawn are fertilized—the worst case scenario. (In practice, any lag between males and females spawning in high flow rate tanks will impede fertilization.)

There is always a concern that "something drastic could go wrong"—the unexpected, rare event that throws the whole system into disarray, such as a meteor crashing directly into the abalone farm. In this model, the possibility of a rare event is incorporated in the form of a total washout of all particulate matter from the grow-out tanks; that is, whatever fertilized spawn is present gets washed straight out to sea without the benefit of a settling tank. The likelihood of such a rare event is a beta-pert distribution with the following parameters: Minimum 0.0000001; most likely

0.000001; Maximum 0.000005. That is, the event is most likely to occur with a 1 in a million probability, but could be as high as 5 in a million, or as low as 1 in ten million. This parameter is included simply to allow for the occurrence of the most extreme adverse event.

Any fertilized eggs (larvae) that escape the system are subject to several events: settlement, survival through the first year, and survival to sexual maturity. Although biologic events, these are treated as probability distributions as follows. Survival to settlement (Shepherd et al. 2000) as a beta-pert distribution: Minimum 0.0002; Most likely value 0.02; Maximum 0.08. Survival for the first year—a uniform distribution between 8 and 16%. Survival from the first year to spawning as a beta-pert distribution: Minimum 0.1; most likely value 0.2; Maximum 0.5. (It should be noted that Shepherd et al. (2000) modeled survival of larvae released directly onto a reef. They also found that survival depended on the density of abalone already on the reef. Our model assumes that any larva escaping from the farm will be carried to a reef area as if it was being released for reseeding. Clearly, this is a worst case scenario.) An abalone reaching maturity and then spawning is considered the end point of the model.

Outputs from the model are:

- total fertilized spawn (larvae) discharged;
- number of abalone surviving in the wild to spawn; and
- probability of a spawning event in aquaculture, and subsequent survival to spawn in the wild.

That is, if a spawning event occurs, and there is no filter, how many larvae escape, how many survive to spawn again, and what probability can be associated with such events occurring?

The model was run for 1,000 iterations, each iteration representing a full grow-out cycle (approximately 3 y). A random number seed is generated internally by @Risk, and this is allowed to vary between runs of the model. Consequently, results vary in detail between runs, but not in order of magnitude. Computationally, the model is a matrix of 17 variables calculated each day for 1,100 days (i.e., 18,700 individual variables determined on each iteration of the model), of which some 9,000 are generated as @Risk statistical distributions. This matrix is treated as an Excel lookup table, and outputs are computed by accumulating looked up values over the total number of iterations. Hence, outputs are not single values, but aggregations of data that are presented as means with upper and lower 95% confidence intervals, where appropriate. Limitations imposed on the model by capacity of @Risk to handle large numbers restricts maximum sizes used in some calculations. This was managed by performing such calculations on a per megaliter of water basis. For example, the use of binomial sampling is limited to population sizes of less than or equal to 32,767—a number far smaller than the expected number of spawn produced by abalone. For the calculation of outputs, the total volume of water is taken into account.

Where appropriate, sensitivity of output variables to the inputs was analyzed using the facility available in @Risk. Suitable input variables are day of spawning, proportion of abalone spawning, and probability of a spawning event, the main drivers of the model. Other variables are not suited to this process, because they are calculated independently each day of the growing cycle (about 1,000 days), and consequently, each daily probability contributes to a very small component of the final output.

The modeled abalone farm is stocked with five million juvenile abalone, of two millimeter (2 mm) length.

TABLE 1.

Outputs from risk model, with a settling pond.

Output	Mean Value	Lower 5% Confidence Interval	Upper 95% Confidence Interval
Total spawn discharged	13,184	3,920	26,800
Abalone surviving to spawn again	10	0	40
Probability of spawning event and survival to spawn in the wild	3.7×10^{-6}	6.1×10^{-7}	9.5×10^{-6}

RESULTS

Details of Output Variables Are Presented in Table 1.

The number of spawn discharged is variable. However, the mean value is under 20,000, with an upper confidence limit of the order of 30,000–40,000. Of these, very few survive, with around 10, but fewer than 100, expected to reach sexual maturity and subsequently spawn again. The likelihood that there will be a spawning event in the grow-out tank, and larvae will escape, developing to sexual maturity, is very low, in the order of 4 in a million, but may be as high as 1 in 100,000. These results include the possibility of a rare, but catastrophic event, as described previously.

The effect of the settling tank, although detectable, was small. The effect of removing the settling tank from the model is given in Table 2. Output graphs for the data in Table 2 follow (Figs. 1–3).

The impact of a "rare event" is small. Because the rare event operates in the same manner as removing the settling pond, the occurrence of a rare event, if it happened at the time of spawning, would be to increase the number of larvae escaping and reaching maturity. Because of the very low probability of a rare event, it does not affect the very low probability that larvae would escape and establish in the wild.

Sensitivity Analysis

As indicated in Tables 1 and 2, the model demonstrates that the presence of a settling pond reduces the quantity of larvae escaping if a spawning event occurs. However, because the effectiveness of the settling pond is not perfect, and its effectiveness decreases in efficiency as flow rates increase, the settling pond does not greatly affect the probability of larval escape.

TABLE 2.

Outputs from risk model, without a settling pond.

Output	Mean Value	Lower 5% Confidence Interval	Upper 95% Confidence Interval
Total spawn discharged	20,796	8,382	38,520
Abalone surviving to spawn again	17	0	80
Probability of spawning event and survival to spawn in the wild	4.0×10^{-6}	6.5×10^{-7}	9.1×10^{-6}

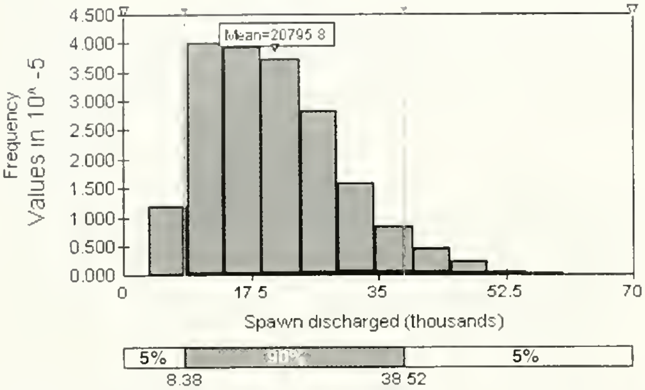

Figure 1. Distribution (frequency) histogram for total spawn discharged. Horizontal bar indicates percentiles.

The built-in facility of @Risk to determine the correlation of input variables to output variables indicates a correlation between total spawn discharged, and the input variables day of spawning (r = 0.8) and proportion of abalone spawning (r = 0.41). Survival of escaped larvae to spawn again subsequently is correlated to day of spawning (r = 0.2) and the proportion of abalone in the growing tank spawning (r = 0.16). Probability of a spawning event in the growing tank, and subsequent survival to spawn again in the wild, is also correlated to the day of spawning (r = 0.40) and probability of a spawning event (r = 0.3).

CONCLUSIONS

The model constructed and described here is a hybrid of risk and biologic models. Biologic components (growth of abalone, deaths, water flows) drive the development of abalone in culture, and risk components drive the events of interest; that is, spawning, settling, escaping, and maturing. Where available, published data have been used to determine probabilities. However, the relative newness of the abalone culture industry is such that much data is either lacking, or dependent on limited production experience.

In regard to the initial question of the importance of a filter to prevent the escape of larvae should a spawning event occur, the incorporation of a filter would seem to be a low utility procedure. The likelihood of a spawning event leading to establishment of abalone in the wild is very small (in the order of 10^{-5}–10^{-6}). The consequences of a spawning event would seem to be likewise small, with very few abalone surviving to spawn again. This result is in accord with practical experience. Most attempts to outplant hatchery-reared juvenile abalone on reefs have failed (Burton & Tegner 2000). It should be remembered that the data used in this model are considered pessimistic; that is, overestimates of the input values, and the outputs can likewise be considered pessimistic. It will require additional research to quantify the variables of concern better. It may also be of benefit to investigate improving the efficiency of settling ponds, to further reduce the numbers of abalone escaping should spawning occur.

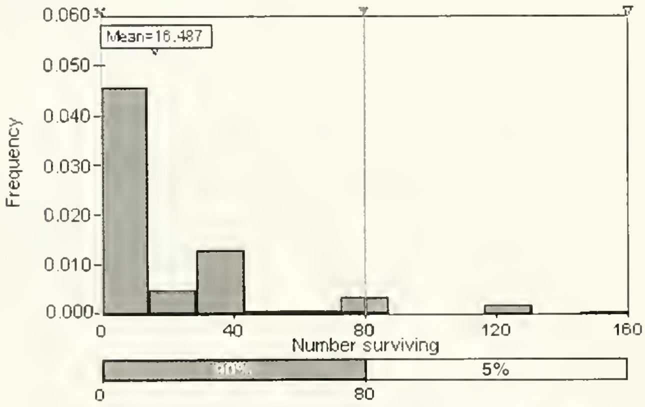

Figure 2. Distribution (frequency) histogram of abalone surviving to spawn in the wild. Horizontal bar indicates percentiles.

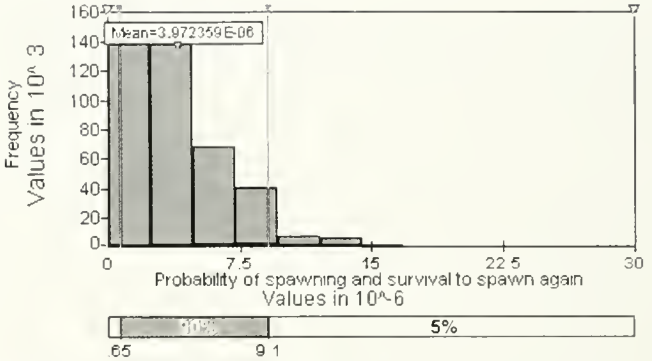

Figure 3. Distribution (frequency) histogram of the probability of a spawning event and survival of abalone to spawn in the wild. Horizontal bar indicates percentiles.

The nature of the model, using a large matrix as a lookup table tends to mask the effects of input variables. This occurs because an input variable may be referred to once in a chain of "events" over a large range (in this case, 3 y, or about 1,000 day-events). The model itself has some 9,000 input variables, which refer back either directly or by way of another variable, to the biologic and stochastic variables that provide the basic inputs for the models. It is difficult to determine relationships between inputs and outputs under these circumstances. However, for the primary driving variables, day of spawning, probability of a spawning event, and the proportion of abalone spawning, positive correlations with output variables were observed. In view of the nature of the model, such correlations strongly suggest close relationships between the outputs and the input parameters, despite traditional statistical thinking on the nature of correlations.

These correlations suggest that it would be beneficial to collate observations associated with spawning events in cultured abalone and to determine what range of triggers influence such an event, with the goal of manipulating these triggers to control or prevent spawning in culture.

Based on the results of this modeling exercise, the source of broodstock for land-based farms is of little importance in terms of genetic impact on wild populations. However, the model does not address disease risks and fine filtration (100 micron absolute) has been applied to systems in Western Australia to reduce disease risks associated with use of stock from interstate.

Similarly, settlement ponds did not greatly influence the risk of larval escape, but this is not their primary purpose. Settlement ponds are used primarily to reduce nutrient release into the pristine coastal areas usually selected as abalone farming intake locations.

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ISOLATION AND CHARACTERIZATION OF MICROSATELLITE LOCI IN THE PACIFIC ABALONE, *HALIOTIS DISCUS HANNAI*

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ABSTRACT Four microsatellite loci, designated *Hdh1321*, *Hdh78*, *Hdh1761*, and *Hdh1457*, were isolated from the Pacific abalone, *Haliotis discus hannai*, using an enrichment method based on magnetic/biotin capture of microsatellite sequences from a size-selected genomic library. Primers designed to amplify via polymerase chain reaction the microsatellite loci were used to screen 30 individuals from a natural Pacific abalone population in Onagawa Bay, Miyagi Prefecture, Japan. The four microsatellite loci were all polymorphic, with an average of 14.3 alleles per locus (range 7–20). The mean observed and expected heterozygosities were 0.48 (range 0.30–0.97) and 0.79 (range 0.60–0.92), respectively. Significant deviations from Hardy-Weinberg expectations were observed at three loci as a result of homozygote excess. The expected heterozygosity values were considerably higher than those previously found for allozymes (range 0.101–0.125), suggesting that these microsatellite loci should provide useful markers for studies of trait mapping, kinship, and population genetics.

KEY WORDS: microsatellite loci, Pacific abalone, *Haliotis discus hannai*, genetic variability

INTRODUCTION

The Pacific abalone, *Haliotis discus hannai*, is distributed along the coastal waters of East Asia, where it is one of the most valuable and popular fisheries resources. Although cultured seeds of the Pacific abalone were produced 20 years ago, the development of abalone culture has long been hampered by the problems of low growth rate and mass mortality during seed production (Hara & Sekino 2001). To resolve these problems and improve animal breeds for aquaculture production, many genetic studies on growth-related traits and temperature tolerance have been performed in the Pacific abalone (Wilkins et al. 1980, Okumura et al. 1981, Hara 1990, Kobayashi et al. 1991, 1992, Kobayashi & Fujio 1994, 1996, Furutono et al. 1995, Kijima et al. 1995, Kawahara et al. 1997, 1999). Allozyme analysis suggested the presence of homozygote excess and inbreeding depression (Kijima et al. 2002); however, genetic control of the target traits in *H. discus hannai* remains unclear. To find genetic markers associated with loci that control economically important traits to assist in selective breeding programs, the development of molecular markers is needed. For this purpose, allozymes are not appropriate because there are too few of them and they are not sufficiently variable (Kijima et al. 1992).

Microsatellites are tandemly repeated arrays of short nucleotide motifs found in all prokaryotic and eukaryotic genomes analyzed to date (Zane et al. 2002). Because they are evenly dispersed throughout genomes, usually characterized by high length polymorphism, and generally inherited in a Mendelian fashion, microsatellite markers have been widely used for genomic mapping, linkage analysis, pedigree analysis, and population genetics of biologic resources (Schlotterer et al. 1991, Knapik et al. 1998, Holland 2001).

The traditional approach to obtain microsatellites is to create a size-selected genomic library in a plasmid or phage vector and then screen clones using oligonucleotide probes containing different repeat motifs. For microsatellite repeats that are less abundant in the genome, it is difficult to isolate them using the method. Recently, different approaches have been developed to enrich for microsatellites (Zane et al. 2002). One method is based on selective hybridization, a protocol that is commonly used in enrichment procedures (Ostrander et al. 1992, Kandpal et al. 1994, Kijas et al. 1994, Refseth et al. 1997, Zane et al. 2002). In the present study, we report the first isolation of microsatellite loci in *H. discus hannai* by magnetic bead hybridization selection and assess polymorphism at microsatellite loci in individuals from a natural population.

MATERIALS AND METHODS

DNA Extraction from Abalone

For constructing a genomic DNA library, high-molecular weight DNA was extracted from a live Pacific abalone from Onagawa Bay, Miyagi Prefecture, Japan. The foot muscle was removed from the abalone and ground with dry ice in a mortar. About 100 mg of the tissue was digested overnight at 37°C in 0.7 mL of lysis buffer (6 M urea, 10 mM Tris-HCl, 125 mM NaCl, 1% SDS, 10 mM EDTA, pH 7.5) and 35 μL of proteinase K (20 mg/mL). The reaction mixture was extracted with phenol:chloroform (1:1), precipitated with isopropanol, and dissolved in TE (10 mM Tris-HCl, 1 mM EDTA, pH 8.0). DNA was treated with DNase-free RNase (20 μg/mL) at 37°C for 1 h, and purified again using a phenol/chloroform extraction.

Digestion of Genomic DNA, Size Fractionation, and Ligation of Adapters

Extracted DNA was digested with *Hae*III, *Dra*I, and *Hinc*II using 100 U each for 50 μg of abalone DNA. The digested DNA (5 μg) was electrophoresed on a 2.5% NuSieve GTG agarose gel (FMC Bioproducts), and fragments of 400–800 bp were excised and purified using a Qiagen column (QIAquick Gel Extraction Kit, QIAGEN). The fragments (1 μg) were ligated with 200 pmol of an *Eco*RI-*Not*I-*Bam*HI adapter (TaKaRa) using a DNA Ligation Kit (TaKaRa), then collected by ethanol precipitation, and resuspended in 20 μL of H_2O.

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Magnetic Isolation of Target Sequences and Adapter Polymerase Chain Reaction (PCR)

One 0.6-mL tube of the Streptavidin MagneSphere® Paramagnetic particles (Promega) was washed according to the manufacturer's instructions, resuspended in 300 μL of 5× SSC (1× SSC = 150 mM NaCl, 15 mM sodium citrate), and mixed with 300 pmol of biotinylated oligoprobe: 5′-$(CA)_{12}$GCTTGA-biotin. The six-base noncomplementary region at the 3′ end was used to prevent the probe, copurified with target DNA during magnetic isolation, from acting as primers in subsequent adapter PCR (Gardner et al. 1999). The beads and probe were incubated for 15 minutes at room temperature, then washed three times in 5× SSC and resuspended in 100 μL of hybridization solution (0.5 M NaCl, 4% polyethylene glycol 8000) at 56°C (Gardner et al.1999). The fractioned DNA (20 μL) was mixed with 80 μL of the hybridization solution, denatured by incubating at 95°C for 5 min, added quickly to the beads, and incubated at 56°C for 20 min. The beads were then washed four times at room temperature in 200 μL 2× SSC followed by four times at 30°C in 200 μL 1× SSC. Immobilized DNA fragments were eluted from the beads in 50 μL 0.15 M NaOH at room temperature for 20 min. The beads were then removed, and the supernatant was neutralized by the addition of 5.5 μL 10× TE, 3.25 μL 1.25 M acetic acid. DNA was purified by a Qiagen column (QIAquick PCR purification kit, QIAGEN) and eluted in 50 μL of TE buffer. PCR amplification was performed in 50 μL volumes containing 5 μL of the released DNA, 1.25 U of AmpliTaq Gold (Perkin Elmer), 5 μL of GeneAmp 10× PCR buffer (Perkin Elmer), 0.2 mM dNTP mix, 1.5 mM $MgCl_2$, and 0.6 μM of the adaptor sequence primer, 5′-CGGCGGCCGCGGATCC-3′. Reactions were denatured at 95°C for 11 min before 35 cycles of 94°C for 1 min, 63°C for 1 min, and 72°C for 1 min, followed by a 5 min, 72°C final extension. PCR products were purified using a QIAquick PCR purification kit (QIAGEN).

Cloning of the PCR-Amplified DNA Fragments

The purified PCR products were digested with *Not*I, inserted into the *Not*I site of the pBluescript II SK(+) vector (Stratagene), and the recombinant plasmid vector was transformed into XL1-Blue MRF′ supercompetent cells (Stratagene) following the manufacturer's protocol. Recombinant clones were selected by plating on LB media containing ampicillin (100 μg/mL), IPTG (100 μL of 10 mM stock solution) and X-Gal (100 μL of 2% stock solution).

PCR Screening of Microsatellite-Containing Clones

A small portion of a white colony was transferred to 10 μL of 10 mM Tris-HCl (pH 8.5) with a toothpick, incubated at 95°C for 10 min, and then used as template (1 μL) in the PCR with two vector primers (T3 and T7) and the nonbiotin-labeled $(CA)_{12}$ primer (Gardner et al. 1999). Each 10-μL reaction mixture contained 0.25 U of AmpliTaq Gold (Perkin Elmer), 1× the supplied buffer (Perkin Elmer), 0.2 mM dNTP mix, 1.5 mM $MgCl_2$, and 0.2 μM of each primer. Screening amplifications were performed as follows: 11 min at 95°C followed by 35 cycles of 1 min at 94°C, 1 min at 57°C, and 1 min at 72°C, followed by 5 min at 72°C. PCR products were electrophoresed on 1.5% agarose gels. Inserts producing two or more bands were considered likely to contain a microsatellite locus. Positive plasmid DNAs were purified using Qiaprep spin columns (QIAGEN), and then double sequenced on a Shimadzu DSQ-2000L DNA sequencer (Shimadzu) using ThermoSequenase cycle sequencing kit (Amersham) in combination with the universal forward (5′-CGCCAGGGTTTTCCCAGTCACGAC-3′) and reverse (5′-GAGCGGATAACAATTTCACACAGG-3′) primers, respectively. PCR primers for each microsatellite locus were designed using the OLIGO software package (National Biosciences Inc.).

Assessment of Polymorphism in Microsatellite Loci

Primer pairs were tested on a random sample of 30 Pacific abalones from Onagawa Bay. Abalone DNA was extracted by the method as described above. PCRs were performed in 10-μL volumes containing 0.25 U of AmpliTaq Gold (Perkin Elmer), 1× PCR buffer, 0.2 mM dNTP mix, 1.5 mM $MgCl_2$, 1 μM forward FITC-labeled primer and reverse primer, and about 100 ng template DNA. The PCR conditions for all loci were 11 min at 95°C followed by 35 cycles of 1 min at 94°C, 1 min at the annealing temperature listed in Table 1, and 1 min at 72°C, with a final extension of 5 min at 72°C. Amplification products were resolved via 6% denaturing polyacrylamide gel on a Shimadzu DSQ-2000L DNA sequencer equipped with DSQ-SA program (Shimadzu), and a pBluescript II SK(+) sequencing reaction was co-electrophoresed as a size marker.

Statistical Analysis

Number of alleles per locus, expected and observed heterozygosities, and χ^2 tests of deviations from Hardy-Weinberg expec-

TABLE 1.

Characterization of four polymorphic microsatellite loci in the Pacific abalone, *Haliatis discus hannai*.

Locus	Repeat Motif	Primers (5′-3′)	Annealing Temperature (°C)	Size (bp)	No. of Alleles	H_O	H_E	GenBank Acc. No.
Hdh1321	$(CGCA)_4(CA)_{18}$	TTCTGAGATGAGACGCACCAC TTGGCAGCAGGCGTCGTGT	62	272–362	20	0.97	0.92	AB084076
Hdh78	$(CACCT)_7$CACTT$(CACCT)_3$	GTCGAAACTAGCACAAACATCT GATCACCGGTCACATCATAC	54	177–332	7	0.33*	0.60	AB084077
Hdh1761	$(CA)_9$TA$(CA)_9$. . .$(CCACA)_{13}$	GTCCAACACAACCAACTCCTC CCTGAGCATGTTTGTGGATAAC	64	405–596	18	0.30*	0.92	AB084078
Hdh1457	$(CGCCA)_{11}(CTCCA)_6$. . . $(CTCCA)_{15}$. . .$(CTCCA)_9$	CACCTGTGTTTCGTACCCAC GGGACAATTCCAAGTAGATGC	62	481–601	12	0.33*	0.71	AB084079

H_O, observed heterozygosity; H_E, expected heterozygosity.

* $P < 0.01$.

tations (HWE) were calculated using GENEPOP 3.1 software (Raymond & Rousset 1995).

RESULTS

Isolation of Microsatellites in H. discus hannai

An enriched library of 350 white colonies was screened using the PCR-based technique, and 84 clones were identified by the presence of two or more bands on the agarose gel. Sequencing the 84 clones gave 46 loci containing microsatellites arrays with a minimum of five repeats, primarily $(CA)_n$, but some in combination with other tetra- or pentanucleotide array motifs. Primer pairs were designed for 10 of the 46 sequences with long, uninterrupted repeats and adequate unique regions flanking the microsatellite array. Of the 10 primer sets developed, four loci were successfully amplified with polymorphisms (Table 1).

Genetic variability of Microsatellites in H. discus hannai

Figure 1 shows the alleles of microsatellite loci *Hdh1321*, *Hdh78*, *Hdh1761*, and *Hdh1457* identified in six Pacific abalone individuals. The presence of minor peaks below the major amplification product ("stutter") was observed. At locus *Hdh1761*, the peaks of longer alleles tended to be smaller than those of shorter alleles. Primer sequences, repeat motif, annealing temperature, number of alleles, amplified product size range, the observed (H_O) and expected (H_E) heterozygosities for the four microsatellite loci are summarized in Table 1. The four microsatellite loci were all highly polymorphic, whereas the degree of variability was different at each locus. *Hdh1321* had the highest number of alleles (20), whereas number of alleles at *Hdh78*, *Hdh1761*, and *Hdh1457* was 7, 18, and 12, respectively. The expected heterozygosity ranged from 0.60 at *Hdh78* to 0.92 at both *Hdh1321* and *Hdh1761*. Significant deviations from HWE for the observed heterozygosities were observed in the *Hdh78*, *Hdh1761*, and *Hdh1457* ($P < 0.01$). Allele frequencies of the four microsatellites for samples from Miyagi Prefecture are shown in Table 2. At loci *Hdh78* and *Hdh1457*, the frequencies of major alleles 267 and 563 were 0.617 and 0.500, respectively. At loci *Hdh1321* and *Hdh1761*, allele frequencies were overall low, with the most common alleles being 0.150 and 0.167, respectively.

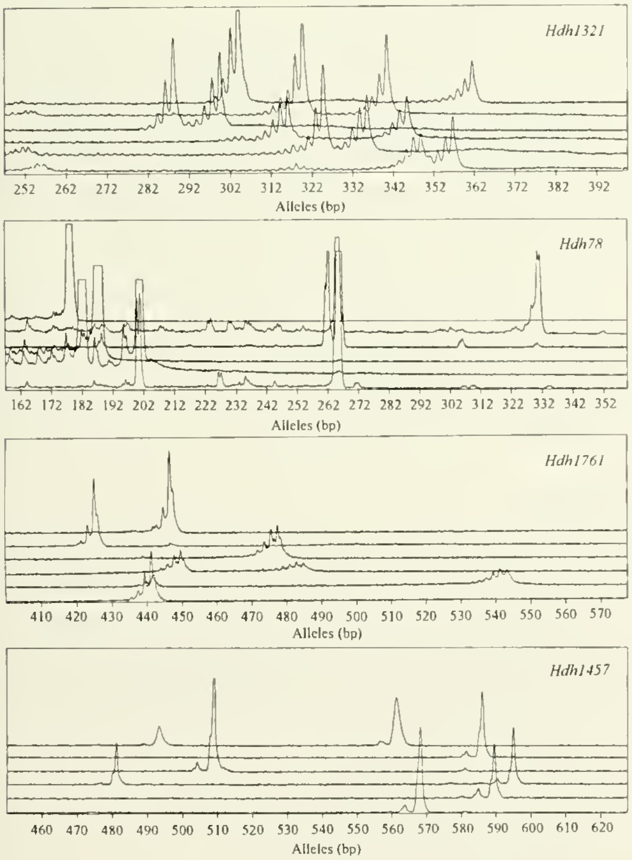

Figure 1. Alleles of microsatellite loci *Hdh1321*, *Hdh78*, *Hdh1761*, and *Hdh1457* identified in six Pacific abalone individuals from a natural population in Miyagi Prefecture.

DISCUSSION

The enrichment efficiency seen here (13.1%) is similar although lower than that reported by Gardner et al. (21%, 1999). By the colony hybridization method, the percentage of positive clones containing microsatellite repeats was 0.52–0.66% in the European flat oyster (Naciri et al. 1995), 0.1% in the quagga mussel (Wilson et al. 1999), and an average of 1.96% in molluscs (Zane et al. 2002). Compared with traditional methodologies, the enrichment procedure using the magnetic bead hybridization selection is more efficient.

The presence of the microsatellite stutter bands seen at all four microsatellite loci is a typical artifact of PCR amplication of microsatellite loci (Johansson et al. 1992). This phenomenon is due to slipped strand mispairing during PCR (Weber 1990). In this study, although the variability observed in the microsatellite loci (average 14.3 allele per locus) is possibly underestimated because of small sample size (30 individuals), it was still much higher than that of allozymes in the Pacific abalone population (Kijima et al. 1992). They surveyed 18 allozyme loci using 445 Pacific abalone individuals in six groups from coastal waters of Japan, and found the average number of alleles per locus in allozymes was 2.01 (range 1.89–2.17). The average expected heterozygosity in total populations was 0.116 (range 0.101–0.125). The high level of length variation found here is similar to that found in *H. discus discus* and other abalone species (Huang & Hanna 1998, Kirby et al. 1998, Miller et al. 2001, Sekino & Hara 2001).

Significant deviations from HWE occurred for the observed genotype frequencies at loci *Hdh78*, *Hdh1761*, and *Hdh1457* because of homozygote excess. The departure from HWE with an excess of homozygotes may be the result of one or more of the following reasons. (1) Large allele "dropout" artifacts in the PCR amplification process: At locus *Hdh1761*, we observed that large allele bands tended to be less intense than small alleles. In heterozygous individuals, preferential amplification of a smaller allele over a larger allele would result in the mis-scoring of heterozygotes for homozygotes even though larger alleles may indeed exist. The problem of large allele dropout during PCR has been well documented for humans (Day et al. 1996), Minke whales (Van Pijlen et al. 1995), and chinook salmon (Banks et al. 1999). (2) Small sample size: As microsatellite DNA has a rapid mutation rate, resulting large number of alleles, a large sample size is needed for accurate reflection of genotypic frequencies (Ruzzante 1998). This sample size was suggested to be at least 50 individuals per population for microsatellite loci studies. (3) Presence of null alleles: Null alleles of microsatellite regions, which occasionally

TABLE 2.

Allele frequencies of four microsatellite loci of Pacific abalone, *H. discus hannai*, from Miyagi Prefecture.

Allele	*Hdh1321*	Allele	*Hdh78*	Allele	*Hdh1761*	Allele	*Hdh1457*
272	0.017	177	0.133	405	0.067	481	0.033
290	0.150	182	0.017	408	0.017	493	0.017
292	0.033	187	0.067	418	0.167	509	0.017
294	0.033	202	0.033	426	0.050	518	0.033
300	0.117	262	0.083	433	0.067	548	0.017
304	0.150	267	0.617	442	0.033	563	0.500
306	0.017	332	0.050	448	0.167	568	0.033
310	0.100			450	0.017	578	0.167
312	0.017			478	0.017	586	0.117
316	0.067			483	0.017	591	0.017
320	0.017			526	0.067	596	0.017
322	0.017			537	0.033	601	0.033
324	0.050			544	0.067		
328	0.100			553	0.033		
336	0.033			570	0.033		
340	0.017			576	0.033		
346	0.017			591	0.083		
348	0.017			596	0.033		
356	0.017						
362	0.017						

fail to yield an amplification product, may arise through mutations such as point mutations in the primer annealing site (Callen et al. 1993, Pemberton et al. 1995). Kijima et al. (1997) have found null alleles at allozyme loci in the Pacific abalone by mating experiment. If null alleles are present but not accounted for, the resulting scoring error of heterozygotes for homozygotes can create an apparent excess of homozygotes in population studies (Jones et al. 1998). (4) Inbreeding effects: Extensive heterozygote deficiency has also been reported at allozyme loci in natural populations of the Pacific abalone (Fujino 1978, Fujio et al. 1986). Hara and Kikuchi (1992) showed an excess of homozygotes in all natural populations of *H. discus hannai* collected from nine different sampling sites, suggesting extensive inbreeding. The excessive homozygotes for microsatellite loci were also described for *H. rubra*, *H. discus discus*, and *H. kamtschatkana* populations (Huang et al. 2000, Miller et al. 2001, Sekino & Hara 2001). (5) Presence of size homoplasy: Size homoplasy is the co-occurrence of alleles that are identical in state (PCR products of the same size) without being identical by descent (Ardren et al. 1999). Mistaking homoplasy for homology can lead to underestimation of the genetic divergence within and among population (Taylor et al. 1999). Microsatellite allele size homoplasy has been confirmed in various animal species (Estoup et al. 1995, Viard et al. 1998, Ardren et al. 1999, Taylor et al. 1999); however, was not reported for abalones. Further studies of natural populations and controlled crosses of *H. discus hannai* will help to clarify this deviation from HWE.

The high variability of the microsatellite markers identified in this study will make them excellent tools for paternity testing, population studies, and the linkage analysis of genes related to traits with economic significance in the Pacific abalone.

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TRANSMISSION OF WITHERING SYNDROME IN BLACK ABALONE, *HALIOTIS CRACHERODII* LEACH

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ABSTRACT Withering syndrome (WS) has been associated with catastrophic declines in black abalone populations in southern and central California. In an effort to identify the etiological agent of WS and to characterize the progression of this disease, we initiated a transmission study in which abalone from Ano Nuevo Island, a location free of WS, shared aquaria with animals from Vandenberg Airforce Base, a location where WS is epizootic. The mean incubation period of WS (time to develop overt signs of the disease) was 245 days with a mean time to death after development of clinical signs of 42 days. Median time to death was 41 wk in the experimentally exposed Ano Nuevo Island abalone and 16 wk in the positive control Vandenberg abalone. Cumulative mortality was significantly different between the negative control (unexposed) Ano Nuevo Island abalone (25% mortality) and both the exposed Ano Nuevo Island abalone (85% mortality; $P = 0.0001$) and the positive control Vandenberg abalone (100% mortality; $P = 0.0001$). In addition, significant differences in prevalences of a recently described Rickettsiales-like procaryote (RLP), "*Candidatus* Xenohaliotis californiensis," were observed between negative control animals (no RLPs) and those with WS (both the experimentally exposed Ano Nuevo Island and Vandenberg abalone were infected with RLPs; $P < 0.001$). All abalone infected with the RLP had signs of WS, including decreased condition indices, foot muscle atrophy, and digestive gland degeneration ($P < 0.05$). No correlation between intensity of RLP infection and degree of WS was observed ($P > 0.05$), suggesting a complex relationship between the RLP and clinical disease in black abalone. Despite this, these data in conjunction with a lack of observation of any other significant pathogens in the abalone provides evidence that the RLP infecting abalone ("*Candidatus* Xenohaliotis californiensis") is the etiological agent of WS.

KEY WORDS: withering syndrome, black abalone, *Haliotis*, rickettsiales, "*Candidatus* Xenohaliotis californiensis"

INTRODUCTION

Withering syndrome (WS) has been associated with catastrophic declines in black abalone populations in southern and central California (Haaker et al. 1992; Steinbeck et al. 1992, Friedman and Haaker unpublished data). Initial studies identified a previously undescribed coccidian parasite, *Margolisiella* (=*Pseudoklossia*) *haliotis* (Friedman 1991; Friedman et al. 1995, Desser & Bower 1997), in black abalone with WS that was subsequently determined to be nonpathogenic as evidenced by field and laboratory studies (Friedman et al. 1993, 1997). VanBlaricom et al. (1993) documented WS on San Nicolas Island in April of 1992. These researchers a observed Rickettsiales-like procaryote (RLP) that was recently described as a new taxon and has been given the provisional status of "*Candidatus* Xenohaliotis californiensis" (Friedman et al. 2000). Two of six abalone with clinical WS harbored RLPs, whereas apparently healthy animals were devoid of the RLP. The authors indicated that Rickettsiales-like bacteria were commonly observed in marine invertebrates and that the pathogenicity of these organisms was unknown. Gardner et al. (1995) also observed RLPs in association with WS in black abalone from San Nicolas and San Clemente Islands in southern California. Healthy abalone from Ano Nuevo Island in central California were not infected with RLPs, suggesting an association between the RLP and WS. Friedman et al. (1997) examined the association between the RLP, degeneration of the digestive gland, and mortality in a laboratory study. No clear associations between intensity of RLP infection and either condition of the digestive gland or time to mortality were observed. Recently, Moore et al. (2000) observed a significant relationship between the intensity of RLP infection and degree of WS in cultured red abalone. These conflicting data indicate further examination of the role of the RLP in WS is warranted. This study was designed to examine the transmissibility of WS and to determine the relationship between RLP infection and WS in black abalone.

MATERIALS AND METHODS

Animals

Healthy black abalone were collected on March 28, 1995 from Ano Nuevo Island, where WS had never been observed. Black abalone with WS were collected from Vandenberg Airforce Base (Vandenberg) and Cayucos on April 24, 1995. Abalone were transported to the Pathology Quarantine Facility at the Bodega Marine Laboratory, where they were placed in an 88-L aquaria and received ambient (8–10°C), flow-through, full-strength seawater. *Macrocystis pyrifera* was collected from Bodega Bay and was surface sterilized by soaking in a tamed iodine solution (Prepodyne: Westagro, Kansas City, MO) for 15 min followed by a freshwater rinse. Animals were fed *M. pyrifera* twice per week. All abalone were tagged and the following data were collected: maximum length, foot length and total volume (TV), and total weight (TW). Animals were bled from the pallial sinus with a tuberculin syringe and a 26-gauge, 0.5-inch needle and the density, cell-type, and condition of circulating hemocytes was determined using a hemocytometer. Visual condition of the abalone was assessed according to the following scale: (3): healthy abalone with a foot and viscera that fills the entire shell volume; (2): visible mantle retraction and moderate atrophy of the foot muscle; and (1): severe atrophy of the foot muscle.

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Histology

Selected tissues were placed in Invertebrate Davidson's solution (Shaw & Battle 1957) for 24 h and processed for routine paraffin histology. Deparaffinized 5-μm sections were stained with hematoxylin and eosin (Luna 1968) and viewed by light microscopy. The intensity of RLP infection was quantified using the following logarithmic scale at 200× magnification: (0): no bacterial foci; (1): 1–10 foci per field; (2): 11–100 foci per field; and (3): >100 foci per field (Friedman et al. 1997). Infection intensity was quantified in both the postesophagus (PE) and digestive gland (DG), and an overall infection intensity was calculated by summing the intensity in the PE and DG (range of 0–6 possible) (Moore et al. 2000). Intensities were examined according to tissue type to determine whether the location of infection was correlated with animal health. Unless otherwise specified, the term RLP infection refers to overall infection intensity. Condition of the digestive gland and foot muscle were assessed using the (1)–(3) scales of Friedman et al. (1997), in which normal was scored as (3), moderate (up to 30%) alteration from normal was scored as (2), and tissue that was severely (>30%) altered was scored as (1). Three specific morphologic changes that characterized observed alterations in digestive gland architecture were individually scored according to the following (1)–(3) scale: (1) normal architecture; (2) moderate (up to 25%) degeneration (characterized by an increase in connective tissue between digestive tubules, the primary tissues responsible for secretion of digestive enzymes and nutrient absorption in abalone) (Voltzow 1994), transport duct metaplasia, or inflammation; and (3) abundant (>25%) transport duct metaplasia, an increase in connective tissues between degenerating tubules, or inflammation.

Transmission Experiment

Groups of 12 abalone from Ano Nuevo Island were randomly placed in each of two negative control (NC) and two experimental aquaria (EA). Groups of 12 abalone with WS (EWS) were randomly added to each of the two experimental aquaria and to each of the two positive control aquaria (PC) (Fig. 1). Animals were maintained on ambient seawater for the first 3 mo of the study. During this time temperatures ranged between 8–10°C ($\bar{X}$ = 9.53°C) for the first 4 wk, 11–15°C ($\bar{X}$ = 12.89°C) for the following 4 wk and 10.5–15°C ($\bar{X}$ = 12.47°C) for the third month. After this time, the animals were acclimated over a 2-wk period to 18 ± 1°C, the temperature at which the abalone were maintained for the remaining 34 wk of the 46-wk study. Physical measurements and hemocyte counts were assessed approximately every 8 wk over the course of the experiment. All moribund abalone or mortalities were sampled as above, including shell weight (SW) and shell volume (SV), and selected tissues (foot, digestive gland, PE, and kidneys) were processed for histology. The intensity of RLP infection and condition of the digestive gland and foot muscle were quantified as described above. The condition of the abalone was also assessed upon death using the body weight condition index of Friedman et al. (1997) = [(TW − SW)/TW]. In addition, the percentage of live tissue volume relative to the entire volume of the animal was determined = [(TV − SV)/TV].

Statistical Analysis

The Fisher's exact test was used to test the independence of exposure to WS and mortality; abalone were grouped as exposed or unexposed and as alive or dead. Chi square contingency table analysis (X^2) was used to test independence between exposure to WS and measured health parameters. Abalone were grouped as exposed (laboratory or field exposed) and unexposed. The following health parameters or responses were grouped as normal (scores of 3 for animal condition and that of the digestive gland and foot and 0 for RLP presence), whereas those with signs of WS and RLP infection were grouped as abnormal. Observed versus expected frequencies in each category were compared using 2 × 2 contingency table analyses. The Fisher's Exact test was used when fewer than five observations were observed in any cells. These analyses

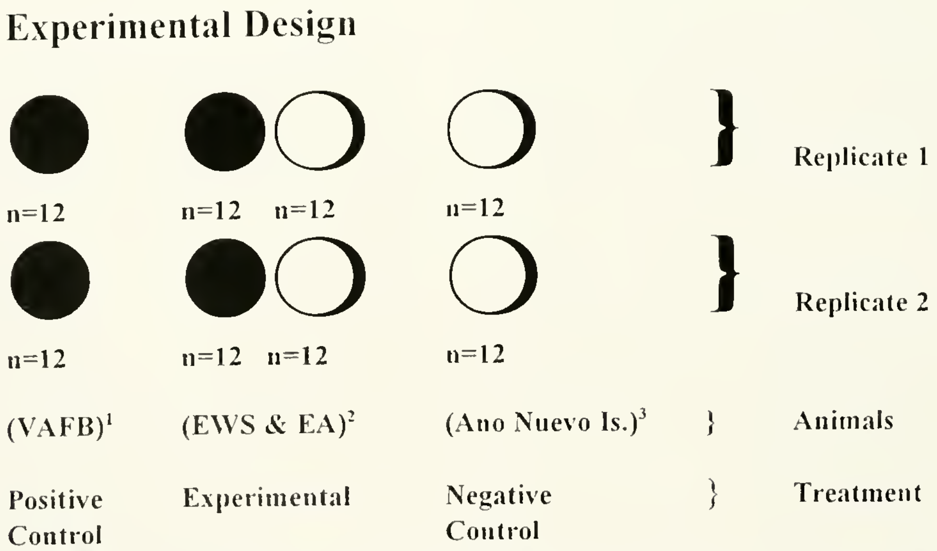

Figure 1. Experimental Design. The dark circles represent black abalone with WS collected from the field (Vandenberg Airforce Base or Cayucos (VBCS)). The open circles represent healthy, naive (no exposure to WS before study) black abalone from Ano Nuevo Island. [1]Animals with WS collected from Vandenberg Airforce Base and Cayucos in the positive control treatments. [2]Animals from VBCS (EWS) or Ano Nuevo Island (EA) with and without WS, respectively, in experimental treatments. [3]Animals in the negative control treatments without WS that were collected from Ano Nuevo Island.

were also used to test the independence of RLP infection and WS. Animals were grouped as infected and uninfected and as above for survival and health parameters. In a separate analysis to further assess the independence of specific lesions and RLP infection intensity animals were grouped as low overall infection levels (0–3) and high overall infection levels (4–6) and the response (e.g., metaplasia) was grouped as present or absent. Spearman rank correlation coefficients were calculated and tested for a (linear) relationship between intensity of RLP infection and condition of the digestive gland and foot, condition indices, density of circulating hemocytes, cumulative mortality, and time of exposure. Stepwise forward and backward regression models were used to predict the intensity of RLP infection in exposed abalone from the following variables: condition of the digestive gland and foot, weight condition index, visual condition assessment, and duration of exposure. Multiple logistic regression analysis was used to predict presence of RLP infection by using combinations of the five independent variables listed above.

RESULTS

All abalone from the PC treatment and all except three animals in the experimental treatment (EWS and EA) that died in this study had visible signs of WS, including weakness, weight loss, and visible atrophy of the foot muscle (Fig. 2). The two EA abalone in the experimental aquaria that died during the first week of the study lacked visual and histopathological signs of WS, RLP infections, or visible injuries. A third abalone from the experimental treatment that died during the 21st week of the study was too decomposed for gross or histologic examination. The six NC abalone that died during the experiment and 18 NC survivors sampled upon termination of the study did not have visible or microscopic signs of WS (Figs. 3 and 4). Both Vandenberg/Cayucos (PC and EWS) and Ano Nuevo Island (EA) abalone with clinical WS had histopathological and hematological signs of this disease, including degeneration and inflammation of and/or metaplastic changes in the digestive gland, depletion of muscle bundles in the foot, (Figs. 3–5), and the presence of necrotic cells, cellular debris, and small hemocytes (~4.5 μm) with a large nucleus to cytoplasmic ratio within the hemolymph. In addition, all PC and EWS animals and all except the two EA abalone that died during the first week of the study were infected with the RLP, whereas none of the NC animals were infected. Other than the nonpathogenic renal coccidian, *Margolisiella* (=*Pseudoklossia*) *haliotis*, no other parasites were observed in any of the abalone examined in this study. In this study, the incubation time for clinical WS is defined as the duration between initiation of the study and development of gross clinical signs such as mantle retraction or visible atrophy of the foot muscle. The mean incubation period for the EA abalone was 245 days ($n = 21$) with a range of 154–301 days. The duration between onset of visible signs of WS and mortality averaged 42 days ($n = 21$) with a range of 6–113 days. The two abalone that died during the initial week of the study from handling stress and the single animal that died at 21 wk did not show signs of WS or were too decomposed for assessment of WS, respectively, and were not included in these calculations. Cumulative mortality approached 100% in the PC aquaria, 85% of the EA animals in the experimental aquaria, and 25% in the NC aquaria (Fig. 6). A significantly higher proportion of abalone died upon exposure to WS (22/24) relative to unexposed animals (6/24; $P < 0.0001$, Fisher's exact test). Median time to mortality was significantly different between the exposed EA (41 wk) and PC (16 wk) abalone ($P < 0.0001$, Mann-Whitney test). As only a few NC abalone died during the study, median time to death was not calculated for this group.

A significantly higher proportion of abalone exposed to WS (EA and PC) had reduced condition indices, morphologic changes, and RLP infections than did unexposed animals (NC). Reduced condition indices were observed in 18/24 EA and 19/24 PC abalone, whereas only 3/24 NC animals lost condition ($P = 0.002$ and $P < 0.001$, respectively, X^2 test). Morphologic changes were observed in the digestive gland of 20/21 EA and 17/24 PC abalone, whereas only 1/24 NC abalone had an abnormal digestive gland architecture ($P < 0.001$, X^2 test). Of these, degeneration was observed in 14/21 EA and 6/10 PC animals, metaplastic changes in 9/21 EA and 4/10 PC abalone, and inflammation in 6/21 EA and 1/10 PC abalone, whereas 1/24 of the unexposed animals only had mild digestive gland degeneration. Pedal atrophy was observed in 15/21 EA, 17/24 PC, and only 1/24 NC abalone ($P = 0.001$ and $P < 0.001$, respectively, X^2 test). Infections with "*Candidatus* Xenohaliotis californiensis" were observed only in EA (22/21) and PC (24/24) treatments ($P < 0.001$, X^2 test and $P < 0.001$, Fisher's exact test). As above, significantly higher proportions of animals with RLP infections died and had clinical signs of WS than did unexposed abalone ($P < 0.001$, X^2 test).

Spearman rank correlation coefficients for relationships between intensity of RLP infection of individuals in each WS-exposed group (EA, EWS, and PC) versus visual condition, condition indices, condition of the foot and digestive gland, and density of circulating hemocytes were low and ranged between −0.275 and 0.486 for the Ano Nuevo Island animals and −0.0175 and 0.0567 for Vandenberg animals. Except for metaplasia and overall

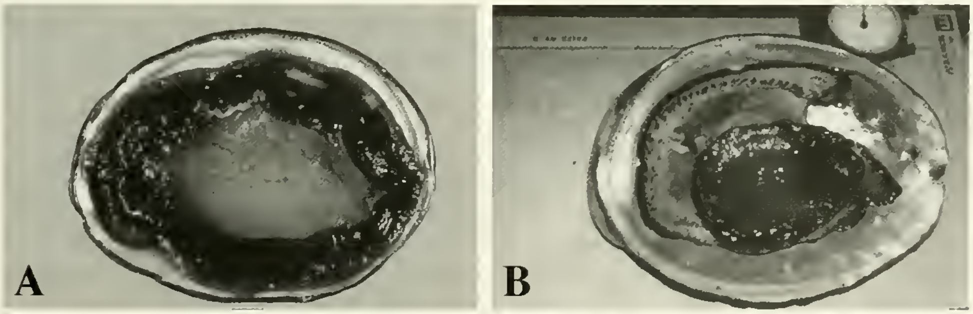

Figure 2. Black abalone with and without WS. A, Healthy animals from Ano Nuevo Island from a negative control treatment. B, An (EA) abalone from Ano Nuevo Island that contracted WS from infected black abalone in an experimental treatment.

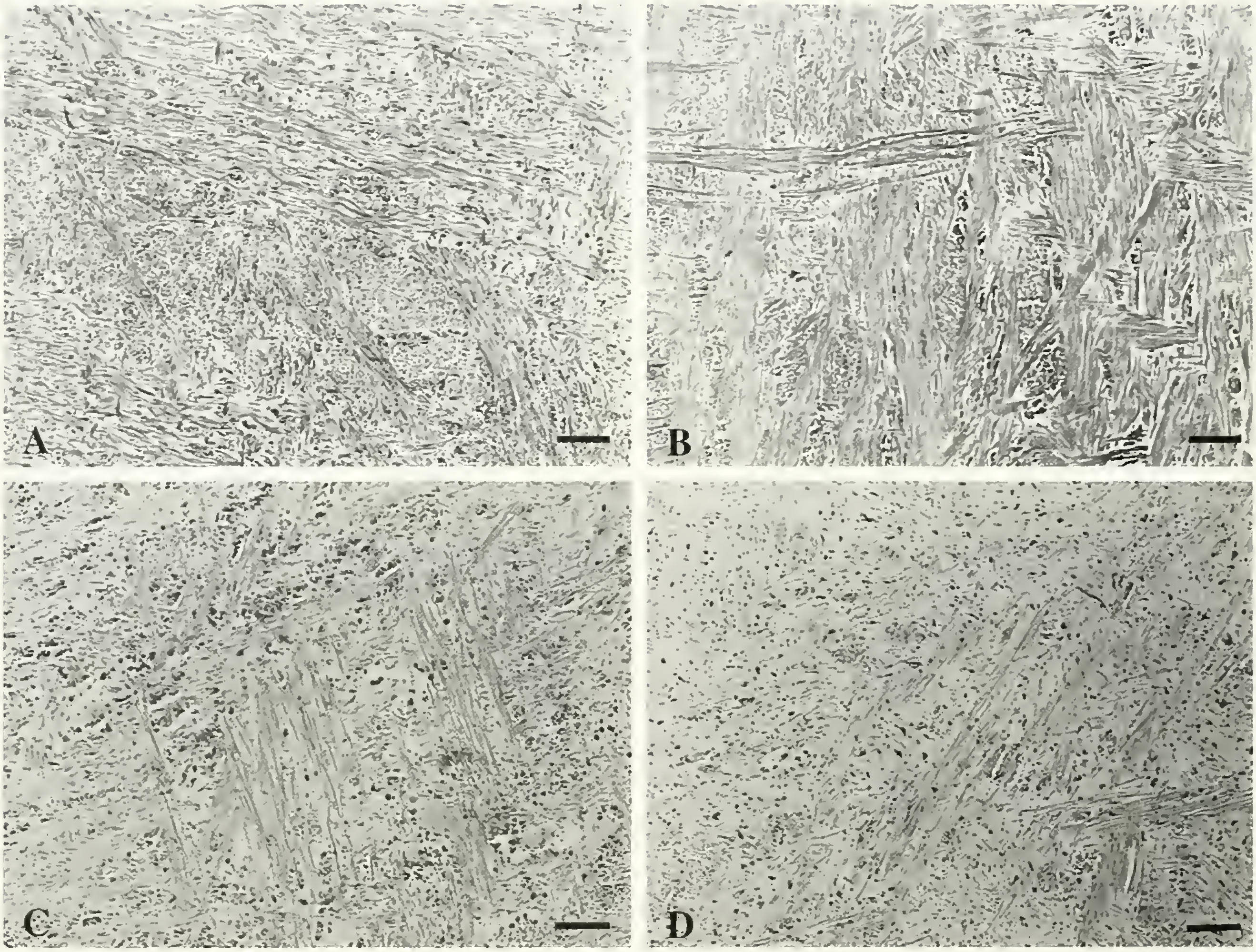

Figure 3. Microscopic anatomy of the foot muscle of black abalone with and without WS. The foot muscle of an uninfected animals are illustrated in A (Vandenberg abalone) and B (Ano Nuevo Island abalone). Note that dense bundles of muscle fibers comprise most the foot. The pedal muscle atrophy of an abalone that contracted WS in the field (C) is also observed in those that acquired WS in this laboratory study (D). Note the severe reduction in muscle fibers and increase in visible connective tissue in affected individuals. Hematoxylin and eosin, bar = 150 µm.

RLP burden in the EA abalone ($P < 0.05$), all coefficients were nonsignificant ($P > 0.05$). This relationship was also mirrored in X^2 analysis in which a higher proportion (8/9) of EA abalone with high overall RLP burdens (scores of 4–6) had metaplastic changes as the sole or partial response to RLP infections than did those with low (scores of 0–3) infections (1/7, $P < 0.01$). Several of the PC abalone were too necrotic to assess specific lesions in the digestive gland, a tissue that degrades more quickly than other tissues (Friedman, personal observation), and resulted in small samples sizes for this specific analysis. Intensity of RLP infection in laboratory EA abalone was predicted from the duration of exposure (time) with weight condition index, visual condition, condition of the foot and digestive gland, and time as independent variables in the model ($P = 0.0156$, Forward and Backward stepwise regressions). No prediction of presence of RLP infection could be made using Multiple logisitic regression analyses using all possible combinations of the five independent variables used in this study ($P > 0.500$). We did observe a significant correlation between hemocyte numbers and weight condition index of the EA abalone ($P = 0.0469$), PC abalone ($P = 0.0016$) and NC abalone ($P = 0.0015$). Correlation coefficients, however, were low to moderate and ranged between 0.2668–0.5612.

DISCUSSION

The present study describes the transmission of WS from black abalone with WS to previously healthy black abalone held in the same aquaria. The similarity in physical, histopathological, and hematological characteristics of WS between black abalone exposed to WS in the laboratory and field, combined with a lack of these signs in the negative control animals, confirmed that the experimental abalone contracted WS in this study (Haaker et al. 1992, VanBlaricom et al. 1993, Gardner et al. 1995, Shields et al. 1996, Friedman et al. 1997). These data also suggest that WS is directly transmissible between sympatric abalone by cohabitation.

WS is a chronic, slow-progressing malady in which clinical signs appear in the final stages of the disease. The presence of advanced microscopic morphologic changes throughout the pedal muscle and digestive gland of affected abalone supports this conclusion (Figs. 2–5). Our data also suggests a long incubation period for WS (~35 wk) followed rapidly by mortality (~42 days) under the conditions used in this study. As shown in Figure 6, once the animals developed clinical WS, the slopes of the mortality curves from the experimentally (EA) and field-exposed (PC) animals were very similar. However, median survival times between

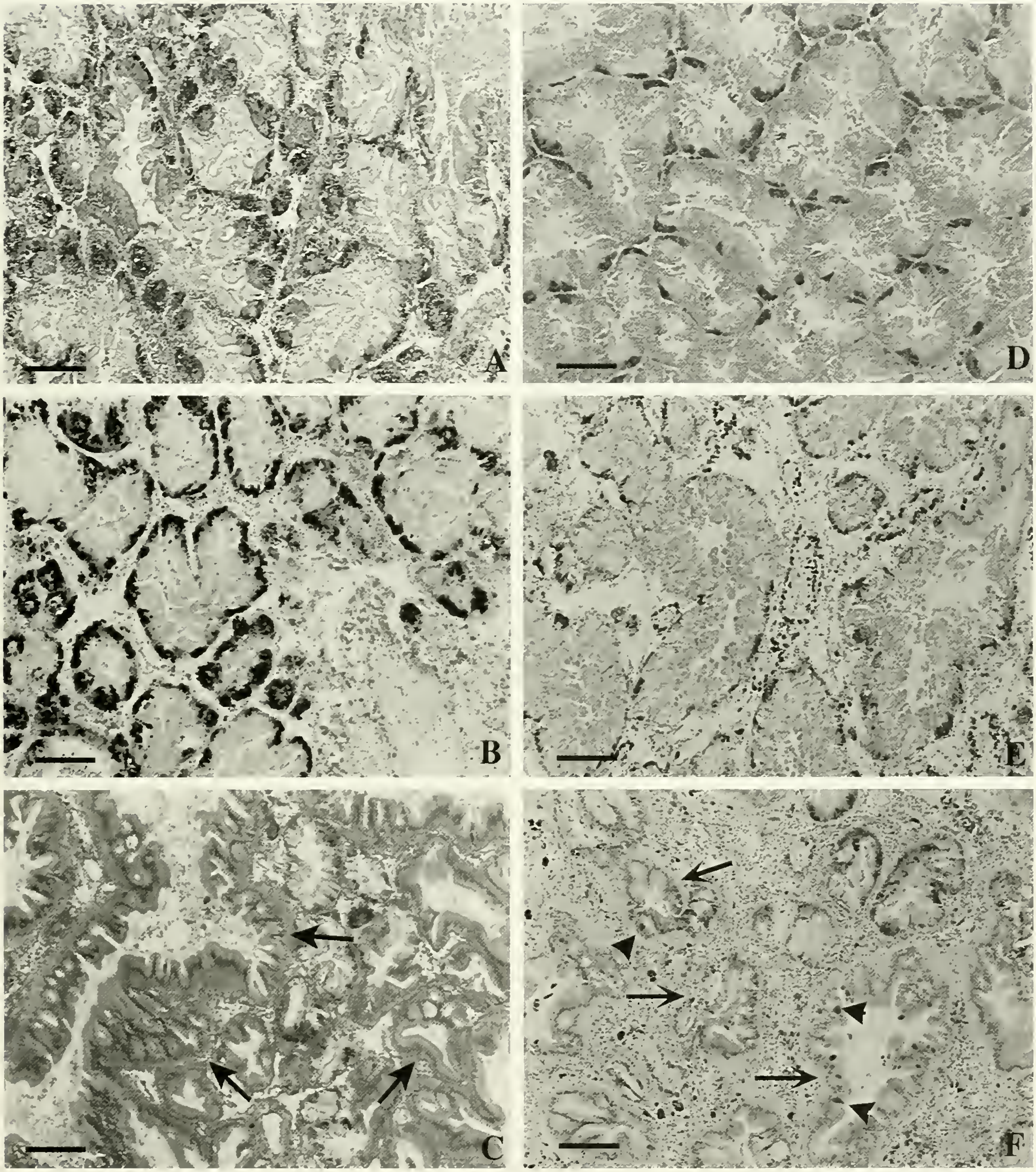

Figure 4. Microscopic anatomy of the digestive gland of black abalone with and without WS. The digestive gland of unexposed abalone from both Vandenberg (A) and Ano Nuevo Island (D) is composed primarily of terminal tubules and little transport/absorptive duct epithelia, whereas those of abalone that contracted WS in the field (B, C) and laboratory (E, F) show a reduction in numbers of terminal tubules and an increase in connective tissue and transport/absorptive duct epithelia. The digestive gland of some abalone with WS is characterized by an atrophy and loss of terminal tubules (B, E), while other individuals respond to RLP infection (arrow heads) with a transport/absorptive duct metaplasia (arrows; C, F). Hematoxylin and eosin, bar = 150 μm.

these two groups were quite different (41 wk for Ano Nuevo and 16 wk for Vandenberg/Cayucos animals) and may be due to a variety of factors. The Ano Nuevo Island EA abalone was uninfected before initiation of the study, whereas the Vandenberg and Cayucos PC abalone were in varying stages of WS. In addition, differences in susceptibility may exist between abalone from these geographically distant locations. In an earlier study in which asymptomatic but previously exposed black abalone were col-

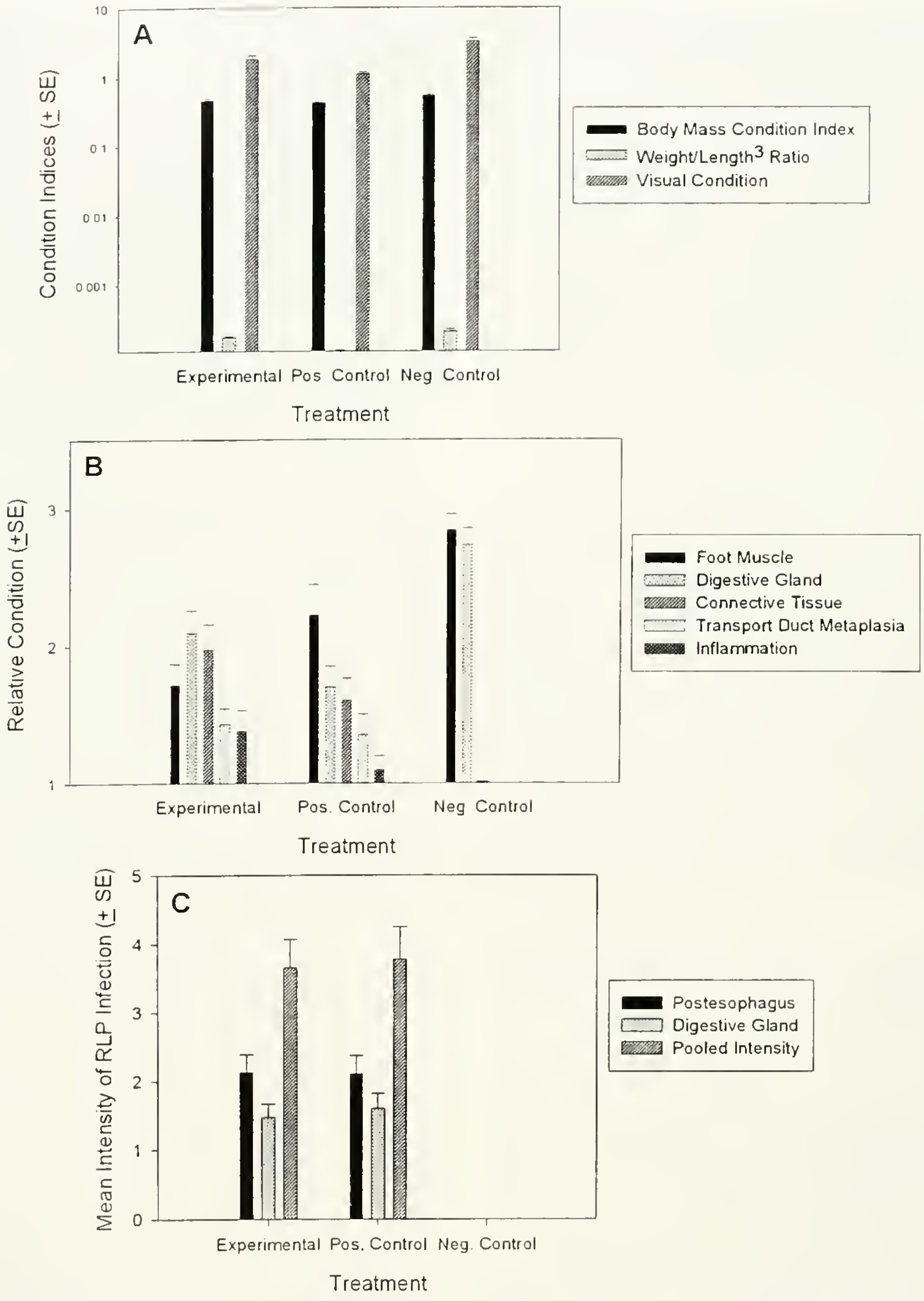

Figure 5. Microscopic morphologic changes of black abalone that acquired WS in the field (Pos. Control) or in the laboratory (Experimental) relative to unexposed (Neg. Control) animals. A, Animal condition; B, relative condition of the foot muscle and digestive gland, C, RLP intensity of infection. Each bar represents the mean of 12 abalone in each of two replicate treatments ± standard error (SE).

lected from Vandenberg and held at 18°C, the initiation of mortality at 15 wk was similar to that observed in the PC and EWS abalone in the current study and also supports a long incubation period for WS (Friedman & Fan 1998).

The observation of RLPs in the EA Ano Nuevo Island animals and not in the NC Ano Nuevo Island animals (Fig. 5) suggests that this bacterium, like WS, is horizontally transmitted by cohabitation and is the etiological agent of this disease. This is further supported by a lack of observation of any pathogens besides "*Candidatus* Xenohaliotis californiensis" in any abalone examined in this study. Transmission of this RLP is thought to be via a water-borne/fecal-oral route because of the presence of bacterial foci in the digestive epithelium and the observation of both intact and lysed RLP foci in lumina of the digestive tract (unpublished observations). Mortalities of the European Saint-Jacques scallop, *Pecten maximus*, have been associated with a branchial RLP infection (Le Gall et al.

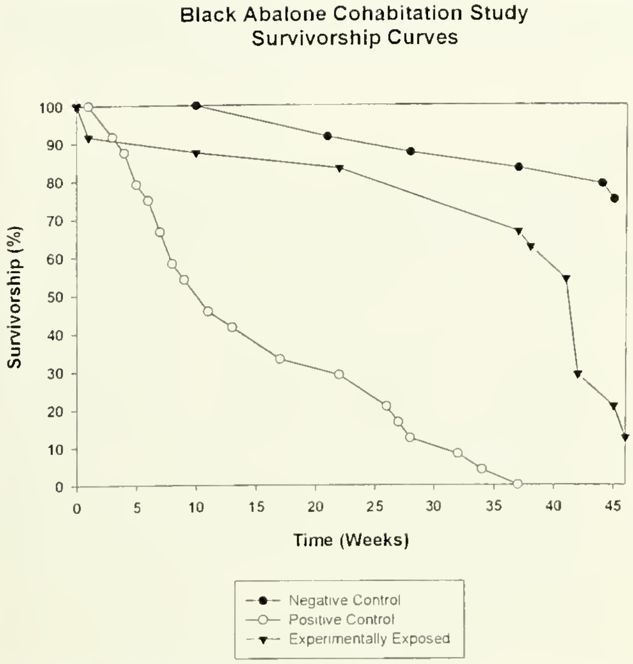

Figure 6. Survivorship curve of abalone in the cohabitation study. The closed circles represent the negative control animals, the open circles represent positive control animals, and the closed triangles represent the (EA) Ano Nuevo Island animals in the experimental treatment.

1988, 1991). Transmission of this scallop-pathogenic RLP via horizontal, water-borne transmission has also been documented (Le Gall et al. 1991). Field and laboratory studies suggested that transmission of the scallop RLP occurred between ~5–28 wk of exposure (Le Gall et al. 1991). Additional field studies reported heavy RLP infections in scallops during the winter months followed by mortalities in the spring (Le Gall et al. 1991), suggesting a relatively long incubation period for the scallop rickettsial disease as we have observed for the RLP-induced WS in this study.

The relationship between the RLP and WS in black abalone is complex as evidenced by higher proportions of mortality and clinical WS in groups of abalone either exposed to WS-affected animals or infected with the RLP. With one exception, a lack of significant correlation existed between intensity of RLP infection and WS in both the experimental and PC animals coupled with a lack of ability to predict intensity of RLP based on gross or histologic signs that characterize WS (regression models). The single significant positive correlation between overall intensity of RLP infection and degree of metaplasia in the EA abalone that responded, in part, with this morphologic change suggests that sustained high RLP burdens may lead to metaplasia in black abalone as has been observed in red abalone (Moore et al. 2000). This relationship was not observed in the small number (10) of PC abalone in which the presence or absence of metaplasia was quantified; the small sample size may account for an inability to detect a relationship. However, when the overall DG condition (alteration from normal, including all three specific morphologic changes) was assessed in the EA and PC, these relationships were not observed. This lack of correlation between RLP infection and DG overall condition in black abalone may relate to the host response to RLP infection (primarily degeneration of digestive tubules) (Figs. 4 and 5) combined with the high turnover rate of the target tissue (digestive epithelia) infected by the WS-bacterium relative to the bacterium's growth rate. Significant correlations between intensity of RLP infection and degree of WS have recently been observed in both field and laboratory studies using wild and cultured red abalone (Moore et al. 2000, Friedman, unpublished observation). The authors also suggested that differences in correlations between intensity of RLP infection and disease in red and black abalone might relate to species differences in host response to infections. Red abalone respond to the RLP infections predominantly by a metaplastic change in which digestive gland tubules are replaced by transport duct epithelium (Moore et al. 2000). Black abalone respond to RLP infection by a combination of digestive tubule degeneration and, to a lesser extent, transport duct metaplasia (Gardner et al. 1995, Friedman et al. 1997) (Figs. 4 and 5). Both of these tissue changes result in a loss of key functional tissue in the digestive gland, the terminal tubules (Voltzow 1994), which may lead to starvation and account for the utilization of foot muscle as an energy source followed by death as observed in abalone with WS (Friedman unpublished data.). As the RLP infects transport duct epithelia and not terminal digestive tubules, this may result in an increase in RLP intensity of infection in red and not in black abalone as the infections progress and clinical disease develops. Figure 5, however, does illustrate alterations in condition indices and changes in the condition of the foot and digestive gland only in abalone with RLP infections. This provides further evidence that the RLP is the etiological agent of WS. RLPs have been associated with atrophy and degenerative changes in other invertebrate species (Min & Benzer 1997).

Infections with RLPs have been reported in a variety of molluscs and crustaceans, including the sea scallop, *Placopecten magellanicus* Gmelin (Gulka & Chang 1984a), the blue mussel, *Mytilus edulis* Linné (Gulka & Chang 1984b), the manila clam, *Tapes japonica* Adams and Reeve and the Japanese scallop, *Patinopecten yessoensis* (Elston 1986), the European flat oyster, *Ostrea edulis* Linné (Friedman et al. 1989), the black abalone, *Haliotis cracherodii* Leach (VanBlaricom et al. 1993), and the penaeid shrimp, *Penaeus marginatus* Randall (Brock et al. 1986). These infections varied greatly in tissue specificity (nonspecific to highly specific), length of incubation period, and pathogenicity, ranging from no apparent harmful effects to lethal effects in the host (Gulka & Chang 1984a, Brock et al. 1986, Frelier et al. 1993, Gardner et al. 1995, Bower et al. 1996). In addition, the pathogenicity of a specific RLP has been shown to vary between host species (Brock et al. 1986). As in these studies, which document that RLPs are pathogenic for marine invertebrates, our data provides evidence that "*Candidatus* Xenohaliotis californiensis," the recently identified RLP observed in abalone in California, is the etiological agent of WS. Future studies that examine the interaction between host gastrointestinal cells and the RLP may provide insight into the cellular physiology of the host and the physiology and disease mechanisms of the bacterium.

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HISTOPATHOLOGICAL EVALUATION OF THE YELLOW ABALONE *HALIOTIS CORRUGATA* AND THE BLUE ABALONE *HALIOTIS FULGENS* FROM BAJA CALIFORNIA, MÉXICO

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ABSTRACT The yellow abalone *Haliotis corrugata* and the blue abalone *Haliotis fulgens* are caught in Baja California. Emerging diseases are affecting the fishery of abalone in several countries around the world. To determine the health status of the yellow and the blue abalone in Isla de Cedros and Islas San Benito in Baja California, México, a histopathological survey of commercial stocks of these species was conducted. The results showed the presence of bacterial foci in epithelial cells of the digestive tract resembling to the intracellular bacterium "*Candidatus* Xenohaliotis californiensis," considered to be the etiological agent of the Withering Syndrome (WS). The prevalence was higher in the blue abalone than in the yellow abalone, 100% and 63%, respectively; and their presence was not correlated with the external signs of WS. These bacteria were found in WS symptomatic and nonsymptomatic abalone. Protozoans and copepods were found between the branchial filaments, with a maximum prevalence of 79% and 37%, respectively; gregarines were also found with a prevalence of 29%. With the exception of the intracellular bacterium, the other organisms seem to be innocuous for the studied abalone.

KEY WORDS: abalone, *Haliotis fulgens*, *Haliotis corrugata*, histopathology, Rickettsia, withering syndrome

INTRODUCTION

Abalone production in Baja California (México) was 365 metric tons in 1998, reaching a value of about 36 million US dollars. The abalone fishery has been one of the main sources of economic resources in the Peninsula of Baja California. Currently, there are more than 1,300 direct employees involved in this activity (Ponce et al. 1998). Two species comprise 97% of this fishery, the blue abalone *Haliotis fulgens* and the yellow abalone *Haliotis corrugata*. Other abalone species included in this production are the black abalone *Haliotis cracherodii*, the red abalone *Haliotis rufescens*, and the white abalone *Haliotis sorenseni* (Ramade et al. 1998). During the last few years, an important decrease in the production has been recorded, which has been related to overfishing, inefficient application of management regulations, and environmental fluctuations that have favored the development of some diseases (Haaker et al. 1992, Vanblaricom et al. 1993, Olivas-Valdéz & Cáceres-Martínez 2002). In 1984, dramatic mortalities of California black abalone were recorded after the occurrence of El Niño. Lately, it has been suggested that the agent responsible for those mortalities was an intracellular bacteria from the order Rickettsiales (Gardner et al. 1995), tentatively named "*Candidatus* Xenohaliotis californiensis" (Friedman et al. 2000, Moore et al. 2000, Friedman et al. 2002). External signs in the abalone include reduction of the foot muscle in relation to shell size, loss of adherence capacity, and death. Because of the foot muscle appearance, these symptoms are called withering syndrome (WS) (Haaker et al. 1992). It has been suggested that very high temperatures may increase the mortality of abalone affected by the WS (Friedman et al. 1997). Other parasites that have been recorded in abalone species are the protozoan *Margolisiella* (= *Pseudoklossia*) *haliotis*, which infect the kidney and was first associated with the WS (Friedman et al. 1995), and the eukaryotic protist *Labyrinthuloides haliotidis*, which is pathogenic to juvenile abalone of *H. kamtschatkana* and *H. rufescens* (Bower 1987). Metazoan parasites such as the nematode *Echinocephalus pseudouncinatus* have been detected in *H. corrugata*, *H. fulgens*, and *H. cracherodii* (Milleman 1963). Damage to the host by these parasites varies and some of them cause severe mortalities (Bower et al. 1994).

Studies of the parasites and symbionts in abalone from México are scarce. These studies revealed a trematode belonging to the family Opecolidae in *H. fulgens* (Romero 1996), a boring clam in *H. fulgens* and *H. corrugata* (Álvarez-Tinajero et al. 2001), and the parasitic load of *H. rufescens* from a culture facility (Cáceres-Martínez & Tinoco-Orta 2001). The present study was conducted as part of a health evaluation of a commercial catch of *H. fulgens* and *H. corrugata* from Isla de Cedros and Islas San Benito, Baja California.

MATERIALS AND METHODS

In the first sampling (November 1997), 19 individuals of *H. corrugata* and 16 individuals of *H. fulgens* were obtained from Punta Norte, north of Isla de Cedros; in March 1998, 26 *H. fulgens* were studied from Islas San Benito; finally, in June 1998, 21 *H. corrugata* and 14 *H. fulgens* were obtained from San Agustín, south of Isla de Cedros (Fig. 1). Abalone species were obtained from commercial catches (mean size of 145 mm ± 1.73 SD) during commercial season when abalones are not in the reproductive stage. The external appearance of the abalone was recorded from a visual examination using a modification of the WS scale of Friedman et al. (1997) as: (0) no external symptoms, (1) from 1 to 10% reduction of the foot muscle in relation to the shell, (2) from 11 to 20% reduction of the foot muscle, (3) from 21 to 30% reduction, and finally (4) from 31 to 50% reduction of the foot muscle.

The visceral mass of the abalone was fixed whole in the Davidson's fixative (Shaw & Battle 1957) for at least 24 h. Seven transverse sections that contained portions of the digestive tract (pos-

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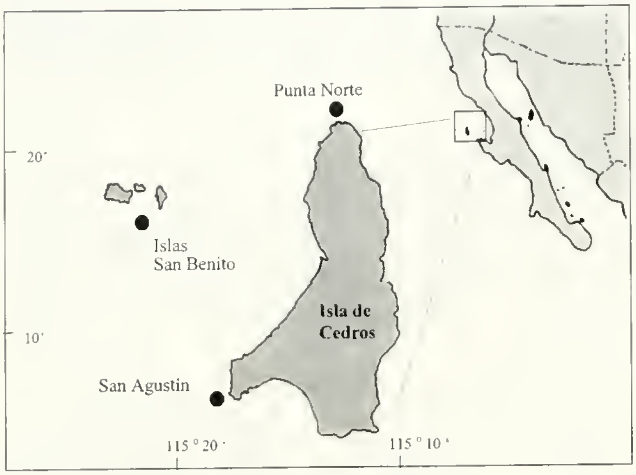

Figure 1. Map showing the sampling sites in Isla de Cedros and Islas San Benito, Baja California, México.

terior esophagus included), kidney, gonad, muscle, epipode, and gills were processed for histology. Sections of 5 μm were stained with iron hematoxylin and eosin (Gray 1954). The rickettsia-like prokaryotes (RLPs) infection intensity was estimated considering the number of bacterial foci in each histologic preparation at 200× magnification, which were enumerated using a modification of the scale of Friedman et al. (1997): (0+) no RLP, (1+) 1 to 10 RLP, (2+) 11 to 100 RLP, (3+) 101 to 1000 RLP, and (4+) >1000. Moreover, following the criteria of Friedman et al. (1997), the appearance of the digestive gland was marked as (1) normal tissues (see Antonio et al. 2000), (2) moderate tissue degeneration, and (3) severe tissue degeneration. Protozoa in the digestive tract and gill branchiae were counted in each histologic preparation. Prevalence of the other organisms was estimated as (number of infested abalone/number of abalone examined) × 100. The Mann-Whitney U Test was used to compare differences in the intensity of organisms per abalone species and the Spearman rank order correlation test was used to determine the relationship between degeneration of the digestive gland, intensity of RLP, and external signs of WS (Zar 1984).

RESULTS

RLPs

The RLPs were found in *H. fulgens* and *H. corrugata* in the three study sites. These bacteria infected the epithelial cells of the digestive tract, including the posterior esophagus, stomach, digestive diverticula, and intestine (Fig. 2a and b). The RLPs formed colonies inside a large vacuole in the cytoplasm of the infected cells. These colonies varied in shape from round to elongated and their size ranged from 17.5 × 10.8 μm to 24.3 × 10.2 μm. The host cell nucleus was displaced to the limit of the cytoplasm, the host cell became hypertrophied, and in a heavy infection there was a metaplasia of the epithelium within the digestive gland. Some host cells were disrupted and bacterial colonies were expelled to the lumen of the digestive tract (Fig. 2a).

Table 1 shows the RLP intensity of infection in *H. fulgens* and *H. corrugata* per studied site. The yellow abalone from Punta Norte showed a RLP prevalence of 63.2%; of these, 52.6% had a severe infection (3+ to 4+), including a moderate degeneration (2) of the digestive gland in 47% of the animals, whereas the remaining 52.6% of the abalone did not show signs of degeneration of the digestive gland. Blue abalone from the same area showed a RLP prevalence of 43.7%, with severe infection (3+ to 4+) in 31.3% of the animals. Only 12.4% of the infected abalone showed a moderate degeneration of the digestive gland (2). The results showed a higher grade of infection in yellow abalone than blue abalone, but this was not significant statically (U Mann-Whitney, $P = 0.33$). There was no significant correlation between the degree of degeneration in the digestive gland and the intensity of infection by RLP in the yellow and blue abalone species (Spearman rank order correlation $r = -0.19$, $P = 0.93$ and $\mathbf{r} = 0.34$, $P = 0.18$, respectively). This correlation was neither significant between the external signs and degeneration degree of the digestive gland (Spearman rank order correlation $r = 0.36$, $P = 0.12$ and $r = -0.23$, $P = 0.37$).

The blue abalone from Islas San Benito showed a RLP prevalence of 96.2%, and the infection was severe (3+ to 4+) in 92.4% of infected abalone. In 56.2% of these abalone the digestive gland was normal whereas the remaining 43.8% showed a moderate

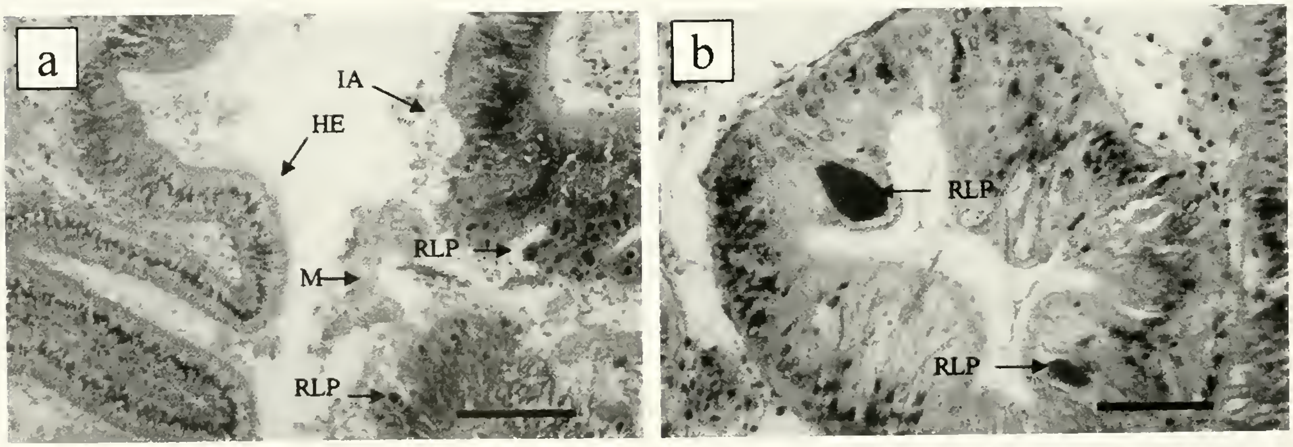

Figure 2. (a) RLP in the intestinal epithelium. An RLP-infected area is shown where the epithelium of the post-esophagus has lost its structure by hypertrophy of infected cells and rupture (IA). The epithelium in front is in normal condition (HE). There is mucus or cellular debris (M) in the intestinal lumen. Scale bar = 40 μm. (b) RLP in digestive diverticula. The hypertrophy of infected cells is compressing the neighbor cells. Scale bar = 20 μm.

TABLE 1.

Intensity and prevalence of RLP in *H. corrugata* and *H. fulgens* from different localities studied.

	Punta Norte			San Agustín	
Scale	*H. corrugata* (%)	*H. fulgens* (%)	Islas San Benito *H. fulgens* (%)	*H. corrugata* (%)	*H. fulgens* (%)
0	36.8	56.3	3.8	38.1	0
1	5.3	6.2	0	4.8	21.4
2	5.3	6.2	3.8	42.8	21.4
3	42.1	18.8	46.2	14.3	35.8
4	10.5	12.5	46.2	0	21.4
Prevalence	63.2	43.7	96.2	61.9	100
n	19	16	26	21	14

TABLE 2.

External signs scale of the WS in *H. corrugato* and *H. fulgens*.

	Punta Norte			San Agustín	
Scale WS	*H. corrugata* (%)	*H. fulgens* (%)	Islas San Benito *H. fulgens* (%)	*H. corrugata* (%)	*H. fulgens* (%)
0	89.4	75.0	88.5	47.6	71.6
1	5.3	18.8	0	4.8	7.1
2	5.3	6.2	7.7	28.6	0
3	0	0	3.8	19.0	14.2
4	0	0	0	0	7.1
n	19	16	26	21	14

degeneration (2). There was no significant correlation between the presence of RLP and degeneration of the digestive gland (Spearman rank order correlation $r = 0.34$, $P = 0.08$) or between WS external signs and the degeneration degree of the digestive gland (Spearman rank order correlation $r = 0.33$, $P = 0.08$).

The yellow abalone from San Agustín showed a RLP prevalence of 61.9% and the infection intensity was medium (2+) in 42.8% of the organisms. In relation to the degeneration of the digestive gland, 57.1% of the infected organisms had a moderate degeneration (2), 14.28% had a severe degeneration (3), and 28.6% had a normal digestive gland. There was no significant correlation between the RLP intensity and degeneration degree of the digestive gland (Spearman rank order correlation $r = -0.03$, $P = 0.86$). However, a sign correlation was observed between the external signs of WS and the degree of digestive gland degeneration (Spearman rank order correlation $r = 0.75$, $P < 0.001$). The blue abalone from the same area showed a RLP prevalence of 100%, with severe infections (3+ to 4+) in 57.2% of these abalone. Of the severely infected abalone, 28.5% showed a moderate degeneration of the digestive gland, 14.28% showed severe degeneration, and 57.14% showed a normal appearance of the digestive gland. In both species, the correlation between the RLP intensity and degeneration of the digestive gland was not significant (Spearman rank order correlation $r = 0.08$, $P = 0.75$), but it was significant between the WS external signs and the degeneration of the digestive gland (Spearman rank order correlation $r = 58.36$, $P < 0.001$). The infection prevalence from San Agustín was significantly higher in the blue abalone (U Mann-Whitney $P < 0.01$) but not from Punta Norte (Table 1).

Table 2 shows the percentage of both abalone species with external signs of the WS. At the microscopic level, all these organisms showed some tissue disorders, such as an increase in connective tissue between digestive tubules; lack of gonadal maturation and in a few occasions duct metaplasia or inflammation. The foot muscle showed a reduction in muscle fibers and loss of muscle bundle orientation, increase in connective tissue and infiltration by hemocytes. There was no correlation between the WS external signs and the presence of RLP in tissues (Table 3); in some individuals with WS external signs, infection by RLP was not detected (Fig. 3).

Ciliated Protozoan

A ciliated protozoan (Fig. 4a) was found in the mantle cavity and gill filaments of the yellow and blue abalone. The unidentified ciliates measured 26.5 μm × 12.7 μm. No histologic disorders or lesions were associated with these organisms. Prevalences were low in both species with 10.5% of the yellow and 6.3% of the blue abalones from Punta Norte (Table 4, Mann Whitney U, $P = 0.66$). Prevalences were higher at the other two locations where over 40% of the blue abalone from Islas San Benito and over 70% of both yellow and blue abalones from San Agustín were infested (Table 4, Mann Whitney U, $P = 0.25$).

Copepods

Copepods were found among the filaments of the gill where some compression and infiltration of hemocytes in the tissue was detected (Fig. 4b). The mean size of the copepods was 200 μm × 50 μm. Copepod prevalence in the yellow abalone from Punta Norte was 36.5%, there were no copepods in blue abalone from this locality. Copepod prevalence in blue abalone from Islas San Benito was 34.6%. The yellow abalone from San Agustín showed a prevalence of 19.0%, in the blue abalone only one copepod was recorded. There were no significant differences of copepod intensity between both abalone species (pooled data, U Mann-Whitney $P = 0.34$).

Gregarines

Low prevalences of trophozoites of a gregarine protozoan (Fig. 4c) were observed in the branchial epithelium, esophagus epithelium and kidney of the blue abalone (Table 4). The organisms measured 22 μm × 14μm and no host reaction against this protozoan was observed. Prevalences ranged between sites from 0 to a high of 28.7% (Table 4). The limited number of data precluded the use of a statistical test for comparison.

TABLE 3.

Spearman rank correlation between the WS external scale and the abundance of RLP in *H. corrugata* and *H. fulgens*.

	Punta Norte			San Agustín	
	H. corrugata	*H. fulgens*	Islas San Benito *H. fulgens*	*H. corrugata*	*H. fulgens*
R	0.05	0.06	0.18	−0.09	−0.008
P value	0.82	0.79	0.37	0.68	0.97

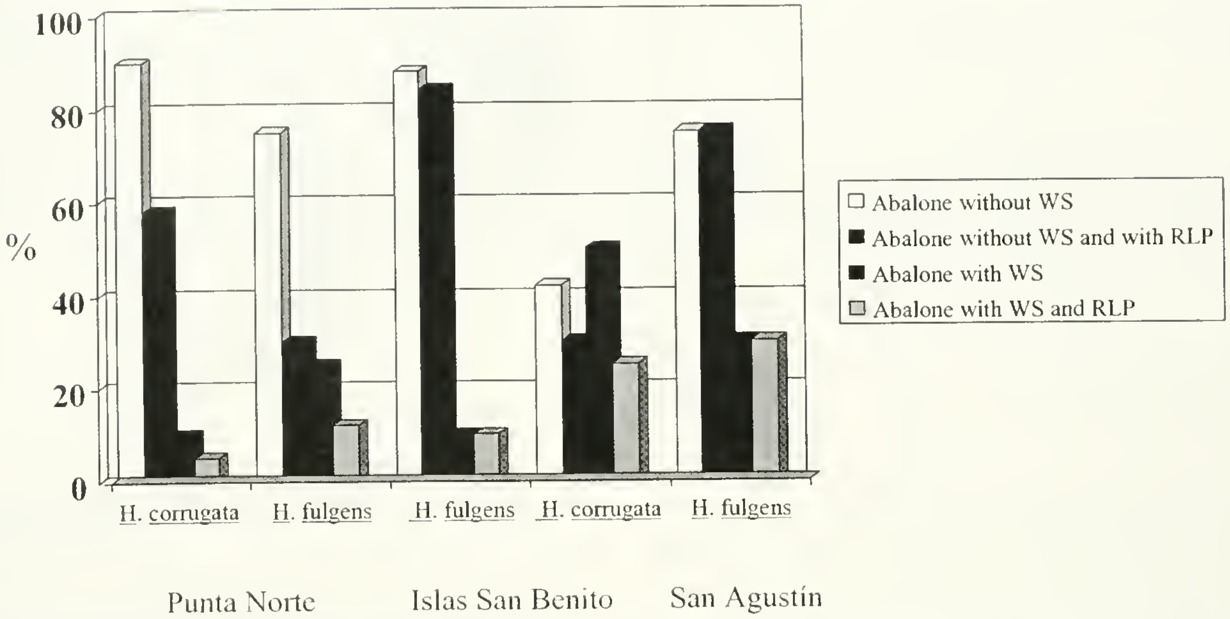

Figure 3. Percentage of the WS symptomatic and nonsymptomatic abalone and the intensity of RLP infection for *Haliotis corrugata* and *Haliotis fulgens*.

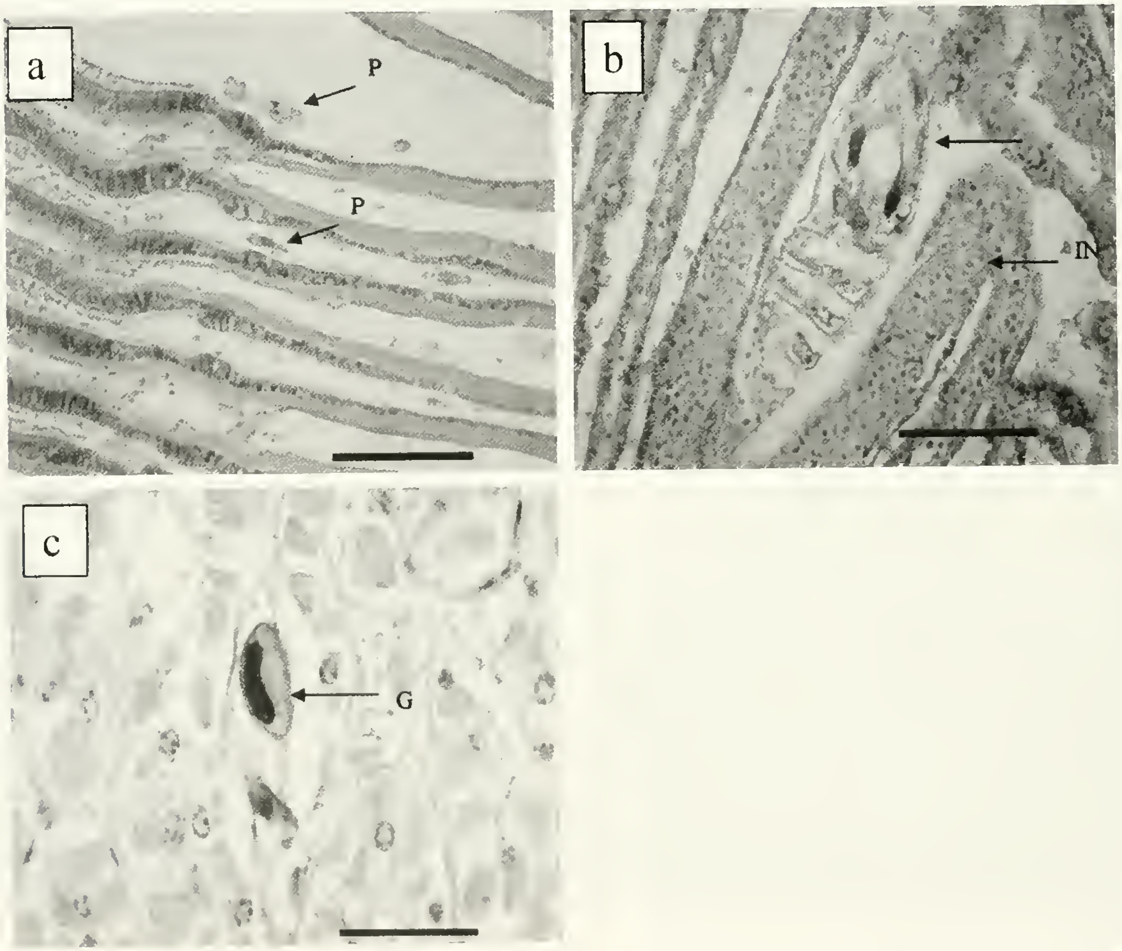

Figure 4. (a) Ciliated protozoan (P) between branchial gill filaments. Scale bar = 20 μm. (b) Copepod between gill filaments (arrow). Note the infiltration of hemocytes in filaments surrounding the copepod (IN). Scale bar = 40 μm. (c) Gregarine protozoan (G) in the right kidney of *H. fulgens*. Scale bar = 15 μm.

TABLE 4.

Other organisms in abalone *H. corrugata* y *H. fulgens* per locality.

Locality	Abalone Species	Mean	Prevalence (%)	Max. Intensity
Ciliated protozoan				
Measured: 26.5 × 12.7 μm				
Location: Mantle cavity and gill filaments				
Punta Norte	*H. corrugata*	0.1	10.5	2
	H. fulgens	0.2	6.3	4
Islas San Benito	*H. fulgens*	4.6	42.3	28
San Agustín	*H. corrugata*	62.7	71.4	594
	H. fulgens	77.2	78.5	346
Copepods				
Measured: 200 × 50 μm				
Location: Filaments of gill				
Punta Norte	*H. corrugata*	1.0	36.5	5
	H. fulgens			
Islas San Benito	*H. fulgens*	0.5	34.6	4
San Agustín	*H. corrugata*	0.3	19.0	3
	H. fulgens	0.1	7.1	1
Gregarines				
Measured: 22 × 14 μm				
Location: Branchial, esophagus, and kidney				
Punta Norte	*H. corrugata*			
	H. fulgens	0.06	6.2	1
Islas San Benito	*H. fulgens*	0.07	3.8	2
San Agustín	*H. corrugata*			
	H. fulgens	0.4	28.5	2

DISCUSSION

Cáceres-Martínez and Tinoco-Orta (2001) and Culver and Richards (1992) mentioned that the WS was observed in the black (*Haliotis cracherodii*) green (*H. fulgens*) and red (*H. rufescens*) abalone from Baja California, México; however, this is the first record of RLP infecting the tissues of *H. fulgens* and *H. corrugata*. The histologic appearance, shape, size, and targeted cells are similar to that of the intracellular bacterium "*Candidatus* Xenohaliotis californiensis" causing the WS in *H. cracherodii* and *H. rufescens* in California, (Friedman et al. 2000). Further characterization is needed to confirm this hypothesis, therefore molecular methods for RLP detection are necessary (Andree et al. 2000, Antonio et al. 2000).

The absence of a relationship between the intensity of RLP infection with the external signs of WS and the degeneration degree of the digestive gland in both abalone species was previously observed in studies on WS in black abalone (Friedman et al. 1997, Friedman et al. 2002). Moreover, Cáceres-Martínez and Tinoco-Orta (2001) found changes of the digestive gland (metaplasia) in red abalone infected by RLP but also in noninfected organisms. Only in San Agustín was the relationship between the WS external signs and the degeneration of the digestive gland significant. Friedman et al. (1997) and Friedman et al. (2002) showed that the WS disease agent requires a long incubation period before the external symptoms become apparent. This could help us to explain this contradictory result. Another possibility is the existence of subspecies or strains of RLP, some pathogenic and other nonpathogenic. Moreover, the external signs of the WS are not exclusive of this disease, other diseases or starvation conditions may result as external symptoms similar to those of the WS (Milleman 1963, Dixon et al. 1991); also, differences in susceptibility between abalone species and individuals are possible. Friedman et al. (2002) noted "studies that examine the interaction between host gastrointestinal cells and the RLP may provide insight into the cellular physiology of the host and the physiology and disease mechanisms of the bacterium."

Vanblaricom et al. (1993) also found a suctorian protozoan in gill squashes from the black abalone *H. cracherodii*, both in healthy specimens and in organisms affected by the WS. Ciliated protozoa are common in marine invertebrates, and most of them are considered as commensals (Lauckner 1983). Our histopathological results did not show any host response to the protozoa, and the low numbers suggested a lack of potential for a health problem.

To our knowledge, this is the first record of a copepod associated with abalone species. Copepoda and mollusk associations have been widely studied, *Mytilicola intestinalis* and *Pseudomyicola spinosus* are two of the most studied copepods associated with bivalve mollusks. These copepods may produce severe damage to the epithelial cells of the digestive tract, they may produce encapsulations in the connective tissue of the digestive gland, and they may produce obstruction and rupture of reproductive follicles (Sindermann 1990, Olivas-Valdéz & Cáceres-Martínez 2002). In this study, no evidence of copepods inside of the abalone digestive tract was found, but some inflammation in the gills of abalone was observed in some cases. Their prevalence suggests a symbiotic association but more studies are needed for determining a kind of interaction between the copepods and the host.

In the blue abalone, we recorded the presence of gregarines in the renal tissue and no histologic evidence of reaction of the host against the protozoan was observed. Some authors (Vanblaricom et al. 1993) found similar protozoa in the black abalone, *Haliotis cracherodii* from California and from Baja California, México. In both studies, no evidence of pathogenic activity was found. A similar gregarine infection has been observed in mussels, oysters and other marine bivalves, in some cases the infection has been associated with a focal, benign inflammatory response, without significant health effects (Bower et al. 1994).

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EFFECT OF EL NIÑO 1997–98 ON THE SNAIL *ASTRAEA UNDOSA* (WOOD) POPULATION ALONG THE BAJA CALIFORNIA WESTERN COAST

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ABSTRACT The effect of the 1997–98 El Niño on the snail Astraea undosa population along the Baja California western coast on biomass changes in June 1997 and July 1998 was evaluated. In 1998, dead snails >40 mm basal diameter were collected and their basal diameter measured. Individual weight and density (snails/10 m^2) decreased significantly in 1998 from those registered in 1997, with an average 47% decrease in biomass. We discuss the possible causes of those changes as a function of the disappearance of the Macrocystis pyrifera forest and of an extended period of positive anomalies of the sea surface temperature that were present previous to July 1998.

KEY WORDS: El Niño 1997–98, snail, *Astraea undosa*, biomass, Baja California.

INTRODUCTION

The 1997–98 El Niño has been considered the most intense of the twentieth century (Kerr 1998, McPhaden 1999). It was detected along the Baja California western coast through positive anomalies of the SST >3°C. In July 1998, we observed dying as well as dead snails *Astraea undosa* (Wood) in the intertidal zone and in fishing banks. No kelp forests *M. pyrifera* (Agardh) were found in Bahía Tortugas from October 1997 to April 1998 (Ladah et al. 1999). This species together with *Eisenia arborea* (Aresch) are the food source and habitat of *A. undosa*, *A. turbanica* (Dall), and other species of herbivores of commercial interest such as the abalone *Haliotis fulgens* (Philippi) and *H. corrugata* (Wood) (Guzmán del Próo et al. 1991).

In July 1998 we monitored the *A. undosa* populations located on the Baja California peninsula western coast to evaluate the effect of the 1997–98 El Niño. This region carries the highest abundance of *A. undosa*, where >80% of its catch is obtained (Gluyas–Millán et al. 2000). The size structure of live and dead snails, average size and weight, density and biomass of July 1998, were contrasted with those obtained from the same fishing banks in June 1997. We assumed that the sampled snails in July 1998 reflect the effect of the 1997–98 El Niño.

MATERIALS AND METHODS

The study area is located between Punta Eugenia (27°51′N–115°04′W) and Bahía Asunción (27°08′N–114°17′W) (Fig. 1). The area was divided into three zones and three fishing banks were selected out of each one (Table 1). We believe the selected geographical area was adequate because it is the main area of abundance of *A. undosa* in its distribution range along the Baja California western coast, and also because of the accessibility of the sampling places, and because this is where the main snail fishing banks are located. Monitoring was done in June 1997 and in July 1998 in the same places. As an environmental indicator of the 1997 to 1998 El Niño conditions we used the SST monthly anomalies of 1996 to 1998 from the deviations of the monthly average of the ten-year period before 1997 for the Bahía Tortugas area. The SST values were extracted from NOAA COADS records (Comprehensive Ocean and Atmospheric Data Set) for the Bahía Tortugas area.

The corners of the polygons that comprise the area fishing banks were estimated using a GPS receiver (Garmin model 45XL) (Table 1). The monitoring in June 1997 and July 1998 consisted of conducting up to 10 dives at each fishing bank (a, b, c), using a Hooka diving equipment at depths between 5 and 20 m. In each dive, all snails inside a 10-m^2-sample unit (SU) were taken and the basal diameter (BD) of each snail was measured, grouping them in intervals of 5 mm to obtain the size frequency distribution. A subsample was obtained by randomly selecting up to five snails of each size interval and measurements were made of the BD of the shell (±1 mm) and the weight (±1 g) of the adductor muscle to estimate the biomass.

The area of each fishing bank was transformed to SUs. Snail density for each fishing bank was stratified according to the snail number frequency distribution in each SU (Cochran 1978). The number of density intervals (DI) was determined with the Sturges rule (Daniel 1984).

An estimate of the population total abundance of snail was obtained according to the estimators of the random stratified sampling technique (Schaeffer et al. 1987, Conquest et al. 1996):

Average population estimate was

$$\bar{y}_{st} = \frac{1}{N} \sum_{i=1}^{L} N_i \bar{y}_i$$

Total abundance population estimate was

$$N\bar{y}_{st} = \sum_{i=1}^{L} N_i \bar{y}_i$$

Where N = total number of SU, N_i = SU number in the fishing bank$_i$, L = number of density intervals, i = density interval average.

From the total population abundance estimation, the biomass was calculated by size interval with the following relationship:

$$BIT = (\alpha T^{\beta}) * \left(\sum_{i=1}^{L} N_i \bar{y}_i \right) * \Phi_T$$

where BIT = size interval biomass, α and β = weight-size relationship parameters, $(\sum_{i=1}^{L} N_i \bar{y}_i)$ = biomass in snail numbers, Φ_T

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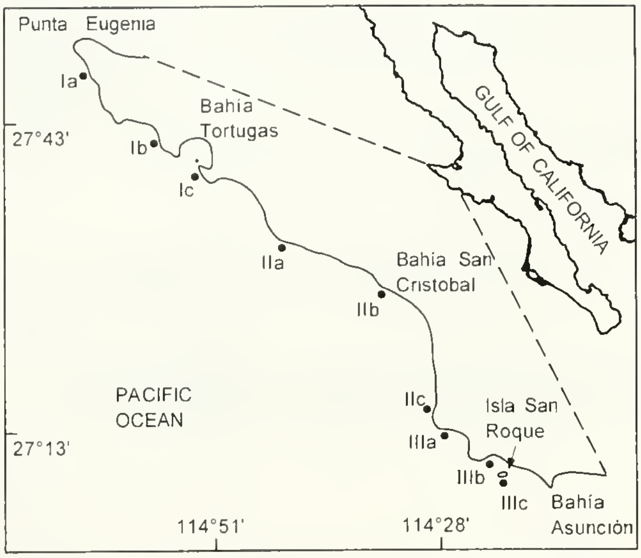

Figure 1. Baja California western coast and location of sampling sites. The names of the sampling locations are in Table 1.

TABLE 1.

Astraea undosa fishing banks by zone on the Baja California western coast. See Figure 1 for geographic location.

Zone	References above the Fishing Banks Location	Fishing Banks Dimensions (m^2)
I a	La Banderita-Punta Quebrada	318,000
I b	Punta Prieta-La Cantina	318,000
I c	Los Morros-Clam Bay	318,000
II a	Cabo Pruneda-Cerro del Calvillo	104,000
II b	El Muerto-Punta Prieta-Salado	124,000
II c	San Pablo	136000
III a	Punta San Pablo-Puerto San Pablo	51,000
III b	Vuelta del Cerro-Reef-Crucecita	63,000
III c	Isla San Roque	75,000

= relative frequency accumulated by size interval from total snails measured.

The total biomass for all the size intervals was estimated by:

$$Bt = \sum_{T} BIT$$

To determine the empty shells of snails from individuals that died in 1998, and that did not come from the commercial catch of that year or earlier, observations of presence-absence of fauna and flora epibiotics were made inside the shell. Additionally, we know that the snails are not killed in the fishing banks, and furthermore the snail-fishing season was suspended in 1998, except in Bahía Tortugas and Isla Natividad. The latter is 7.5 km NW off Punta Eugenia (Fig. 1).

RESULTS

In July 1998 we observed that *A. undosa* snails at depths of 5 to 20 m were dying. In addition empty shells were found in the intertidal zone, and few *M. pyrifera* stalks between Punta Eugenia and Bahía Asunción. July 1998 was preceded by a period of intense warming, the SST rose to 26°C (Fig. 2A). SST positive anomalies >3°C persisted from September 1997 to January 1998 (Fig. 2B) and the *M. pyrifera* forests disappeared in Bahía Tortugas. The June 1997 monitoring was not preceded by conditions of intense warming (Fig. 2A, B), and the snails showed normal conditions, the appearance of the adductor muscle was not flabby as in July 1998; dead snails were not observed, and the *M. pyrifera* forests were present.

Dead snails in the intertidal area in July 1998 showed remains of viscera, a flabby adductor muscle, the opercula fixed to the muscle, and absent epibiotic fauna and flora in the shell internal surface. This indicates that these snails were not the product of fishing and that they had recently died.

In each sampling zone in July 1998, the range and the size frequency distribution of the living as well as of the dead snails were similar ($P > 0.05$, KS-test) (Fig. 3): indicating the 1997–98 El Niño affected proportionally those snails whose BD was >40 mm. This result is reinforced when contrasting the size frequency distribution of 1997 and 1998 (Fig. 3), which showed no significant differences ($P > 0.05$, KS-test). This suggests that the effect of El Niño 1997 to 1998 was not selective for snails >40 mm BD.

In 1997 and 1998 the mean BD of the live snails by zone was not significantly different ($P > 0.05$, t-test) (Fig. 4). This confirms the previous results that indicate an effect on the population without size selectivity. The mean individual weight, density, and biomass were significantly greater in 1997 than in 1998 (Fig. 4) ($P < 0.05$, t-test). This shows a negative effect on individual weight and density of the snail and in consequence the 1998 biomass decreased. The magnitude of the biomass was variable among the zones because of the different area sizes (Table 1). The biomass decreased 47% on average in 1998 as compared with that registered in 1997. Because of this decrease, the commercial catch season was suspended in 1998 in most of the study area.

Observations made in June 1998 of the *A. undosa* stomach contents indicated important proportions of silt and crustose cor-

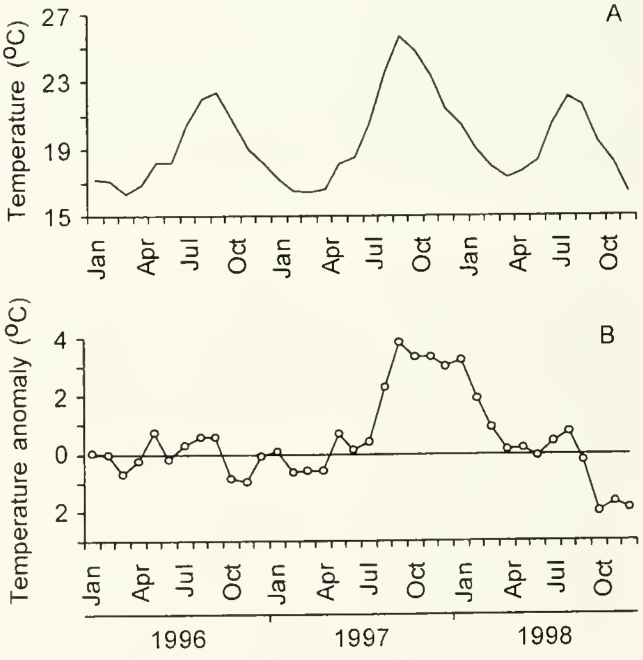

Figure 2. (A) Monthly sea surface temperature (SST), and (B) SST anomalies from 1996 to 1998. Data obtained from NOAA COADS records of the Bahía Tortugas area.

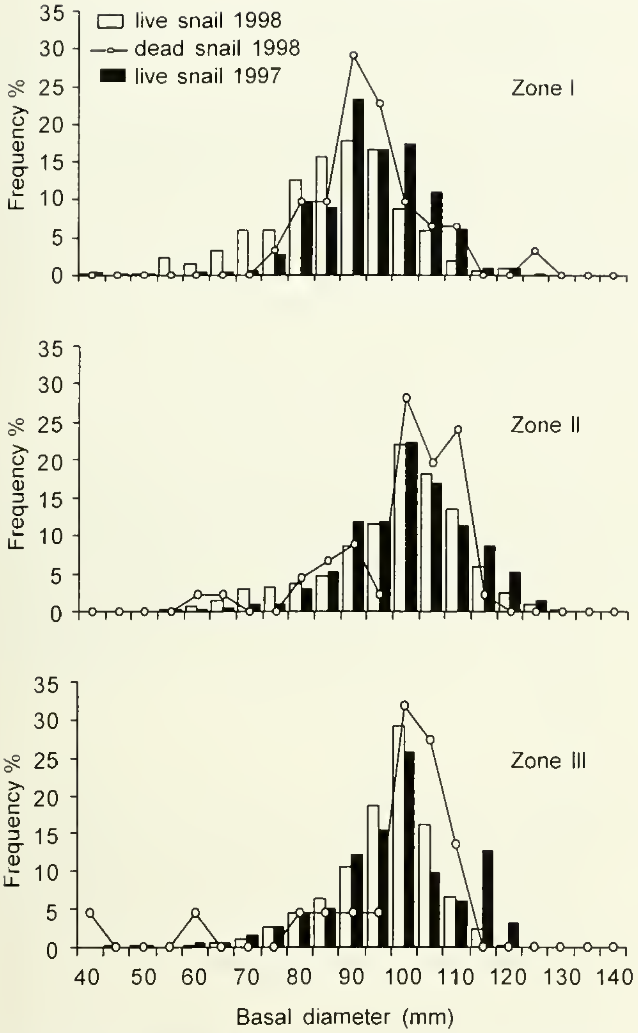

Figure 3. Size frequency distribution of the snails *Astraea undosa* from the Baja California western coast in June 1997 and July 1998.

alline algae (*Lithothamnium* or *Lithophylum*), articulated coralline algae (*Bossiella orbigniana* [(Manza) Johans], and *Corallina officianalis* [L.]). They were the predominant flora then and are of temperate and subtropical affinity (Guzmán del Próo et al. 1991). These algae covered a great extension of the rocky surface at all the levels of the sampling depths. In June 1997 the diet components were varied, and we observed in the snail stomachs an important proportion of *M. pyrifera*, which was still present in the area (Raúl Reyes Tisnado, pers. com.). It was not possible, however, to quantify the stomach contents.

DISCUSSION

Though the 1997–98 El Niño was detected from summer 1997 in the northern Pacific Ocean (Liu et al. 1998, Connolly & Roughgarden 1999), its impact on the *A. undosa* population along the Baja California western coast was not evident until the summer of 1998, when it became obvious that the biomass of snails had decreased and dead snails were seen. These events were also reported at La Bocana, 70 km south of Isla San Roque (Daniel Aguilar Osuna, pers. com.).

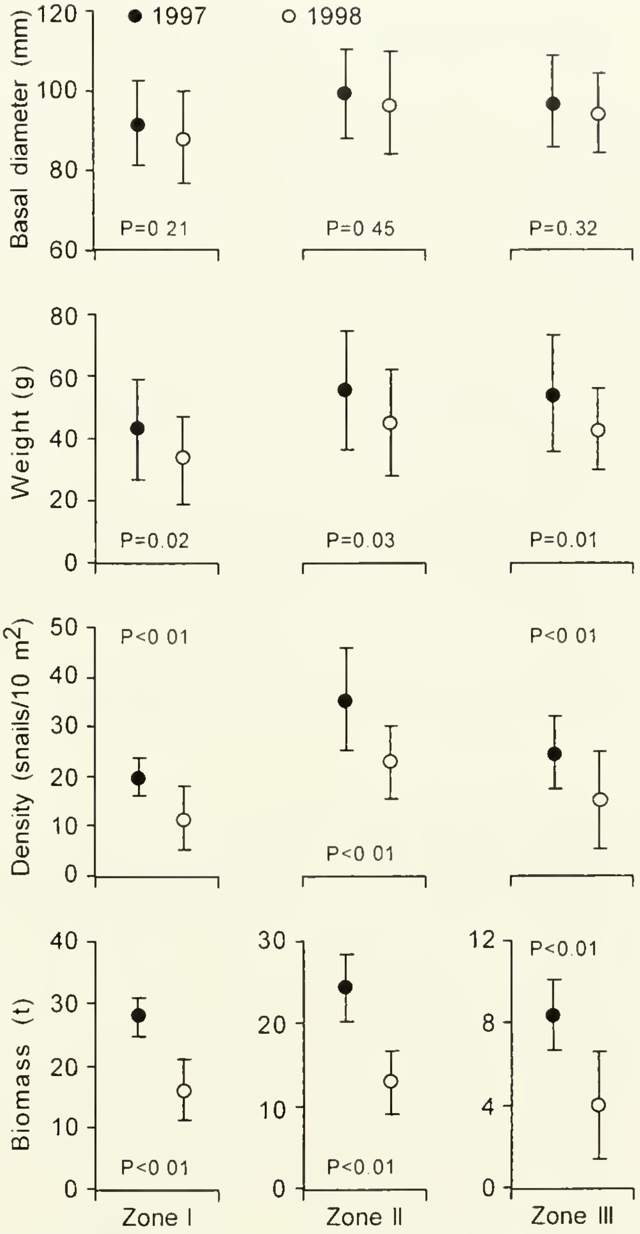

Figure 4. Average and standard deviation of the basal diameter (mm), individual weight (g), density (snails/10 m^2), and biomass (t) by sampling zone of the snail *Astraea undosa* from the Baja California western coast in June 1997 and July 1998.

The results and the observations suggest that the observed mortality and the diminished biomass that occurred in 1998 were caused by weakness and starvation because of the absence of *M. pyrifera*, and a possible long-warming period temperature effect that contributed to the disappearance of kelp forest (Ladah et al. 1999). Nearly all snails >40-mm BD are mature individuals, because the minimum reproductive size is attained at 58-mm BD (Belmar-Pérez et al. 1991). Those snails would be >2-years-old according to the BD-age relationship reported by Gluyas-Millán et al. (2000). The individuals <40 mm-BD are in the cryptic phase (Gluyas-Millán et al. 1999, Gluyas-Millán et al. 2000) and they were not observed in the study period.

The biomass decrease of the *A. undosa* population from 1997 to 1998 was the result of both an increased natural mortality and decreased individual weight and can be attributed to two factors: 1) The prolonged disappearance of the forests of *M. pyrifera* from October 1997 to April 1998 (Ladah et al. 1999) affected the habitat and the food availability of *A. undosa*. This alga is one of the main food sources of *A. undosa* (Guzmán del Próo et al. 1991) and also provides protection against predators; and 2) The physical factors associated with 1997–1998 El Niño had a density-independent influence because their potential effect in each member population >40-mm BD is the same regardless of population size (Sissenwine 1984). Therefore, the increase of SST and the change of the predominant diet components could have affected the mollusk physiology and consequently its growth efficiency.

The decrease of the 1998 snail biomass resulted in an important economic loss for the fishing sector. Because of the low yields and the poor quality of the adductor muscle fishing ceased at most of the fishing banks. These fishing yields decreased from 166 t in 1997 to 44 t in 1998.

El Niño negative effects have been documented in the eastern Pacific Ocean on the productivity, abundance and distribution of invertebrates, the recruitment failure of some fish, and the devastation of the *M. pyrifera* forests (Lenarz et al. 1995, Teagner & Dayton 1987, Ladah et al. 1999). There are also positive effects in the recruitment of the balanus *Balanus glandula* and *Chthamalus* spp. along the California coast attributed to an increase of the transport toward the coast (Connolly & Roughgarden 1999).

For *A. undosa* along the Baja California western coast, the 1997–1998 El Niño negatively affected its survival. The effects of this on the structure and function of the *M. pyrifera* forest ecosystem where *A. undosa* is an important species, cannot be ignored, even when the consequences of these impacts on the structure and function of the ecosystem are difficult to quantify (Gislason et al. 2000). Moving from population to the ecosystem level increases complexity and we lack the general rules to help us predict changes in the abundance of the interacting species (Lawton 1999). However, the annual evaluations of the *A. undosa* biomass from the National Institute of Fishing (INP-Mexico) will allow the evaluation of the recovery of *A. undosa* associated with the *M. pyrifera* forests.

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REPRODUCTION OF THE SPOTTED PINK SHRIMP, *FARFANTEPENAEUS BRASILIENSIS* (DECAPODA: PENAEIDAE)

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ABSTRACT Based on the information from catches in Contoy, QR, Mexico (February–December 1993), the reproductive cycle of the spotted pink shrimp, *Farfantepenaeus brasiliensis*, is described. Population size structure shows that fishery exploitation affects small sizes. Females were more numerous than males, particularly during months of greatest reproductive activity. The period for ripe females period occurs from February to August, peaking in March and April. Main recruitment offshore follows from this spawning population. The estimated size at first reproduction averaged 148 mm. According to this estimation, 65–80% of the females have not been able to reproduce at least once before being caught. We observed a significant relationship (Pearson $P = 0.05$) between the larger size shrimps and a proportionally larger number of ripe females with depth from February to August. Based on catch-per-unit-effort indices (CPUE) of ripe females, we propose the region located between 21°40′ to 22°10′N and 86°30′ to 86°50′W, at depths of approximately 25 to 40 fathoms (33 to 59 m) as the main spawning area for the spotted pink shrimp, *F. brasiliensis* in the northeast region of Contoy, QR, Mexico.

KEY WORDS: spotted pink shrimp, reproductive dynamics, maturity, sex ratio

INTRODUCTION

The spotted pink shrimp, *Farfantepenaeus brasiliensis* Latreille 1817, is a commercially important species in Mexico where the main fishery is located in the Mexican Caribbean along the coast of the state of Quintana Roo. This important commercially area is near the northern end of the species geographic distribution which extends from Florida, USA, to the Rio Grande do Norte in Brazil (Pérez-Farfante 1969, 1988).

In the Mexican Caribbean, *F. brasiliensis* is the target of a trawl fishery. Landings also consist of rock shrimp, *Sicyonia brevirostris*, which is of lesser commercial importance and represents approximately 55% of the total catch. Recorded production of spotted pink shrimp is low compared with the penaeid shrimps harvested in the Gulf of Mexico (*F. aztecus, F. duorarum and Litopenaeus setiferus*). However, fishery exploitation of the spotted pink shrimp represents an important regional activity accounting for more than 45% of the crustacean production of the state (Anonymous 1995).

The annual landings of spotted pink shrimp have declined since 1984–1987, when maximal production was attained (309–474 Metric tons), falling to less than 200 tons during recent years. Similar to other tropical penaeid stocks (Gracía et al. 1997), the spotted pink shrimp, *P. brasiliensis*, has reached its maximal sustainable exploitation levels, or even surpassed them, posing serious problems for the resource. Despite its regional importance, the spotted pink shrimp fishery has not been described adequately, and the available literature about its biology and ecology is scarce. There have been some previous studies about growth (Arreguín Sánchez 1981a), gonad development (Sandoval-Quintero and Gracia 1998), general distribution (Porras-Ruiz et al. in prep.) and some fishery aspects (Arreguín Sánchez 1981b, Soto-Aguirre in prep.), but information about main population processes is still lacking.

Basic information on the reproductive biology of this species is needed to establish adequate management policies to sustain this resource. This study presents data on the temporal pattern of reproductive activity and distribution of females of *F. brasiliensis* in different stages of gonadal maturation in relation to depth.

STUDY AREA

The *F. brasiliensis* fishery in Quintana Roo, Mexico operates for most of the year, except for the closed season which occurs around August and September since 1994. The main fishing location is on the continental shelf of the Yucatán Peninsula, northeast of Contoy Island, between 21°27′ to 22°18′N and 86°34′ to 87°01′W (Porras-Ruiz et al. in prep.). The marine bottom is sandy, with large coralline formations that limit the operation of trawlers to regions locally called "blanquizales" which are free from rocks and corals. It is reported that *F. brasiliensis* is also distributed over coralline bottoms, hence part of the population is not subjected to fishing gears (Soto-Aguirre in prep.).

MATERIAL AND METHODS

Spotted pink shrimp were collected from the fishing area of Contoy from February to December 1993 onboard shrimp trawlers. The monthly information obtained consisted of: sampling coordinates, depth and duration of each of the tows, total length of the shrimp (TL, measured from the tip of the rostrum to the tip of the telson, at a 0.5 mm precision), sex, and gonadal stage of females by visual inspection according to a gonad color key based on a dissecting analysis (Silva Neto et al. 1982, Sandoval-Quintero and Gracia 1998). The calculated appropiate minimum size of the monthly sample taken from the commercial catch was 90 individuals (Daniel, 1987), at a confidence level of 95% ($P = 0.05$). Sex ratio for each month was analyzed through a χ^2 independence test (Méndez et al. 1984).

Periods of greatest reproductive activity during the year as demonstrated from the commercial catch were determined from an analysis of gonadal development of females. Females were grouped according to maturation stage as non-ripe (I and II) or ripe (III and IV) to avoid misclassification of gonadal stage determination. The last two stages indicate proximity of the spawning period, and can only be distinguished precisely by histologic sectioning of the gonads (Sandoval-Quintoro and Gracia 1998).

We used catch-per-unit effort (CPUE) as an index of abundance of these stages to analyze variations in the proportion of non-ripe and ripe females, estimating it monthly from each tow in the following manner: $[\mathrm{CPUE}_{\mathrm{I,M}}] = (n/t)(\%\mathrm{I,M})$, where n = number of females in the sample, t = duration of trawl (hr) and %I,M = percentage of non-ripe (I) or ripe (M) females, expressed in terms of total number of females per hour towed.

Size at first maturity was defined as mean total length of females when 50% had reached maturity. The relationship between size distribution and depth was analyzed through a linear regression analysis (Zar 1974, Bhattacharya & Johnson 1977). We calculated the correlation coefficient (r) to determine whether there was a statistical association between the gonad stage and depth for those months in which the variables were not independent (Everitt 1977). Localities were classified arbitrarily according to whether they yielded >50% non-ripe or ripe females.

RESULTS

Population Size Structure

Total length for entire study ranged from 35 to 205 mm both sexes, with females typically reaching greater maximum lengths than males (Fig. 1). Both sexes size distribution was unimodal during most of the months. When two modes were found they corresponded to large females and small males groups.

Male and female size distribution varied along the period studied (Table 1). Usually female modal sizes were greater than 140 mm TL. Female average total length varied from 129 mm (inmature females) to 166 m (ripe females). Male average lengths varied from 116 to 133 mm TL. Modal frequencies were close to the average in most of the months. May and September modes suggest the presence of two cohorts (Fig. 1).

Sex Ratio

Sex ratio varied in the months sampled (Table 2). Females prevailed in the months of March to May and in July. Males prevailed in February and August and from October to December. The χ^2 test for independence revealed significant differences (χ^2_{obs} = 276.8; χ^2_{tab}, 0.05 = 18.307) in the sex proportions during the year.

Size of First Reproduction

The size at which 50% of the females are mature was estimated as 148 mm TL (Fig. 2). However, mature females with a minimum size of 95 mm were found in February and March, while a minimum size of 105 mm was observed in April. In the other months, minimum sizes of mature females ranged from 110 to 125 mm TL.

Distribution of Females on the Fishing Ground

A significant correlation (Pearson $P < 0.05$) between increasing mean size of spotted pink shrimp females and increasing depth of capture (Table 2, Fig. 3) was found from February to August. In most cases the relationship was positive, except in August when large females were found at shallower depths.

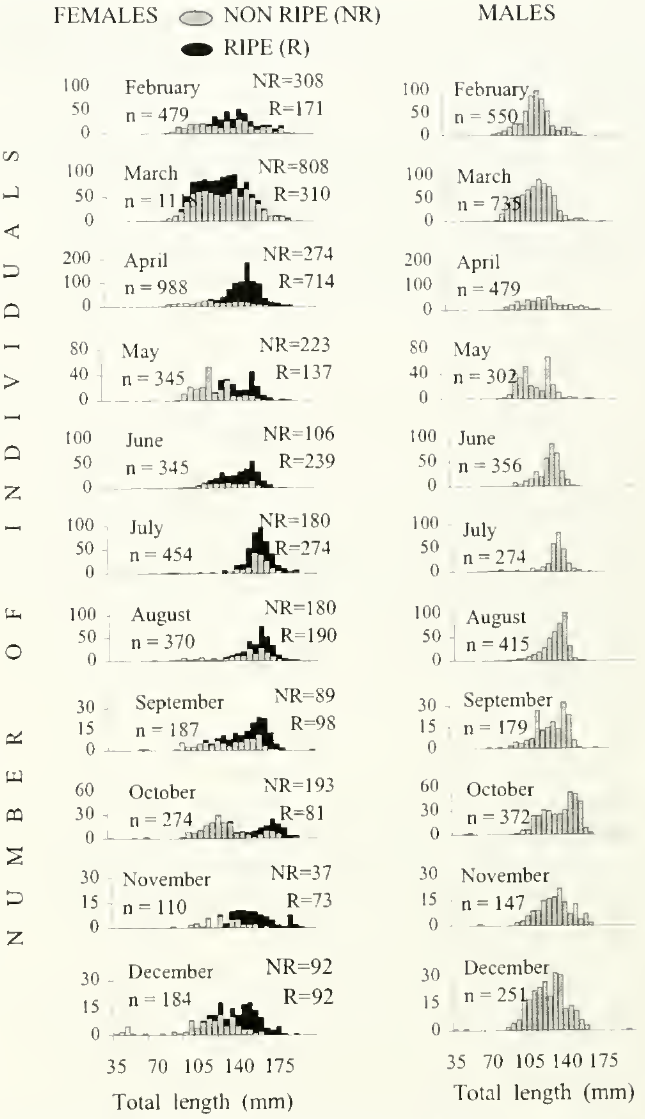

Figure 1. Size structure of the spotted pink shrimp, *F. brasiliensis*, stock from February to December 1993.

The degree of ripeness of the females and the depth showed a significant linear relationship (Pearson $P \leq 0.05$) from February to May and during July and August. April, May, July, and February yielded positive correlations between the degree of ripeness and depth. The inverse condition was observed in March and August.

During all months, except March, at least one area was found in which more than 50% of the females were ripe. From February to June ripe females were more widely distributed. In March, no area had high percentages of ripe females; however, the CPUEs in some of the areas were among the highest (17, 20, 28 ripe females/hr towed) along with those observed in April (13–20 ripe females/hr towed).

Sites with larger CPUE for non-ripe females occurred in February (8–15 and 15–20 non-ripe females/hr towed) and March (35, 45, 52 non-ripe females/hr towed). During other months, these values ranged from 1–13 non-ripe females/hr towed.

One main area which contained ripe females (more than 50%) during almost all months was delimited (Fig. 4). Based on this we propose that the main spawning area within the general fishing ground is located at 21°40′ to 22°10′N and 86°30′ to 86°50′W. In subarea B, determined only for April, with depths of 25 to 40 fathoms (approximately 42–67 m), the CPUE was estimated at 13

TABLE 1.

Female and male size distribution.

	Average Size	Maximum Size	Minimum Size	Mode	Size Range 95%	Observations
Immature females	129–157	195	35	110–160	85–195	
Ripe females	146–166	205	95	130–170	115–180	February and March mean length 130 and 136 mm (recruits)
Males	116–133	200	35	110–145	80–170	May and September, two modes
Both sex	124–147	205	35	120–160	80–185	July, August and October, two modes

to 20 ripe females/hour towed. Subarea C located between 20 to 35 fathoms depth (approximately 33–59 m) was also present for most of the year with a CPUE of 2 to 11 ripe females/hr towed.

Reproductive Periods

Ripe females were present throughout the whole year, indicating that reproduction may be continuous. The highest percentages were found in the months of April (72.3%), June (69.3%), and November (66.4%) (Fig. 5a). Female percentages registered in May and July to October were lower (less than 50%) than the previous months. These differences are also related to the catch of this species within the year, which reaches a maximum in April (Soto-Aguirre in prep.). Catches yield a relatively high number of females in April, suggesting that the greatest reproductive activity occurs during this month.

If we analyze the reproductive activity using CPUE as an index of abundance (ripe females/hr towed), the pattern obtained differs from that of percentages (Fig. 5a and 5b), mainly on the last part of the year. Ripe females are better represented in March and April, with a CPUE of 8.3 ripe females/hr towed, indicating that these months are most important in terms of reproduction. June, also considered as an important month for reproductive activity in terms of percentage of mature females, had lower CPUE values of 5.6 females/hr towed. February, May, July, and August, varied from 4.6 to 4.8 ripe females/hr towed. November registered as important in terms of percentage of ripe females had one of the lowest values based of CPUE. From September to December the number of females decreased from 3.2 to 1.6 ripe females/hr towed (Fig. 5c).

TABLE 2.

Coefficient correlations (r) of female pink spotted shrimp (n) size and depth, and monthly sex ratio (calculated on the basis of females) in the Contoy fishing ground.

Month	r	n	Sex Ratio (Male/Female)
February	0.246*	479	1.17
March	0.414*	1118	0.66
April	0.471	988	0.48
May	0.220*	360	0.83
June	0.185*	345	1.03
July	0.351*	454	0.60
August	−0.197*	370	1.12
September	0.060	187	0.95
October	0.096	274	1.35
November	0.047	110	1.33
December	−0.073	184	1.38

* Significant at 5% level.

DISCUSSION

Ninety-five percent of the spotted pink shrimp catches consisted of organisms that ranged from 80 to 185 mm TL, which suggests that shrimp are recruited at small sizes about 80 mm TL. This agrees with the emigrating size (80–100 mm TL) of juveniles of other penaeid shrimps from the nursery areas in the Gulf of Mexico (Bielsa et al. 1983, Gracia 1989a, Gracia & Soto 1990, Gracia 1995). Juveniles of *F. brasiliensis* seem to leave the nursery areas at 67–84 mm TL according to data recorded in coastal lagoons near the study area (May 1999).

Sex Ratio

In general, the months with the greatest ratio of males/females are within the main reproductive period (February to August). In contrast, the months of October to December, which are not considered as part of the main reproductive period, had a higher proportion of males (1.33–1.38 per female). Guitart and Hondares (1980) also found a difference in the female/male ratio of *F. duorarum* and *F. aztecus* in the Campeche Bank.

Female Spatial Distribution Pattern

Some relationships between size of females and gonad stage were found; however, no consistent pattern was observed. Several authors have reported that mature female shrimp migrate toward deeper regions during the spawning time (Bielsa et al. 1983, Brusher et al. 1972, García and Le Reste 1986, Gracia 1992). This may be the case for *F. brasiliensis*, since the reproduction period established in this paper (February to August) coincides with a significant relationship of size and gonad stage with depth.

The best correlation between size of females and depth of catches was observed during March and April that are the months of greatest reproductive activity. If we also consider that female size can be related to maturity, one could expect a similar behavior

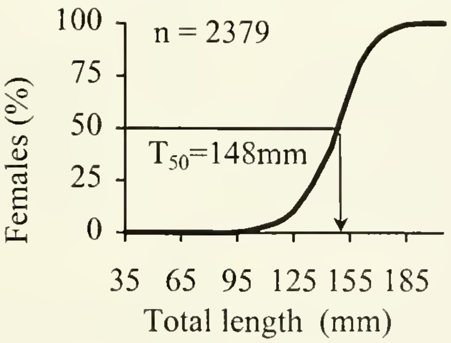

Figure 2. Size of first maturity of *F. brasiliensis* with the criterion of 50%.

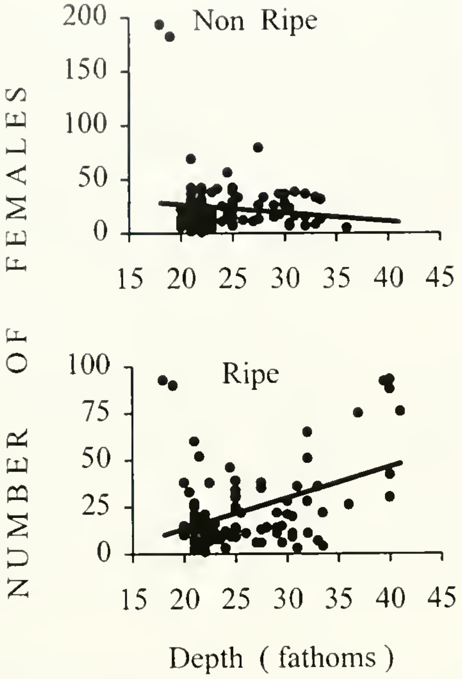

Figure 3. Number of ripe and non-ripe females of *F. brasiliensis* vs depth.

for the relationship between ripeness and depth. This was found for April but did not occur in March, when no significative correlation was observed. This correlates well with the fact that March is a month of marked recruitment to the fishing ground (Porras-Ruiz et al. in prep.) which can mask this relationship.

In August, when smaller organisms were found at greater depths, the relationship of size and depth depicts a negative slope. This finding is related with the second recruitment period of the population towards the fishing grounds (Soto-Aguirre in prep.) during summer. In general terms, the results suggest that there may be a relationship of size and female gonad stage with depth of the fishing ground, especially during reproduction periods.

Nonetheless, monthly variations of non-ripe and ripe females ocurrence in the fishing ground suggest an area of recruitment with small shrimp troughout the year (21°30′ to 21°50′N and 86°30′ and 86°40′W) and a spawning area (21°40′ to 22°10′N and 86°30′ to 86°50′W) at depths of 20 to 40 fathoms (approx. 33–67 m) which can be useful as reference areas for the fishery.

This study suggests that the reproductive period for pink spotted shrimp occurs throughout the whole year, but the highest reproductive activity in terms of number of ripe females occurs from February to August, peaking in March and April. According to García and Le Reste (1986), variation in reproductive activity of shrimps is typically linked to seasons of the year, with two reproductive peaks. Other authors (Crocos and Kerr 1983, Crocos 1987a and b, García 1988) have also observed a bimodal pattern in egg

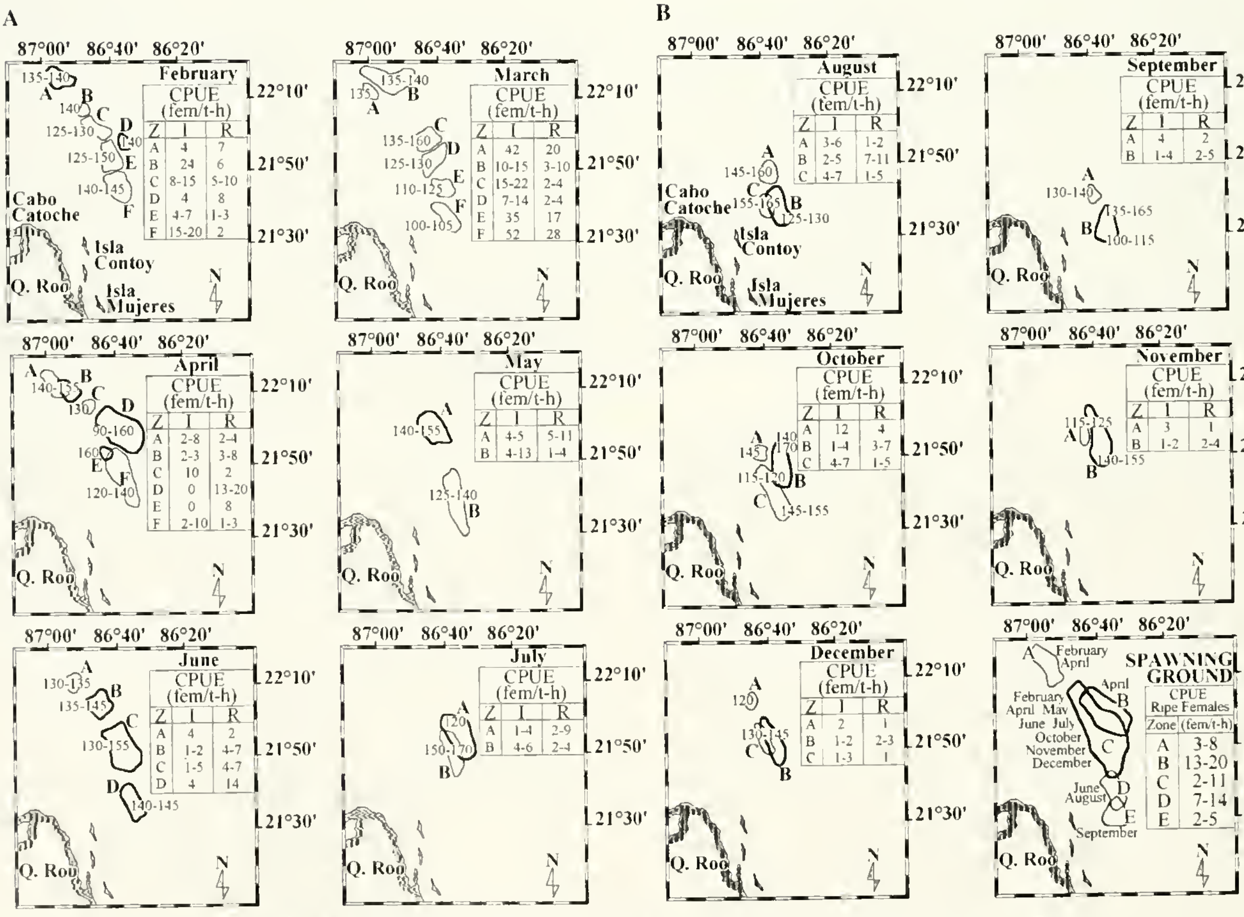
A
B
87°00' 86°40' 86°20'
22°10'
21°50'
21°30'
Cabo Catoche
Isla Contoy
Isla Mujeres
Q. Roo
N
February
CPUE (fem/t-h)
Z I R
A 4 7
B 24 6
C 8-15 5-10
D 4 8
E 4-7 1-3
F 15-20 2
135-140
140
125-130
140
125-150
140-145
March
CPUE (fem/t-h)
Z I R
A 42 20
B 10-15 3-10
C 15-22 2-4
D 7-14 2-4
E 35 17
F 52 28
135
135-140
135-160
125-130
110-125
100-105
April
CPUE (fem/t-h)
Z I R
A 2-8 2-4
B 2-3 3-8
C 10 2
D 0 13-20
E 0 8
F 2-10 1-3
140-135
130
90-160
160
120-140
May
CPUE (fem/t-h)
Z I R
A 4-5 5-11
B 4-13 1-4
140-155
125-140
June
CPUE (fem/t-h)
Z I R
A 4 2
B 1-2 4-7
C 1-5 4-7
D 4 14
130-135
135-145
130-155
140-145
July
CPUE (fem/t-h)
Z I R
A 1-4 2-9
B 4-6 2-4
120
150-170
August
CPUE (fem/t-h)
Z I R
A 3-6 1-2
B 2-5 7-11
C 4-7 1-5
145-160
155-165
125-130
September
CPUE (fem/t-h)
Z I R
A 4 2
B 1-4 2-5
130-140
135-165
100-115
October
CPUE (fem/t-h)
Z I R
A 12 4
B 1-4 3-7
C 4-7 1-5
140
145
170
115-120
145-155
November
CPUE (fem/t-h)
Z I R
A 3 1
B 1-2 2-4
115-125
140-155
December
CPUE (fem/t-h)
Z I R
A 2 1
B 1-2 2-3
C 1-3 1
120
130-145
SPAWNING GROUND
CPUE Ripe Females
Zone (fem/t-h)
A 3-8
B 13-20
C 2-11
D 7-14
E 2-5
February April
April
February April May June July October November December
June August
September

Figure 4. Monthly main distribution spawning area of the spotted pink shrimp *F. brasiliensis*.

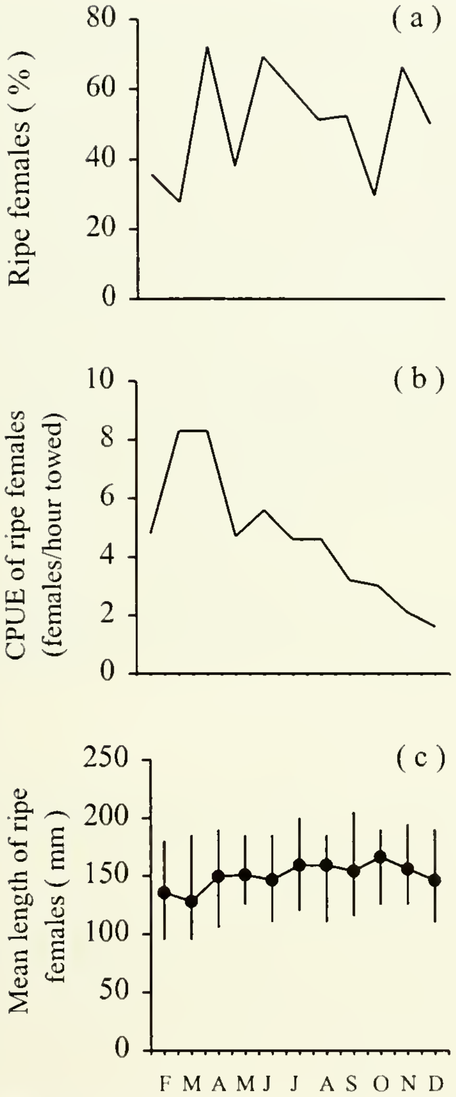

Figure 5. Monthly variation of ripe percentage (a), ripe female CPUE (b) and mean length (c) of *F. brasiliensis* females in the fishery area of Contoy, QR, Mexico.

production, as well as abundance of larval stages of various penaeid species (Rothlisberg et al. 1987). Gracia (1989a) reports that the *L. setiferus* population of the Campeche Sound presents two reproductive peaks of variable magnitude: the most important one occurs late spring and early of summer and a second less intensive period occurs in autumn.

We observed only one reproductive peak during spring for *F. brasiliensis*. However, it will be necessary to determine whether this reproductive pattern is typical of the spotted pink shrimp or whether it is related to environmental factors or high fishing effort levels that might have suppressed the second reproductive peak during the year evidenced only from ripe female percentage. Spawning becomes unimodal, even for species that reproduce all year round, when they occur near the temperate limits of its geographical distribution (Dall et al. 1990). The study area is located in a tropical latitude, so it could be expected that *F. brasiliensis* would fit better to a spawning bimodal pattern.

Protracted spawning period allows a high reproductive potential for *F. brasiliensis* and also allows a protracted recruitment through the year. This behavior agrees with the opportunistic reproductive strategy proposed for white shrimp *L. setiferus* by Gracia (1989a). Maintaining a high reproductive potential year-round could permit pink spotted shrimp to cope with environmental fluctuations, thereby adopting an opportunistic strategy to make good use of microscale variations, mainly in nursery areas which constitute a critical stage (Gracia 1989a, 1991). Unfortunately, in spite of its importance, no data are available on this stage which can confirm the presence of postlarvae year round or that can give details about the behavior of *F. brasiliensis* in the nursery areas.

On the other hand, shrimp spawning during the main peak reproductive period seems to have a high importance with respect to the relative contribution to the fishery recruitment. The main shrimp recruitment that occurs in February-March arises from female population left at the end of the fishing season of the previous year (November–December). This may suggest that the decimated spawning population at the end of the year is more important for stock renewal than the large female population of younger small-sized females present during March and April. This behavior conforms to a common pattern of penaeid shrimp spawning in which some of the individuals of a cohort breed at earlier ages and then have a massive spawning at about 10–12 mo of age (Crocos 1987a and b, Gracia 1989a). During this time a large proportion of the spawning stock is removed by the fishery. Other shrimp species like *P. merguiensis*, *P. semisulcatus*, *L. notialis*, and *L. setiferus* (Rothlisberg et al. 1985, Mathews et al. 1987, Lhome and García 1984, Gracia 1989a) present this type of reproduction where the main recruitment originates from a minor spawning stock. The success of these spawnings are related to envinronmental factors which propitiate postlarvae recruitment to the nursery areas, affect juvenile survival and subsequent recruitment offshore (Gracia 1991).

On the other hand, pink spotted shrimp size caught by the fishery is reducing, which can also influence the reproductive dynamics of the stock. Gracia (1989b, 1995) points out that considering shrimp reproductive strategy, where the recruitment is highly variable due to environmental conditions, the protection of juvenile stages has greater influence on increasing population fecundity than protecting spawning population. For *F. brasiliensis* females, 60 to 85% of the catches consist of sizes with a total maximum length of 140 to 150 mm. The size for the first reproduction averages 148 mm TL and the high fishing mortality, suggest that most of the females have no chance of reproducing at least once before being caught. This may induce recruitment over-

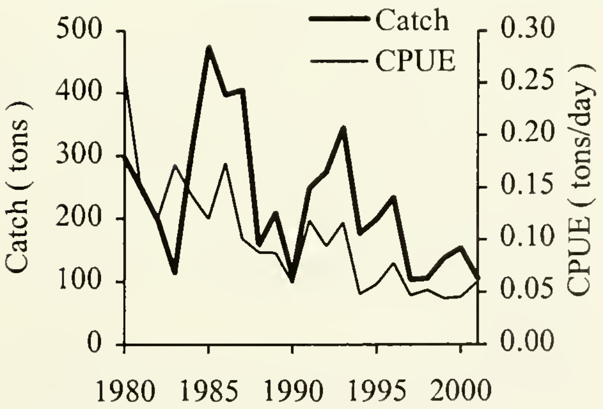

Figure 6. Catch variations of *F. brasiliensis* in Contoy, Q. Roo

fishing and lead to serious problems for the recovery of the population.

This could be one of the reasons for the decreasing shrimp production which shows a sustained negative trend since 1980. Both annual shrimp yield and commercial CPUE have shown a steady decreasing trend to around 20% of maximum records (Fig. 6). It has been reported that shrimp can support exploitation levels which can reduce spawning stock in a wide range up to around 20% of virgin biomass without affecting seriously the recruitment (Gracia 1996). However, it also has been demonstrated that a depleted spawning stock could reduce recruitment and decrease yield. Fishery statistics seem to indicate that this is the case and maximum sustainable yield of the resource has been surpassed affecting the reproduction rate and hence recovery of the population.

A closed season during August–September was implemented to protect spotted pink shrimp and also rock shrimp *S. brevirostris*. The fishery regulation was mainly designed to reduce fishing pressure on *F. brasiliensis* spawning stock. This seems reasonable because location of the main spawning area, as well as the periods of highest reproductive activity, coincide with the areas and periods of greatest fishing activities on *F. brasiliensis*, which could impact negatively this critical stage for the population renewal. However, according to our data, this closure does not fill this objective, as it does not protect the more important spawning at the end of the year or the more abundant ripe female population earlier in the year. Neither does this regulation reduce growth overfishing as it does not protect the main recruitment period of pink spotted shrimp. A more effective regulation would be to close the fishery between November–December to protect the remaining and more important spawning stock. A measure like this would be more important to enhance spawning potential if a second closure is considered in March–April. A combination like this could help to reduce fishing effort on main spawners and pre-spawners of red spotted pink shrimp which could result in a higher reproductive potential.

Information provided in this study could serve as a basis to design management strategies which can help to improve spawning stock and shrimp yield. It is clear that a closure oriented to improve spawning potential of spotted pink shrimp should be directed to the more important spawning stock; however, other combinations of fishery regulations can also be done.

ACKNOWLEDGMENTS

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INFLUENCE OF THE REPRODUCTIVE CYCLE ON THE BIOCHEMICAL COMPOSITION OF DEEP-SEA DECAPOD *PARAPENAEUS LONGIROSTRIS* (LUCAS, 1846) IN THE PORTUGUESE SOUTH COAST

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ABSTRACT The reproductive cycle and biochemical composition of the muscle, ovary, hepatopancreas (HP) of *Parapenaeus longirostris* were studied during a period of one year (October 2000–September 2001) in the Portuguese south coast. Gonado-somatic index (GSI) increased significantly in May and June and during the maturation process, suggesting that spawning may start in late spring or summer. Hepato-somatic index (HSI) also increased throughout the ovarian maturation, suggesting that the HP resources are not depleted. Ovarian lipid levels increased with maturation, but no concomitant decrease occurred with HP lipids. The muscle showed very low lipid levels and higher percentages of polar lipids than ovary and HP. On the other hand, these two tissues presented higher proportions of neutral lipids, mainly triacylglycerols (TAG). Because both ovarian and HP cholesterol increased with maturation, the mobilization of HP cholesterol stores to the build-up of ovarian cholesterol was not clear. Protein and glycogen contents in the muscle, ovary, and HP did not vary as a function of ovary maturity stage. Among the different tissues analyzed, the glycogen is mainly stored in the HP and to a lesser extent in the muscle. In both ovary and HP, the major fatty acids were 16:0, 18:1(n-7), 18:1(n-9), 20:5(n-3), and 22:6(n-3), and significant increases in the levels of monounsaturated fatty acids (MUFA) were observed in the ovary during sexual maturation, which indicates that these compounds act as the major sources of energy during embryonic and early larval development.

KEY WORDS: biochemical composition, *Parapenaeus longirostris*, reproductive cycle

INTRODUCTION

The deep-water rose shrimp *Parapenaeus longirostris* (Lucas 1846) is the second most valuable species caught by the Portuguese crustacean trawl fleet, operating mainly along the south coast of Portugal (Caramelo et al. 1996). It has a wide geographical distribution in the Eastern Atlantic, from the north of Spain to southern Angola, as well as in the Mediterranean and its adjacent seas (Pérez-Farfante 1982). Its growth, reproduction, morphometry, and fecundity have been studied by Ribeiro-Cascalho and Arrobas (1983, 1984, 1987) and Ribeiro-Cascalho (1987) in the south Portuguese waters. However, there are no biochemical data associated with the reproductive biology of *P. longirostris*.

Knowledge of the biochemistry and metabolism processes that occur during the reproductive cycle are essential for a complete understanding of reproduction. Biochemical changes during maturation, moulting, and reproduction in gonads, hepatopancreas, and muscle have been examined for a number of crustacean species (Pillay & Nair 1973, Guary et al. 1974, Gehring 1974, Galois 1984, Castille & Lawrence 1989, Mourente & Rodriguez 1991, Palacios et al. 2000). Many of these studies concentrated on lipid dynamics. The accumulation and mobilization of these organic reserves constitutes one of the most significant metabolic events in the physiology of crustaceans (Teshima et al. 1989). Carbohydrates are important in the Krebs cycle, in glycogen storage, in chitin synthesis, and in formation of steroids and fatty acids (New 1976). Synthesis of several proteins, including peptide hormones, enzymes, high-density lipoproteins (HDLs), and glycoproteins is especially important in maturation and reproduction (Yehezkel et al. 2000). These two biological processes are also regulated by terpenoid, peptide, and steroid hormones (Quackenbush 1986), and a particular consideration in steroidogenesis is the metabolism of the steroid precursor, cholesterol. Crustaceans are not capable of *de novo* synthesis of the steroid ring (Van den Oord 1966, Teshima & Kanazawa 1971), and the dietary intake of cholesterol or mobilization of previously consumed cholesterol reserves is required to support production of steroid hormones (Middleditch et al. 1980).

The purpose of the present study was to follow the biological changes (gonad and hepatosomatic indices) and the biochemical composition of the muscle, ovary, and hepatopancreas (HP) of *P. longirostris* over a one-year period and link these to the shrimps' reproductive cycle.

MATERIAL AND METHODS

Samples

The research was performed over a period of one year, between October 2000 and September 2001. Monthly samples (30–40 shrimps taken from a commercial trawl vessel catches—*Costa Sul*) were collected in Algarve (Portuguese south coast). The fishery of *Parapenaeus longirostris* was mainly between 200 and 300 m. All the specimens analyzed (a total of 452) had a carapace length between 20–28 mm. For each shrimp, the following parameters were recorded: sex, total weight, gonad and HP weight, and maturity stage (only for females). The maturity stage was based on Ribeiro-Cascalho (1987). Females were classified as immature (S1: ovary thin and transparent or translucid white, very small and with no signs of granulation); maturing (S2: thick ovary, yellowish or greenish); and mature (S3: stout ovary, olive green or bluish green colored). Gonad (GSI—gonad wet weight/ body wet weight ×100) and hepatosomatic indices (HSI—hepatopancreas weight/ body wet weight × 100) were only calculated for females. To study the seasonal biochemical changes in the muscle, the monthly samples were pooled in triplicate taking sex into account. To elucidate how biochemical composition may be associated with maturation process (namely, oogenesis), females tissues (muscle, ovary, and HP) were pooled in triplicate taking maturation stage into account.

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Proximate Chemical Composition and Lipid Class Analysis

Water, protein, fat, and ash contents were determined according to AOAC procedures (1998). Total lipids were extracted using the Bligh and Dyer (1959) method. Lipid classes were resolved by analytical thin-layer chromatography (TLC) on plates coated with 0.25 mm silica gel G and developed with hexane: diethylether: acetic acid (65:35:1 by volume). The developed plates were sprayed with 10% phosphomolybdic acid in ethanol. Lipid class identification was made by comparing with standards (Sigma, St. Louis, MO). Quantification was performed using a scanner and the software Quantity One (version 2.4) from PDI, Inc. (New York).

Total Lipid Fatty Acid Analysis

The percentage distribution of fatty acids was based on the experimental procedure of Lepage and Roy (1986) modified by Cohen et al. (1988). The fatty acid methyl esters were analyzed in a Varian 3400 gas chromatograph, equipped with an autosampler and fitted with a flame ionization detector. The separation was carried out with helium as the carrier gas in a fused silica capillary column Chrompack CPSil/88 (Middleburg, The Netherlands) (50 m × 0.32 mm inner diameter [id]), programmed from 180–200°C at 4°C/min, held for 10 min at 200°C, and heated to 210°C for 14.5 min, with a detector at 250°C. A split injector (100:1) at 250°C was used. Fatty acid methyl esters were identified by comparison of their retention times with those of Sigma chromatographic standards. Peak areas were determined using the Varian software (Sunnyvale, CA).

Cholesterol and Glycogen Analyses

The quantification of cholesterol content was based on the experimental procedure of Naemmi et al. (1995) modified by Oehlenschläger (1998). The cholesterol was analyzed in a Hewlett-Packard 5890 gas chromatograph. The separation was carried out with helium as the carrier gas in a column HP5 (Wilmington, DE) (30 m × 0.5 mm id). The temperatures of the oven, injector, and detector were 280°C, 285°C, and 300°C, respectively. Cholesterol was identified and quantified by comparing with standards (Sigma) from which a standard curve was prepared. Glycogen concentrations were determined according to Viles and Silverman (1949). Glycogen was measured by the anthrone-reagent method, and the absorbance read at 620 nm. A calibration curve was prepared using glycogen (Sigma, St. Louis, MO) as a standard.

Statistical Analysis

Data were analyzed using a one-way and a two-way analysis of variance (ANOVA), after the assumptions had been met (normality and homogeneity of variances were verified by Kolmogorov–Smirnov and Bartlett tests, respectively). When data did not meet the assumptions of ANOVA, the nonparametric ANOVA equivalent (Kruskal–Wallis test) was performed. Whenever significance was accepted at $P < 0.05$, the Tukey (parametric) and Dunn (nonparametric) multiple comparison tests were used (Zar 1996).

RESULTS

Biological Changes

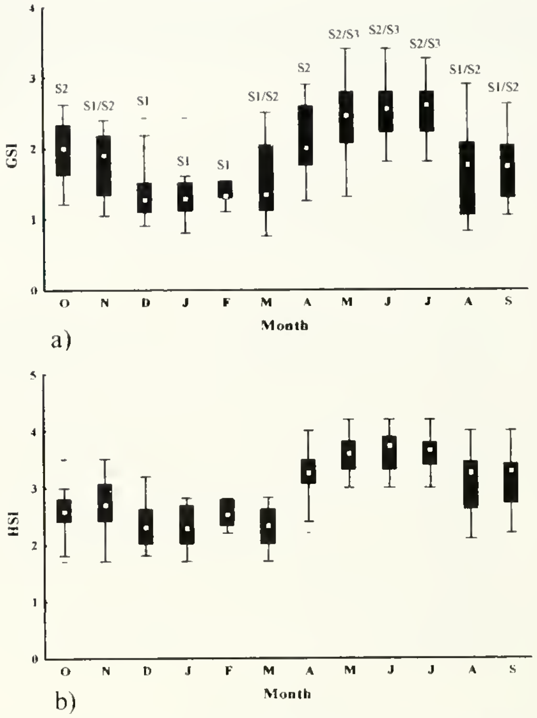

Figure 1. Box plots of the monthly distribution of gonadosomatic index (GSI) (a) (with the indication of the dominant maturity stages found in each month) and hepatosomatic index (HSI) (b) of *Parapenaeus longirostris* females.

The seasonal (intra-annual) patterns of the GSI and HSI observed in *Parapenaeus longirostris* females ($n = 325$) can be seen in Figure 1. GSI and HSI are the highest in May, June, and July.

Proximate Chemical Composition, Cholesterol, and Glycagen Contents of Muscle, Ovary, and Hepatopancreas

The biochemical composition of the muscle of *Parapenaeus longirostris* exhibited seasonal variations in the water, protein, and lipid contents (Fig. 2). The water content ranged from 72.9–75.7% in females and 73.6–75.5% in males, revealing significant temporal variations. The protein content ranged from 20.1–22.3% in females, and 19.5–21.3% in males. The statistical analysis revealed a significant increase from November/December to April/May in both genders (Females: $F_{11,24} = 4.63$, $P < 0.05$; Males: $F_{11,24} = 4.25$, $P < 0.05$). The lipid content ranged from 0.1–0.5% in females and 0.1–0.4% in males, exhibiting a considerable rise in the spring. The ash levels varied between 1.8–2.1% in females and 1.9–2.3% in males. The cholesterol content in the muscle ranged from 56.1–72.3 mg/100 g in females and 50.5–67.9 mg/100 g in males (Fig. 2), exhibiting significant seasonal variations between winter and summer months (Females: $F_{11,24} = 4.55$, $P < 0.05$; Males: $F_{11,24} = 4.09$, $P < 0.05$). The glycogen content varied from 0.8–2.6 mg/100 mg in females and from 1.2–2.8 mg/100 mg in males (Fig. 2) (Females: $F_{11,24} = 4.32$, $P < 0.05$; Males: $F_{11,24} = 4.25$, $P < 0.05$), being the highest between March and May for both genders.

The protein content of the muscle, ovary, and HP of the females as a function of ovary maturity stage showed no significant variations (Table 1). Statistical differences were obtained when comparing the different tissues; the protein content varied from 20.6–22.3% in the muscle, 42.1–45.3% in the ovary, and from 8.5–9.7% in the HP. The cholesterol content increased significantly from S1

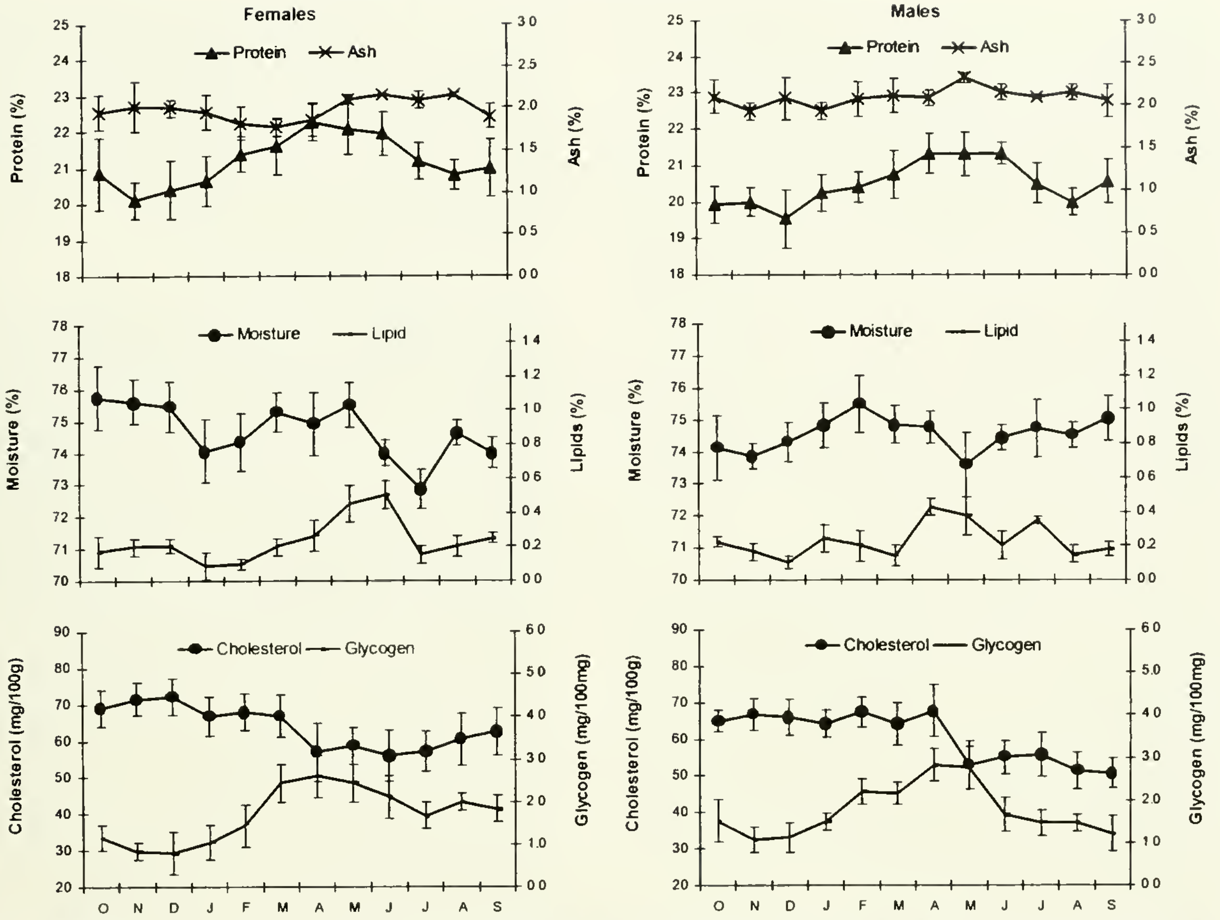

Figure 2. Monthly variations in proximate chemical composition (% wet wt.), cholesterol (mg/100g wet wt.), and glycogen (mg/100mg wet wt.) contents in the muscle of *Parapenaeus longirostris* females and males (values are means of three pooled samples ± SD).

to S3 (62.3–73.5 mg/100 g) in the ovary and from S1 to S3 (150.2–181.9 mg/100 g) in the HP. In the muscle, this content decreased significantly from S1 to S3 (70.0–56.7%) (Table 1). On the other hand, muscle, ovary, and HP glycogen content did not show significant variations throughout the maturation process (Table 1).

Lipid Class Distribution Among Female Tissues

To elucidate how lipids may be associated with maturation and reproduction processes, lipid content was determined in the muscle, ovary, and HP of females as a function of ovary maturity

TABLE 1.

Variations in protein (% wet wt.), glycogen (mg/100 mg wet wt.), cholesterol (mg/100 g wet wt.), total lipids (mg/100 mg dry wt.), lipid class content (% total lipids) in the muscle, ovary, and hepatopancreas of *Parapenaeus longirostris* females at different stages of ovarian development.

	Muscle			Ovary			Hepatopancreas		
	Stage 1	Stage 2	Stage 3	Stage 1	Stage 2	Stage 3	Stage 1	Stage 2	Stage 3
Protein	20.6 ± 0.7^{a}	21.2 ± 0.5^{a}	22.3 ± 1.0^{a}	42.1 ± 1.5^{b}	45.6 ± 1.6^{b}	45.3 ± 1.3^{c}	8.5 ± 1.2^{c}	8.4 ± 1.0^{c}	9.7 ± 1.0^{c}
Glycogen	1.9 ± 0.2^{a}	2.1 ± 0.2^{a}	2.4 ± 0.3^{ab}	2.7 ± 0.3^{b}	2.6 ± 0.5^{b}	2.5 ± 0.6^{b}	2.6 ± 0.4^{b}	2.7 ± 0.3^{b}	2.5 ± 0.3^{b}
Cholesterol	70.0 ± 2.1^{a}	68.8 ± 2.8^{a}	56.7 ± 3.2^{b}	62.3 ± 4.5^{b}	64.4 ± 3.7^{b}	73.5 ± 3.4^{a}	150.2 ± 6.7^{c}	173.3 ± 5.8^{d}	181.9 ± 5.3^{d}
Total lipids	2.9 ± 0.3^{a}	3.1 ± 0.2^{a}	3.4 ± 0.4^{a}	19.8 ± 1.9^{b}	25.2 ± 1.7^{bc}	29.1 ± 2.2^{c}	37.8 ± 2.1^{b}	44.6 ± 2.5^{d}	48.3 ± 2.6^{d}
Triacylglycerols	8.3 ± 3.1^{a}	8.9 ± 2.8^{a}	9.5 ± 1.5^{a}	40.5 ± 6.5^{b}	49.8 ± 5.3^{b}	58.7 ± 4.5^{c}	53.6 ± 5.5^{b}	60.9 ± 4.2^{c}	71.1 ± 3.5^{d}
Phospholipids	25.1 ± 3.5^{a}	25.6 ± 3.7^{a}	26.2 ± 4.4^{a}	12.5 ± 3.6^{b}	9.3 ± 4.2^{b}	6.1 ± 3.2^{b}	2.8 ± 0.5^{c}	1.6 ± 0.4^{c}	1.6 ± 1.1^{c}
Diacylglycerols	6.9 ± 0.7^{a}	6.6 ± 0.4^{a}	6.3 ± 0.8^{a}	4.7 ± 1.2^{b}	5.6 ± 1.1^{b}	6.9 ± 1.5^{a}	3.5 ± 0.3^{c}	2.6 ± 0.7^{c}	3.0 ± 0.2^{c}
Monoacylglycerols	2.5 ± 0.2^{a}	2.8 ± 0.1^{a}	3.2 ± 0.2^{a}	5.5 ± 0.5^{b}	4.7 ± 0.4^{b}	4.6 ± 0.4^{b}	1.8 ± 0.6^{ac}	2.4 ± 0.2^{a}	0.8 ± 0.1^{c}
Free fatty acids	18.1 ± 1.4^{a}	17.0 ± 1.6^{a}	15.9 ± 1.1^{a}	7.0 ± 0.8^{b}	4.3 ± 0.8^{c}	2.8 ± 1.8^{c}	13.4 ± 1.3^{d}	15.2 ± 0.2^{d}	6.6 ± 0.5^{b}
Free cholesterol	29.3 ± 3.3^{a}	28.6 ± 3.8^{a}	27.8 ± 2.6^{a}	9.7 ± 2.4^{b}	10.8 ± 1.4^{b}	10.2 ± 1.2^{b}	6.5 ± 1.0^{c}	5.1 ± 1.1^{cd}	3.7 ± 0.7^{d}
Cholesterol esters	5.7 ± 1.2^{a}	6.0 ± 0.9^{a}	6.3 ± 1.7^{a}	11.0 ± 2.7^{b}	7.9 ± 1.4^{a}	6.2 ± 3.5^{a}	16.1 ± 4.0^{c}	9.9 ± 2.9^{b}	10.8 ± 0.9^{b}
Hydrocarbons	4.1 ± 1.9^{a}	4.4 ± 1.6^{a}	4.8 ± 1.8^{a}	6.7 ± 1.7^{b}	7.4 ± 0.5^{b}	4.5 ± 0.5^{a}	2.3 ± 1.4^{c}	2.3 ± 3.5^{c}	2.3 ± 4.9^{c}

Values are the means ± SD of three pooled samples. Different superscript letters within rows represent significant differences ($P < 0.05$).

stage (Table 1). The lipid levels did not vary significantly in the muscle (from 2.9–3.4 mg/100 mg), but in the other tissues showed significant variations; namely, between S1 and S3 (ovary: 19.8–29.1 mg/100 mg; HP: 37.8–48.3 mg/100 mg).

Different patterns of distribution of the polar and neutral lipids, (expressed as % total lipids) among the muscle, ovary, and HP can be found throughout the maturation period (Table 1). Neutral lipids were dominated by triacylglycerols (TAG), which are by far the largest fraction in the ovary and HP. TAG increased significantly from stages 1 to 3 (ovary: 40.5–58.7%; HP: 53.6–71.7%). The free fatty acids (FFA) percentage decreased during the maturation process, and the neutral lipids, diacylglycerols (DAG), and monoacylglycerols (MAG) showed no clear trends in the different tissues. Similar trends of variation were shown by the other classes. In the muscle, the polar lipids attained higher percentages, because phospholipids (PL) reached about 25% during the maturity process. Significant differences in PL percentages were also detected between the ovary and HP.

Total Fatty Acid Composition of Female Tissues

The fatty acid composition in the muscle of females at different stages of ovarian development is shown in Table 2 (only the quantitatively most important fatty acids are represented in the table). Saturated fatty acids (SFA) content ranged from 6.9–7.1 mg g^{1} dry weight. The most predominant was 16:0, attaining 4.6–4.7-mg g^{-1}. The monounsaturated fatty acids (MUFA) content ranged from 6.8–8.0 mg g^{-1}. Most of this content was present as 18:1. Polyunsaturated fatty acids (PUFA) were the major group attaining the highest values in stage 3 (12.2 mg g^{-1}) because of the increase of ARA (20:4n-6), EPA (20:5n-3), and DHA (22:6n-3) in that period. These fatty acids accounted for almost 80% of the PUFA.

The fatty acid composition of the ovary and HP of females in relation to ovary maturity stage is also presented in Table 2. With respect to the SFA fraction, there were significant differences among the developmental stages. A similar trend was obtained in the most predominant saturated fatty acid (16:0). The MUFA fraction increased significantly during oogenesis and represented more than half of the total fatty acids. The significantly higher values of the MUFA in the ovary and HP compared to muscle were attributable to the significantly higher contribution of 18:1(n-9), 18:1(n-7), 20:1(n-9), and 20:1(n-7). PUFA ranged from 46.1–58.5 mg g^{-1} in the ovaries and from 72.9–90.0 mg g^{-1} in the HP. The major PUFA were ARA, EPA, and DHA, and significant differences were obtained between the tissues analyzed.

DISCUSSION

Oogenesis and primary vitellogenesis usually correspond to the juvenile and prepubescence phases, which are characterized by a slow increase in ovary weight. The onset of puberty is distinguished by a rapid deposition period, the secondary vitellogenesis (Adiyodi & Adiyodi 1970, Aiken & Waddy 1980). In this study, the maturation of *P. longirostris* ovary, indicated by GSI, seemed to show this basic phase pattern, with a slow increase up to the S2, followed by a rapid increase in S3. The temporal trend of GSI seems to be concordant with the seasonal spawning pattern observed by Ribeiro-Cascalho & Arrobas (1987) in Portuguese waters, which suggests two peaks of reproduction, one at the end of spring and another at the beginning of autumn in October.

On the other hand, the variations of HSI throughout the year and during the maturation process did not seem to corroborate the general pattern among the decapods; namely, the storage of organic reserves in the HP and the utilization of these reserves in the ovarian development (Gibson & Barker 1979, Kyomo 1988) or in the formation of a new exoskeleton (Adiyodi & Adiyodi 1970). The fact that both GSI and HSI increased with the ovarian maturation suggests that the HP resources are not depleted and, according to Tuck et al. (1997), if resources are mobilized from this organ, then these resources seem to be compensated by those gained from feeding.

The increase in lipid levels in the ovary occurs as a result of the maturation process. In fact, neutral lipids, particularly TAG, are the major energy source, and the predominant form of energy

TABLE 2.

Fatty acid composition (mg g^{-1} dry wt.) in the muscle, ovary, and hepatopancreas of *Parapenaeus longirostris* females at different stages of ovarian development (only the quantitatively most important fatty acids are represented).

	Muscle			Ovary			Hepatopancreas		
Fatty Acids	**Stage 1**	**Stage 2**	**Stage 3**	**Stage 1**	**Stage 2**	**Stage 3**	**Stage 1**	**Stage 2**	**Stage 3**
14:0	0.2 ± 0.0^{a}	0.2 ± 0.0^{a}	0.3 ± 0.1^{a}	2.6 ± 0.9^{b}	3.2 ± 1.3^{b}	4.0 ± 2.0^{bc}	6.4 ± 1.2^{c}	8.3 ± 1.5^{d}	8.7 ± 1.9^{d}
16:0	4.6 ± 0.9^{a}	4.7 ± 0.7^{a}	4.6 ± 1.1^{a}	24.3 ± 4.2^{b}	32.7 ± 3.7^{c}	38.7 ± 5.1^{c}	54.7 ± 4.9^{d}	61.7 ± 6.4^{de}	73.9 ± 7.9^{e}
18:0	1.7 ± 0.5^{a}	1.7 ± 0.4^{a}	1.4 ± 0.8^{a}	5.4 ± 1.3^{b}	9.0 ± 1.7^{c}	9.5 ± 1.9^{c}	9.2 ± 2.1^{c}	10.2 ± 2.7^{c}	11.6 ± 3.0^{c}
Σ saturated	7.1 ± 1.3^{a}	7.2 ± 1.5^{a}	6.9 ± 1.2^{a}	34.3 ± 5.9^{b}	47.5 ± 6.8^{c}	57.4 ± 7.9^{d}	76.4 ± 10.3^{e}	87.4 ± 12.1^{ef}	98.2 ± 9.9^{g}
16:1(n-7)	1.5 ± 0.8^{a}	1.6 ± 0.9^{a}	1.5 ± 0.6^{a}	8.5 ± 2.2^{b}	11.0 ± 4.5^{b}	19.5 ± 3.6^{c}	28.6 ± 8.9^{d}	33.3 ± 7.6^{de}	37.6 ± 6.3^{e}
18:1(n-9)	4.3 ± 1.6^{a}	3.6 ± 0.9^{a}	3.6 ± 1.2^{a}	44.3 ± 8.2^{b}	61.1 ± 10.9^{c}	74.3 ± 12.4^{cd}	83.1 ± 12.5^{d}	107.8 ± 18.1^{de}	116.8 ± 20.6^{e}
18:1(n-7)	1.1 ± 0.5^{a}	1.1 ± 0.6^{a}	1.3 ± 0.4^{a}	12.1 ± 3.2^{b}	13.5 ± 2.8^{b}	16.0 ± 3.3^{bc}	29.0 ± 7.5^{c}	28.4 ± 6.0^{c}	26.0 ± 5.4^{c}
20:1(n-9)	0.7 ± 0.3^{a}	0.2 ± 0.0^{b}	0.2 ± 0.1^{b}	8.3 ± 1.5^{c}	10.3 ± 2.3^{c}	18.0 ± 2.7^{d}	16.9 ± 4.2^{cd}	20.1 ± 3.4^{d}	21.5 ± 5.0^{d}
20:1(n-7)	0.1 ± 0.0^{a}	0.1 ± 0.0^{a}	0.1 ± 0.0^{a}	1.5 ± 0.5^{b}	2.2 ± 0.8^{b}	7.7 ± 3.6^{c}	5.1 ± 1.3^{c}	6.1 ± 2.1^{c}	6.6 ± 2.4^{c}
Σ monounsaturated	8.0 ± 1.9^{a}	6.8 ± 1.6^{a}	7.0 ± 0.8^{a}	83.0 ± 15.6^{b}	109.0 ± 25.3^{bc}	140.7 ± 19.8^{c}	169.0 ± 23.5^{cd}	204.0 ± 18.7^{de}	224.6 ± 26.6^{e}
18:2(n-6)	0.3 ± 0.1^{a}	0.3 ± 0.2^{a}	0.2 ± 0.1^{a}	1.5 ± 0.3^{b}	1.3 ± 0.5^{b}	2.5 ± 0.8^{bc}	3.2 ± 0.9^{c}	4.2 ± 2.1^{c}	3.3 ± 1.3^{c}
20:4(n-6)	1.5 ± 0.8^{a}	1.5 ± 0.6^{a}	1.8 ± 0.9^{a}	3.4 ± 0.8^{b}	4.9 ± 1.7^{b}	7.7 ± 3.4^{c}	11.1 ± 3.2^{d}	12.9 ± 2.2^{d}	14.9 ± 4.4^{d}
20:5(n-3)	3.8 ± 1.2^{a}	3.8 ± 1.5^{a}	4.0 ± 1.0^{a}	11.3 ± 2.8^{b}	14.2 ± 4.4^{bc}	16.5 ± 3.0^{c}	23.2 ± 4.2^{d}	27.2 ± 4.0^{de}	30.1 ± 3.9^{e}
22:6(n-3)	4.4 ± 1.7^{a}	5.4 ± 2.1^{a}	5.7 ± 1.9^{a}	24.0 ± 5.8^{b}	30.3 ± 7.1^{c}	19.7 ± 6.3^{b}	24.5 ± 3.3^{b}	29.5 ± 4.8^{c}	31.4 ± 5.2^{c}
Σ polyunsaturated	10.9 ± 2.5^{a}	11.8 ± 0.9^{a}	12.2 ± 0.9^{a}	46.1 ± 15.6^{b}	58.5 ± 12.4^{bc}	51.4 ± 12.0^{b}	72.9 ± 11.7^{c}	87.0 ± 14.5^{d}	90.0 ± 12.6^{d}

Values are the means ± SD of three pooled samples. Different superscript letters within rows represent significant differences ($P < 0.05$).

storage in the adult, egg, and prefeeding larva (Xu et al. 1994, Nates & Mckenney 2000), comprising primarily 16:0 and omega-9 family fatty acids (Teshima et al. 1988). PL, DAG, and sterols are the other main lipid classes found in marine shrimps and are also associated with the maturation of oocytes (Teshima 1997, Ravid et al. 1999, Wouters et al. 2001).

The HP is the major lipid storage and processing organ for postembryonic stages (Voght et al. 1985), but during maturation the ovary becomes an additional center for lipid metabolism, including lipogenesis—TAG synthesis (Teshima et al. 1988). In the present study, and like some previous studies in decapods (Castille & Lawrence 1989, Cavalli et al. 2001), the increase in ovarian lipids is not accompanied by a decrease in HP lipids. Under these circumstances, the lipid requirements of the developing ovary seem to be more dependent on the ingestion of dietary lipids than on HP reserves.

The profile of fatty acids in the ovaries of *P. longirostris* is a reflection of the fatty acid requirement of this tissue or of what is required for transfer to the developing embryos after fertilization. Alava et al. (1993), Cahu et al. (1994), and Cahu et al. (1995) demonstrated the benefits of high levels of highly unsaturated fatty acids in the diet on reproductive parameters and on offspring quality of penaeid shrimps. In fact, the long-chain fatty acids are necessary for vitellogenesis of crustaceans (Middleditch et al. 1980, Millamena & Pascual 1990) and ARA and EPA are precursors of eicosanoids in marine animals (Lawrence et al. 1979, Sargent, 1995). The high values of MUFA in the ovary and HP are consistent with previous findings of Clarke et al. (1990) and Roustaian et al.(1999), which indicated that these compounds are the major sources of energy during embryonic and early larval development.

In the present study, the protein content of the ovary and HP did not vary significantly. On the other hand, the seasonal (intra-annual) variation of protein content of the muscle may be linked with changes in feeding activity. Muscle protein loss during starvation has been observed in other deep-sea decapod species (Dall 1981).

Because cholesterol is a precursor of steroid hormones (Kanazawa & Teshima 1971), the increase of ovarian cholesterol levels with maturation in the *P. longirostris* was not surprising. This increase can also be related to the role of cholesterol as precursor of ecdysteroids, as these compounds are known to increase during gonadal maturation (Wilder et al. 1991). Similar trends were observed in HP cholesterol, which differs from the results obtained by other authors (Adiyodi & Adiyodi 1970, Lautier & Lagarrigue 1988), where the decrease in HP cholesterol during vitellogenesis suggests that mobilization of HP cholesterol stores may contribute to the build-up of ovarian cholesterol. The explanation of our findings could be found in Teshima et al. (1988), which indicated that cholesterol is sequestered to the ovaries from the muscle stores. In fact, in the present study, the muscle cholesterol content decreased significantly from S1 to S3, and the seasonal variation of the cholesterol content also seems to confirm the conclusions of Teshima et al. (1988). On the other hand, because cholesterol stores within the HP and gonads are derived from the diet (Middleditch et al. 1980), because of the incapacity of *de novo* synthesis of the steroid ring, if the steroid resources are mobilized from the HP to the gonad, then the HP resources seem to be compensated by those gained from feeding.

Glycogen did not show significant variations throughout the maturation process, in contrast to what was stated by Kulkarni and Nagabhushanam (1979). Moreover, because carbohydrates have specific roles in the production of nucleic acids, are precursors of metabolic intermediates in the production of energy and nonessential amino acids, and as a component in ovarian pigments (Harrison 1990), they have to be especially important for the maturation process and for embryogenesis. Among the different tissues analyzed in this study, the glycogen is mainly stored in the HP and to a lesser extent in the muscle, but according to the studies by Hagerman et al. (1990) and Baden et al. (1994), on the decapod *Nephrops norvegicus* (Linnaeus 1758), the occurrence of glycogen depletion in the muscles, following hypoxia and starvation, suggests that the muscle contains a particularly important store of glycogen, because it is more readily accessible when there is a shift to anaerobic metabolism or when there is decrease in the feeding activity during winter. This can explain the seasonal variation of glycogen content in the muscle of *P. longirostris*, because the lowest values were obtained in the winter. Although there is no evidence of a decreasing feeding activity during this period of the year, the diet composition of *P. longirostris* should vary significantly between seasons like the other deep-sea crustaceans species (Cartes & Sardà 1989, Labropoulou & Kostikas 1999), because these changes correspond basically to the period of abundance of the different dietary group in the deep-sea environment (Cartes 1994).

In summary, it is evident that reproductive cycle has profound effects upon the biochemistry of *P. longirostris*. Reproduction and gonadal maturation have large associated energy costs attributable to the increase in biosynthetic work, which will support the lecithotrophic strategy (reliance on egg yolk nutrition) of the embryos and prefeeding larval stages. Moreover, these processes seem to be influenced or even synchronized with seasonal feeding activity or food availability. Despite not being mentioned or discussed in this study, moult cycle can also have an important effect in the biochemistry and physiology of decapod crustacea, manifested in changes in their physiological ecology and behavior. Lack of data on this matter in *P. longirostris* indicates that further work will be necessary to understand better the biochemistry and physiology of this species.

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A COMPARISON OF GROWTH PERFORMANCE ACROSS THE SQUID GENUS *ILLEX* (CEPHALOPODA, OMMASTREPHIDAE) BASED ON MODELLING WEIGHT-AT-LENGTH AND AGE DATA

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ABSTRACT A new approach to study growth performance in squid is described, based on modeling the ratio of body weight to mantle length (BW/ML) as a function of age (squid "condition"). This approach has several advantages above traditional size-at-age modeling, including the concurrent use of two size indices, the fact that it is not constrained by theoretical considerations regarding the form of growth in absolute size, and that it is also less severely affected by sampling bias. It was tested on three species of the genus *Illex* using raw data sets for two species as well as BW/ML values calculated from sizes predicted by models available in the literature for all three species. Several experimental models were explored, but the specific logistic model was found to be the most suitable, especially when most of the life cycle was sampled. This model was successfully applied to all species and case studies, thereby elucidating common features of growth performance throughout the genus *Illex*. It provided similar or superior model fits when compared with corresponding models of length-at-age or weight-at-age. Comparisons within and among species indicated some effects that have also been suggested from independent length-at-age studies, including a positive effect of temperature on growth performance. This new approach also indicates a relationship between growth performance and sexual maturation.

KEY WORDS: squid, *Illex*, growth performance, age

INTRODUCTION

Three species of short-finned squids of the genus *Illex* (Teuthoidea, Ommastrephidae) are commercially exploited (Roper et al. 1998); *Illex illecebrosus* (Lesueur 1821), *I. argentinus* (Castellanos 1960), and *I. coindetii* (Verany 1839). The fisheries they support have recently gained increasing attention because of concerns regarding potential for overexploitation (Haimovici et al. 1998, O'Dor & Dawe 1998, Sánchez et al. 1998).

Effective squid fisheries management, however, is hampered by uncertainties concerning the life cycle. As is typical of most squid, *Illex* species show great and unpredictable variability in growth, maturation, and spawning patterns over very short time periods (Mangold 1987, Forsythe 1993, O'Dor & Lipinski 1998). Such great variability in life history parameters largely accounts for the great variability evident in size-at-size (e.g., weight-at-length) and size-at-age relationships observed in *Illex* sp. and the difficulty in applying length-frequency methods (Caddy 1991, Jereb & Ragonese 1995) conventionally used for estimating growth rates in other fisheries resources (Pauly & Morgan 1987). Recent direct ageing techniques based on squid statolith microstructure (Jereb et al. 1991; Jackson 1994), despite the inevitable existence of ageing bias (Pauly 1998, Gonzalez et al. 2000), indicate very high growth rates, even higher than those observed in fast growing pelagic fish such as the scomberoids (Longhurst & Pauly 1987, Jarre et al. 1991).

Despite the many studies of the past decade, there is no consensus on which model is the most suitable to describe squid growth. Among those used for various species and portions of the life cycle, the simple linear, power, log linear, piece-wise, exponential, seasonally oscillating von Bertalanffy, double exponential (or Gompertz), and logistic (Jackson 1994) have most frequently been applied. To date, five models have been used to describe absolute growth in either length or weight within the genus *Illex* (Table 1).

Until now, size-at-size and size-at-age relationships have been analyzed separately, despite the strong correlation existing between body mass and mantle length. Here, these relationships are analyzed jointly by relating body mass to mantle length and investigating trends in this ratio with age. The main purpose of our study was to combine two indices of size (both important factors in ecology and evolution studies; Peters 1983, La Barbera 1989) into a new index, which may better indicate variation in growth performance than either length or weight alone and thereby facilitate broad-scale comparison within and among species. Potential advantages of this approach include that variability surrounding models of weight-at-length on age may be lower than for more familiar models of size on age. Weight-at-length on age models may be simpler, more consistent, and provide better fits than single size variable on age models. They may also better reflect suitability of the biotic environment than growth in either length or weight alone.

The models we developed were applied to three congeneric squid species (*Illex* spp.) to test the utility of this approach for comparison of growth performance patterns.

MATERIALS AND METHODS

The weight-at-length index (WaL) is defined as the ratio of whole body weight (BW; g) to dorsal mantle length (ML; mm), i.e., WaL = BW/ML; it is, therefore, analogous to other more conventional condition indices, derived from the classic Huxley's allometric formula $y = ax^b$ (La Barbera 1989) and widely used in aquaculture and fisheries science (Dawe 1988, Bolger & Connolly 1989, Scott Cone 1989).

Two kinds of size-at-age databases were used (Tables 1 and 2). The first one consisted of original individual estimates of size (ML and BW) and age. Such data sets were available for two micro-cohorts of *I. coindetii* from the Strait of Sicily (Arkhipkin et al.

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TABLE 1.

Size-at-age models used to predict weight and mantle length and calculate the weight-at-length ratio from predicted size values for *Illex* species and populations.

Species	Area	Case	Sex	Hatching Group or Location	Age Range (days)	Mantle Length (y) at Age (x)	Weight (y) at Age (x) or Length (z)	Source and Models
I. coindetii	Central Mediterranean	A1	f	Oct.–Dec.	75–240	y = 191.6/1+exp–0.032(x–119.2)	y = 168.7/1+exp–0.054(x–154.0)	Arkpikin *et al.* (2000)
		A2	f	May–July	74–181	y = 158.2/1+exp–0.050(x–92.8)	y = 107.8/1+exp–0.077(x–117.2)	Logistic
		A3	m	Oct.–Dec.	76–230	y = 144.4/1+exp–0.034(x–98.7)	y = 125.8/1+exp–0.048(x–145.3)	
		A4	m	May–July	81–191	y = 130.3/1+exp–0.054(x–85.5)	y = 93.7/1+exp–0.083(x–113.9)	
I. coindetii	W Mediterranean	B1	f	Oct.–March	220–475	y = 37.6+0.351*x	y = 0.00005297+z^2.886	Sanchez (1995)
		B2	f	Apr.–Sept.	160–460	y = 31.1+0.379*x	y = 0.00005297+z^2.887	Simple Linear and Power
		B3	m	Oct.–March	160–430	y = 11.2+0.357*x	y = 0.00003783+z^3.0184	
		B4	m	Apr.–Sept.	160–370	y = 1.9+0.423*x	y = 0.00003783+z^3.0185	
I. coindetii	Galician (Atlantic)	C1	f	pooled animals	90–450	y = –5.9+0.65*x	y = 0.000000138*x^3.74	Gonzales *et al.* (1996)
		C2	m	pooled animals	90–390	y = –53.6+1.01*x	y = 0.000000138*x^3.80	Simple Linear and Power
I. coindetii	NW African Coasts	D1	f	Sierra Leone	120–255	y = 0.051*exp(826*(1–exp(0.0225*x)))	y = 170.8*(1–exp(–0.0117*(x–92.4)))	Arkpikin (1996)
		D2	f	W. Sahara	90–300	y = 6.092*exp(3.99*(1–exp(0.0119*x)))	y = 0.01079*exp(9.4*(1–exp(0.095*x)))	Gompertz, Logistic
		D3	m	Sierra Leone	105–210	y = 168.9*(1–exp(–0.0087*(x–24.9))	y = 121.4*(1–exp(–0.0099*(x–89.3)))	and von Bertalanffy
		D4	m	W. Sahara	90–255	y = 194.6/(1+((194.6–9.3)/9.3)*exp–0.0294x))	y = 00003134*exp(16.4*(1-exp(0.018*x)))	
I. illecebrosus	NW Atlantic	E1	f	March	115–250	y = 84.0+0.71*x	$\log_{10}y = -1.39+1.60*\log_{10}x$	Dawe & Beck (1997)
		E2	f	April	100–235	y = 46.1+1.04*x	$\log_{10}y = -3.22+2.48*\log_{10}x$	Simple Linear and Power
		E3	f	May	120–210	y = 26.5+1.29*x	$\log_{10}y = -4.50+3.12*\log_{10}x$	
		E4	m	March	115–220	y = 108.2+0.53*x	$\log_{10}y = -1.01+1.42*\log_{10}x$	
		E5	m	April	115–220	y = 105.6+0.60*x	$\log_{10}y = -2.07+1.93*\log_{10}x$	
		E6	m	May	135–210	y = 110.8+0.75*x	$\log_{10}y = -2.60+2.27*\log_{10}x$	
I. argentinus	Patagonian Shelf	F1	f	June	180–360	y = –13.2+0.99*x	$\log_{e}y = -13.0+3.40*\log_{ex}$	Rodhouse & Hatfield (1990)
		F2	m	June	180–360	y = 46.1+0.70*x	$\log_{e}y = -14.3+3.68*\log_{ex}$	Simple and Log Linear

TABLE 2.

Ranges of age, mantle length (ML), and weight-at-length ratios (WaL) for data sets based on individual observations and on predicted size values for *Illex* species and populations. For cases A and E, the predicted values were calculated only for the age ranges sampled (i.e., without extrapolation).

				Original (individual) Observations					Theoretical (mean) Observations				
Species-Area	Case	Sex	Group	Age Range (days)	ML Range (mm)	WaL (min)	(g/mm) (max)	N	Age Range (days)	ML Range (mm)	WaL (min)	(g/mm) (max)	N
I. coindetii	A1	f	Oct.–Dec.	75–240	41–202	0.073	1.284	189	70–230	33–186	0.054	0.891	17
Central Mediterranean	A2	f	May–July	74–181	52–175	0.102	1.027	144	70–180	31–156	0.089	0.687	12
	A3	m	Oct.–Dec.	76–230	46–171	0.078	1.154	218	80–230	50–143	0.105	0.866	16
	A4	m	May–July	81–191	56–140	0.117	0.857	98	100–180	89–129	0.251	0.720	9
I. coindetii	B1	f	Oct.–March						220–475	115–204	0.407	1.206	18
W Mediterranean	B2	f	Apr.–Sept.						160–460	92–205	0.266	1.218	21
	B3	m	Oct.–March						160–430	68–165	0.191	1.127	19
	B4	m	Apr.–Sept.						160–370	69–158	0.198	1.042	15
I. coindetii	C1	f	pooled animals						90–450	37–401	0.099	4.160	25
Galician (Atlantic)	C2	m	pooled animals						90–390	53–248	0.053	2.773	21
I. coindetii	D1	f	Sierra Leone						120–255	113–192	0.418	0.757	10
NW African Coasts	D2	f	W. Sahara						90–300	84–294	0.282	2.522	15
	D3	m	Sierra Leone						105–210	84–135	0.206	0.627	8
	D4	m	W. Sahara						90–255	80–192	0.187	1.823	12
I. illecebrosus	E1	f	March	116–247	154–299	0.383	1.753	32	115–250	166–261	0.488	1.070	10
NW Atlantic	E2	f	April	108–228	159–286	0.396	1.734	32	100–235	150–291	0.366	1.574	10
	E3	f	May	112–195	175–281	0.491	1.697	12	120–210	181–297	0.535	1.869	7
	E4	m	March	116–204	152–227	0.409	1.128	48	115–220	169–225	0.488	0.922	8
	E5	m	April	115–216	152–246	0.357	1.447	35	115–220	174–237	0.463	1.189	8
	E6	m	May	141–200	217–265	0.917	1.620	17	135–210	212–268	0.812	1.749	6
I. argentinus	F1	f	June						180–360	149–311	0.377	2.206	13
Patagonian Shelf	F2	m	June						180–360	172–298	0.589	2.567	13

1998, Arkhipkin et al. 2000; Case A: Tables 1 and 2) and three micro-cohorts of *I. illecebrosus* from the Northwest Atlantic (Dawe & Beck 1997; Case E; Tables 1 and 2). *I. coindetii* samples were collected during an experimental depth-stratified random bottom trawl survey (stretched mesh size in the cod-end of 31 mm), whereas *I. illecebrosus* samples were collected from the Newfoundland inshore jig fishery during July to November 1990.

The second kind of database consisted of size values (ML and BW) at age predicted by the specific size-at-age models reported by sex for different hatching groups or populations (pooled over hatching groups) of *Illex* spp. in the literature (Tables 1 and 2). When only ML-at-age models were available (Case B), the corresponding BW-at-age data were roughly approximated from published BW-ML relationships obtained for the same season. There-

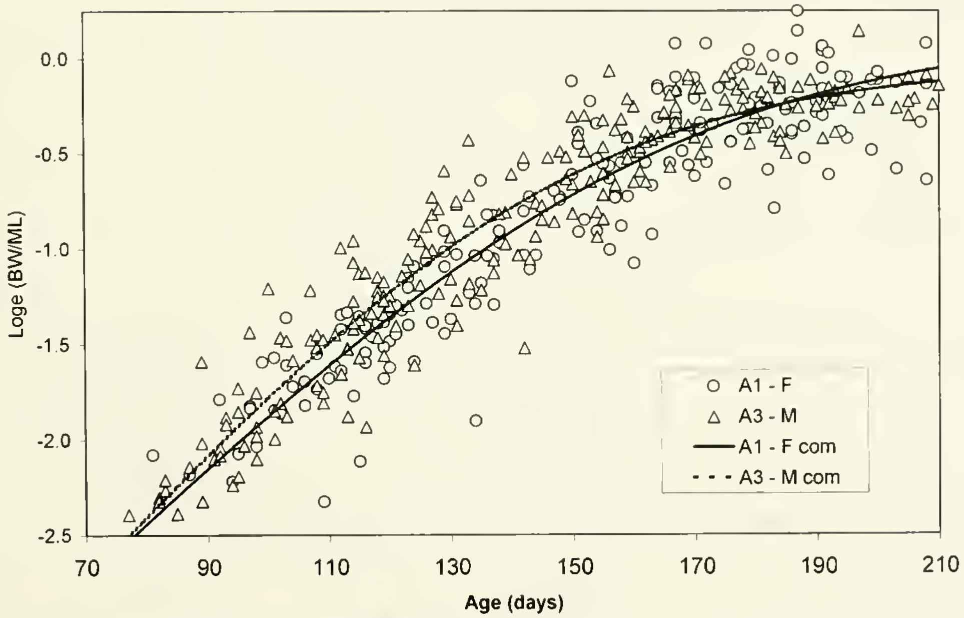

Figure 1. Individual $\log_e$ of body weight (BW) at mantle length (ML) ratio vs. age and computed (com) specified logistic model for A1 and A3 cases, *Illex coindetii* females (F) and males (M) of the Central Mediterranean (see Table 1 for case specifications).

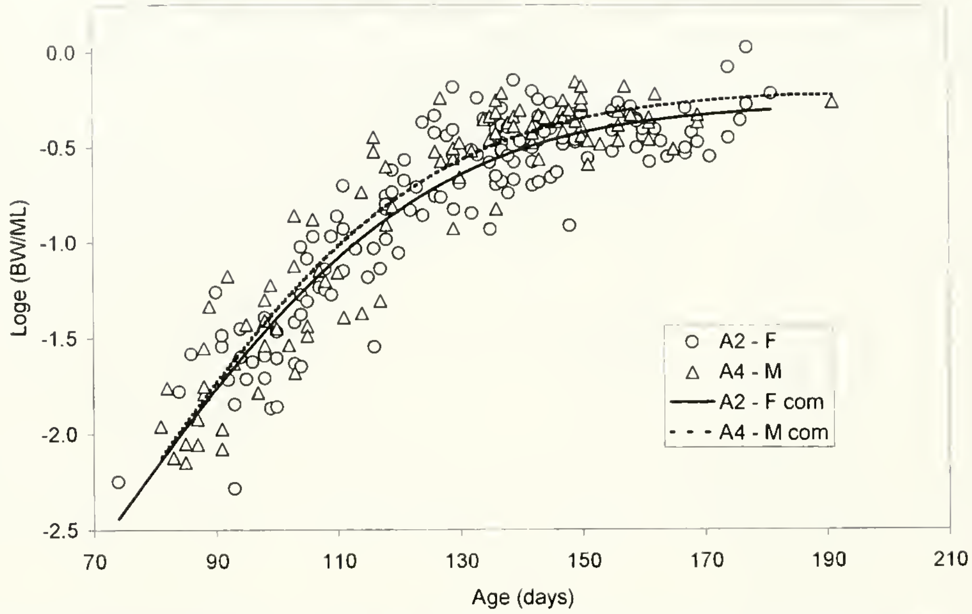

Figure 2. Individual $\log_e$ of body weight (BW) at mantle length (ML) ratio vs. age and computed (com) specified logistic model for A2 and A4 cases, *Illex coindetii* females (F) and males (M) of the Central Mediterranean (see Table 1 for case specifications).

fore, they have to be considered only as representing the form of growth.

To explore the most suitable models to apply, WaL-at-age scatter plots were analyzed by points interpolation with a model-fitting procedure (Systat 1992). A variety of available models were applied to the untransformed WaL and age data, as well as to data sets with the dependent variable only and with both variables transformed (using natural, base e, logarithms). This exploratory analysis indicated that the relationship of WaL on age (t) was best described by the power function in some cases (WaL $= at^b$) and by asymptotic models in other cases. Among the asymptotic models, the sigmoidal logistic model was preferred (Ratkowsky 1983).

Therefore, the following two models (applied to log transformed data to stabilize the variance and normalize the residuals) were tested:

the **allometric** model, hereafter referred to as the **log-log model**, which represents the linear transformation of the power model:

TABLE 3.

Estimated coefficients of the log-log and specific logistic models by using individual weight-at-length (WaL) at-age observations: log = natural ($\log_e$) logarithm; r^2 = coefficient of determination; MSE = mean squared error; N = sample size.

					Log-log Model (i)					Specific Logistic Model (ii)					
Species-Area	Case	Sex	Group		$\log_e a$	b	r^2	MSE		A	B	C	r^2	MSE	N
I. coindetii	A1	f	Oct.–Dec.		−13.552	2.549	0.859	0.059		0.1335	0.0314	159.4	0.950	0.054	189
Central Mediterranean				s.e.	0.378	0.076			s.e.	0.0856	0.0021	6.2			
	A2	f	May–July		−11.754	2.260	0.777	0.055		−0.2708	0.0508	114.4	0.953	0.041	144
				s.e.	0.493	0.101			s.e.	0.0499	0.0040	2.6			
	A3	m	Oct.–Dec.		−13.134	2.479	0.887	0.050		−0.0373	0.0360	143.0	0.968	0.041	218
				s.e.	0.298	0.060			s.e.	0.0515	0.0017	3.5			
	A4	m	May–July		−13.118	2.552	0.840	0.057		−0.2046	0.0527	114.4	0.961	0.041	98
				s.e.	0.547	0.113			s.e.	0.0602	0.0045	3.2			
I. illecebrosus	E1	f	March		−9.934	1.883	0.685	0.040		Model failure					25
NW Atlantic				s.e.	1.359	0.266									
	E2	f	April		−14.893	2.877	0.777	0.039		0.7126	0.0335	175.3	0.839	0.037	28
				s.e.	1.542	0.302			s.e.	0.2702	0.0082	17.3			
	E3	f	May		−13.280	2.618	0.827	0.013		Model failure					11
				s.e.	2.052	0.400									
	E4	m	March		−6.288	1.164	0.536	0.024		Model failure					47
				s.e.	0.814	0.162									
	E5	m	April		−7.827	1.488	0.416	0.090		Model failure					35
				s.e.	1.549	0.307									
	E6	m	May		−9.154	1.819	0.590	0.019		Model failure					15
				s.e.	2.168	0.420									

$$\log_e WaL = \log_e a + b\log_e t + e$$

and;

the **specific logistic** (Weatherley 1972):

$$\log_e WaL = A - \log_e(1 + e^{-B(t-C)}) + e'$$

where $\log_e$**a** and **b** are the intercept and the slope parameters respectively, **A** denotes the asymptote ($\log_e WaL_\infty$), **B** is the slope, **C** is the inflection point (days), **t** is the age (days), considered as the independent variable, and **e** and **e′** are the error terms.

A possible disadvantage of the specific logistic model is the apparent lack of a direct biologic interpretation for the asymptotic parameter (A). However, a direct biologic meaning may be found for the parameter C, which, when modeling growth in length, represents the inflection point at the end of the initial exponential growth phase and the beginning of the logarithmic growth phase. We investigate the possible relationship of the age at inflection point (i.e., the C parameter) with the approximate mean age of all mature animals of a specific group, as derived from the literature.

Model coefficients were estimated by the ordinary (Type I) simple least squares method, using both linear (Model i) and non-linear (Model ii; "Quasi Newton" and "Simplex") iterative procedures. The coefficient of determination (r^2) and the mean square errors (MSE) were used as approximate indices of goodness of fit. The former statistic, in particular, was used to compare results derived by modeling WaL with those derived by modeling ML and BW separately (original size models, Table 1). Probability levels for acceptance of significance of the regression coefficients and the overall regressions were fixed at $P < 0.05^*$ (significant) and $P < 0.01^{**}$ (highly significant). Statistical analysis, model fitting, and computations were performed using the package Systat (1992).

RESULTS

Models for Data Sets from Individuals

I. coindetii

The log-log transformation (Model i) was not successful in achieving linearity between WaL and age for *I. coindetii* from the Strait of Sicily, with the exception of females of the October to December group (A1). However, the scatter-plots of $\log_e$ transformed WaL values vs. age according to Model ii (Figs. 1 and 2), showed an acceptable homogeneity of variance. Male and female curves overlapped, but the two seasonal groups did not. October to December specimens (A1–A3) showed lower $\log_e$WaL values for a given age than did their May to July counterparts (A2–A4), suggesting a seasonal effect with higher growth performance of spring-summer group.

Considering cases A and Model i (Table 3), all regressions were significant, but the regression parameters were rather imprecise, as reflected by high standard errors. The Model i, therefore, did not properly fit the data sets, as already indicated by failure to achieve linearity. Improvement in both r^2 and MSE was achieved by fitting Model ii (Table 3). For both hatching groups and sexes convergence was rapid, and various starting values led to very similar final estimates of regression parameters, regardless of which non-linear fitting procedure was used. The precision of the regression parameters was acceptable. Model ii parameter values were similar between sexes of the May–July group (A2 and A4: Table 3, Fig. 2), whereas some sex effect was detected in the October to December group (A1 and A3, Fig. 1). In that fall to winter group, females exhibited higher asymptotic values than males and a small difference in the slope parameter and inflection points (Table 3, Fig. 1). Overall, *I. coindetii* specimens hatched in May to July showed a higher growth performance than their October to December counterparts.

I. illecebrosus

For this species (E cases), parameter estimation was achieved only using Model i (Table 3), except for the E2 case. The precision in the estimation of parameter A (Model ii) was very low in that case, however, as reflected by the ratio of that parameter to its standard error (0.27/0.71, i.e., 38% of the variation).

The Model i (log-log regression) coefficients (Table 3) indicate that the slopes were higher for May and April than for March, and higher for females than males.

Models for Data Sets of Predicted Values

Results obtained from fitting both models to the data sets of predicted values (all cases) are presented in Table 4. The consistently higher model fits (reflected by higher r^2 and lower MSE) than for models fitted to data sets of individuals (Table 3), reflects the much lower variance in the data sets of values predicted by other (single size-at-age) models than in raw empirical data sets. A comparison of specific logistic models among cases is shown for females (Fig. 3) and males (Fig. 4).

It is interesting to initially compare the A and E cases for which models were also fitted to the data sets based on individuals.

Case A: *I. coindetii*

The $\log_e$WaL-at-$\log_e$-age plots (Model i) were nearly linear for the October to December group (cases A1 and A3) and more strongly curvilinear, with an asymptotic trend, for May to July hatched squid (cases A2 and A4). The $\log_e$WaL-at-age plots (Model ii) showed a curvilinear trend in October to December specimens and a clear asymptote in May to July animals.

Application of Model (i) resulted in clear differences in models between seasonal groups and sexes. Model fits were better (higher r^2 and lower MSE) and slopes were higher for October to December than for May to July models and also for female than for male models (Table 4).

The application of Model ii (Table 4) resulted in rapid convergence and good agreement between empirical and predicted values. The asymptotic (A) and inflexion (C) parameters were higher in the October to December group; with the exception of the A parameter (which showed great variability), males and females within the each group showed very similar inflection and steepness parameters (Table 4).

Case E: *I. illecebrosus*

Linearity was not fully achieved by the log-log transformation and the application of Model i resulted in different parameter values among groups (cases E1 to E6; Table 4); however, the fit was good (high r^2 and low MSE) with only slight differences between sexes, except for E3 and E6 cases (higher slopes for females than males in the May-hatched group).

The $\log_e$WaL-at-age plots (Model ii) resulted in very similar (virtually coincident) curves for both sexes of the March (E1, E4) and May (E3, E6) groups while larger values of $\log_e$WaL-at-age were observed in females than in males of the April group, (E2, E5); the slopes consistently increased from March to May for both sexes, suggesting increase in growth rate throughout the spring.

TABLE 4.

Estimated coefficients of the log-log and specific logistic models by using predicted weight and mantle length to derive weight-at-length (WaL)-at-age observations: log = natural ($\log_e$) logarithm; r^2 = coefficient of determination; MSE = mean squared error; N = sample size.

				Log-log Model (i)				Specific Logistic Model (ii)					
Species-Area	Case	Sex	Group	$\log_e a$	b	r^2	MSE	A	B	C	r^2	MSE	N
I. coindetii	A1	f	Oct.–Dec.	−14.029	2.628	0.964	0.037	0.002	0.038	148.7	0.997	0.004	17
Central Mediterranean	A2	f	May–July	−11.912	2.288	0.918	0.048	−0.301	0.054	108.5	0.996	0.003	12
	A3	m	Oct.–Dec.	−11.551	2.152	0.953	0.026	−0.065	0.036	140.0	0.998	0.001	16
	A4	m	May–July	−8.650	1.639	0.799	0.031	−0.315	0.078	108.4	0.999	0.000	9
I. coindetii	B1	f	Oct.–March	−8.569	1.418	0.999	0.0001	0.7747	0.0065	443.0	0.999	0.0000	18
W Mediterranean	B2	f	Apr.–Sept.	−8.729	1.452	0.999	0.0003	0.5215	0.0083	360.0	0.999	0.0002	21
	B3	m	Oct.–March	−10.825	1.803	1.000	0.0001	0.8973	0.0085	443.0	0.997	0.0010	19
	B4	m	Apr.–Sept.	−11.680	1.982	1.000	0.0000	0.4756	0.0119	323.1	0.999	0.0002	15
I. coindetii	C1	f	pooled animals	−13.337	2.405	0.997	0.0041	1.5600	0.0140	355.9	0.997	0.0037	25
Galician (Atlantic)	C2	m	pooled animals	−15.041	2.689	1.000	0.0000	1.0460	0.0181	297.0	0.995	0.0083	21
I. coindetii	D1	f	Sierra Leone	−4.258	0.727	0.941	0.0024	−0.2693	0.0251	110.8	0.994	0.0003	10
NW African Coasts	D2	f	W. Sahara	−9.506	1.845	0.995	0.0020	1.0167	0.0196	198.5	0.999	0.0007	15
	D3	m	Sierra Leone	−8.214	1.473	0.889	0.0185	−0.4887	0.0482	118.3	0.994	0.0012	8
	D4	m	W. Sahara	−10.972	2.126	0.961	0.0229	0.5799	0.0327	152.9	0.996	0.0026	12
I. illecebrosus	E1	f	March	−5.947	1.010	0.999	0.0001	0.3350	0.0131	161.8	0.999	0.0000	10
NW Atlantic	E2	f	April	−8.848	1.705	1.000	0.0000	0.8022	0.0180	189.3	0.999	0.0002	10
	E3	f	May	−11.324	2.235	1.000	0.0000	1.2347	0.0205	202.0	0.999	0.0001	7
	E4	m	March	−5.364	0.981	0.999	0.0000	0.2224	0.0139	146.6	0.999	0.0000	8
	E5	m	April	−7.662	1.454	1.000	0.0000	0.5779	0.0164	178.5	0.999	0.0000	8
	E6	m	May	−8.728	1.737	1.000	0.0000	1.1411	0.0171	196.4	0.999	0.0000	6
I. argentinus	F1	f	June	−14.200	2.548	1.000	0.0000	1.2977	0.0140	333.2	0.999	0.0002	13
Patagonian Shelf	F2	m	June	−11.606	2.130	1.000	0.0001	1.6207	0.0113	358.9	0.999	0.0000	13

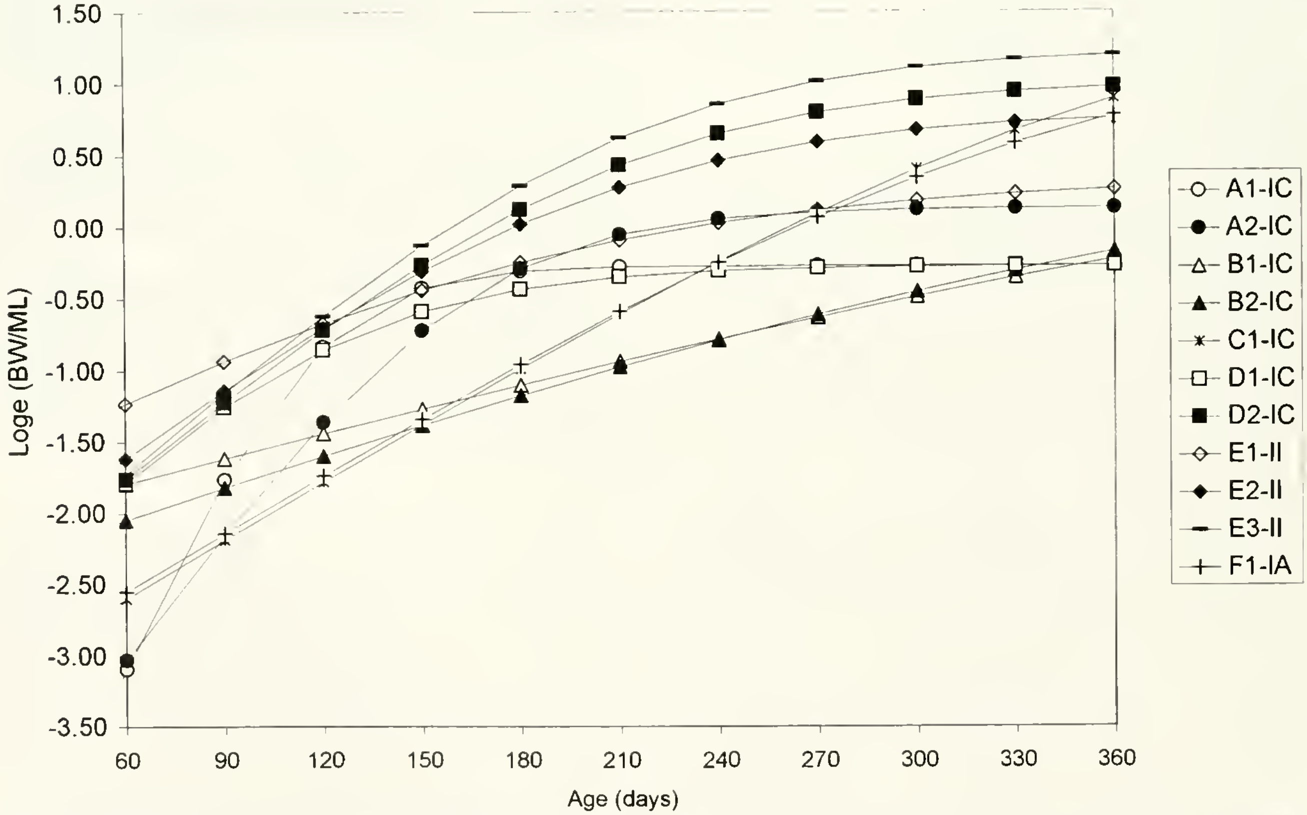

Figure 3. Log_e of body weight (BW) at mantle length (ML) ratio (based on predicted values, symbols) vs. age and computed specified logistic models (lines) for *Illex* females (see Table 1 for case specifications).

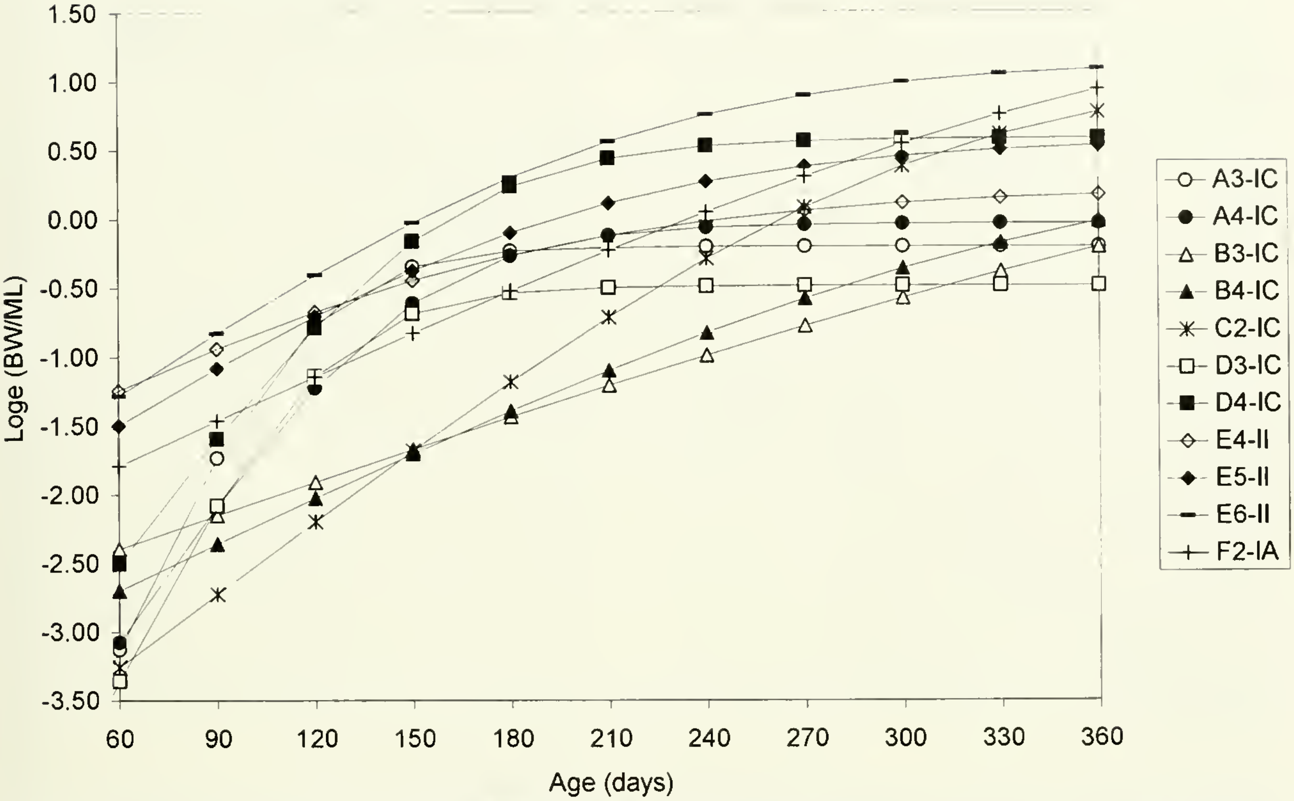

Figure 4. Log$_e$ of body weight (BW) at mantle length (ML) ratio (based on predicted values, symbols) vs. age and computed specified logistic models (lines) for *Illex* males (see Table 1 for case specifications).

Application of Model ii resulted in rapid convergence and satisfactory agreement between predicted and empirical values (Table 4). Both the slopes (B) and asymptote (A) increased with month of hatching in each sex. Differences in these parameter values between sexes were slight for all hatching groups.

Case B, C, D, and F: Illex spp.

For these cases, for which no raw data were available, both models performed quite well (Table 4), but Model i did not achieve complete linearity in D cases. There were some strong differences in model parameters between the sexes but none that were consistent among groups and between Models i and ii. All cases for all three species are compared using Model ii for females (Fig. 3) and males (Fig. 4). Two patterns emerged: relationships with a slight curvature (Cases B1–B4; C1–C2; F1–F2) and relationships with a pronounced curvature and asymptotic trend (all D cases).

Clear differences in model slopes between sexes were evident in *I. coindetii* off Sierra Leone (D1, D3) and in *I. argentinus* (F1, F2), but in the former the slope was higher for females than males, whereas the opposite was true in the latter.

A comparison among and within species indicates that B cases (*I. coindetii* from the Catalonian Sea; Figs. 3 and 4) are unique. They are consistently different from the other curves, suggesting relatively slow and near linear growth, regardless which model is considered.

The Inflection Point and Maturity

The relationship between age-at-the-inflection point (i.e., C parameter; Table 4) and the mean age of mature animals of a specific group (as directly computed or roughly estimated from the literature) was examined (Fig. 5). Despite the rough approximation, a positive and significant linear trend between the two parameters was evident, suggesting that C values reflect to a certain extent ages at maturity. The estimated slope (1.7), however, is higher than the value expected in case of direct proportionality (close to 1). The inflection parameter tended to be relatively lower than (or equal to) the age at maturity for groups where almost all the life stages were represented in the samples (i.e., A1–A4 and D1–D4). It tended to be relatively higher in cases in which mature specimens were underrepresented in the samples, i.e., mean age at maturity was probably underestimated (i.e., C1–C2 and F1).

DISCUSSION

The most appropriate model for describing growth in cephalopods is currently a subject of strong debate. One argument is that squid are fundamentally different from fish and so any of the several available empirical growth models may be appropriate and applied (Jackson 1994). The alternative argument is that most principles of fish population biology do apply to squid; therefore the most appropriate growth function to use is the von Bertalanffy Model (Pauly 1985, Longhurst & Pauly 1987, Pauly 1998).

There is no need of entering into this matter here because an index of growth performance was modeled, not growth in absolute size itself. Therefore we were justified in embracing, the view of Ricker (1979), that "the only criteria for choosing a growth curve that have proven valid are goodness of fit and convenience." It is, however, of considerable interest to compare trends in growth performance obtained here with trends in growth from studies of absolute size-at-age.

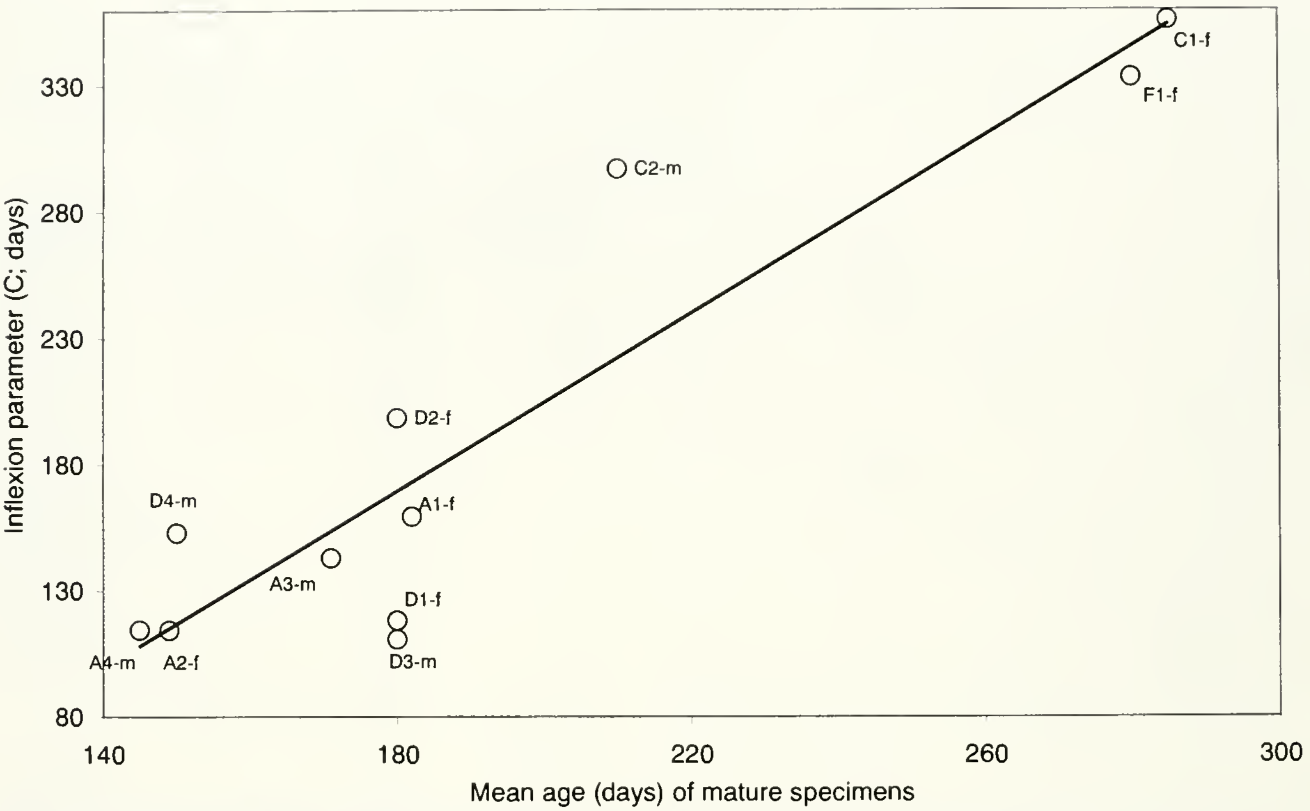

Figure 5. Relationship between age at inflexion point (C parameter of the specific logistic model) and mean age of mature *Illex* females (see Table 1 for case specifications). The fitted straight line is significant ($r^2 = 0.835$; df = 9).

Trends in Growth Performance

A single curve has seldom been suitable to describe the whole life cycle when modeling size-at-age (Ricker 1979, Jackson 1994); therefore, a single model is more often a compromise that deals with different growth phases, each of which could be better described independently.

In the case of *Illex* spp, at least three phases of growth can be identified: an early (larval) exponential phase, a "juvenile" (power or log linear) phase and a "mature and post mature" phase. Because of this, the exponential and the power models are often defined as "early growth curves" (Peters 1983).

Linear modeling of length-at-age data fits satisfactorily some restricted portions of the life cycle (Rodhouse & Hatfield 1990, Gonzalez et al. 1996, Dawe & Beck 1997), but clearly does not apply to the entire cycle (Dawe & Beck 1997, Gonzalez et al. 1998).

A logistic model was already used quite satisfactorily to represent growth in length and weight of A1–A4 squids (Arkhipkin et al. 2000).

However, several different models can fit the same set of data (Arkhipkin et al. 1998) and in some situations both linear and curvilinear models could perform quite well, as was recently reported for length-at-age of *I. argentinus* (Uozumi & Shiba 1993).

In our study of growth performance, the logistic model was quite suitable for cases in which most of the ontogenetic development was represented in the samples (i.e., individual and predicted data for cases A and values based on predicted sizes for cases D). Nevertheless, some imprecision in the estimate of the A (asymptotic) parameter was evident, likely as a consequence of the poor representation in the samples of spent males and females, i.e., a poor representation of the last portion of the life cycle.

Besides the problem related to sampling the whole life cycle, poor model fits obtained with raw data may be caused by several different biases that are related to the different aspects of the methodology applied, starting with the partitioning of specimens into groups or "microcohorts" based on periods of hatching (e.g., month). Statolith increment counts in fact, can be affected by systematic and measurement errors due to preparation techniques and reader's interpretation (Jackson 1994, Dawe & Beck 1997, Arkhipkin et al. 1998, Gonzalez et al. 1998, Gonzalez et al. 2000). Thus, the hatching month to which any individual is assigned may be largely a function of ageing error (Gonzalez et al. 2000).

The use of mean values, despite statistical problems in fitting models, reduces the incidence of these errors thus reflecting in a better model fit.

Comparisons of absolute growth can also be biased by differences in the sampling gear used. Although little is known about the catching capacity of gear such as trawls, traps and jigs, it is most likely that their selectivity by size do differ, so that none of them individually would provide a truly representative sample. Our approach facilitates comparisons when such sampling biases are present, since different gears are likely to be much more highly selective for absolute size than for physical condition.

Evaluation of Models

One of the immediate questions to answer, i.e., "Is there any advantage to modeling WaL above modeling ML and BW separately?" requires comparisons to be answered. Such comparisons were possible only for individual *I. coindetii* (A1–A4 cases) and *I.*

illecebrosus (E1–E6) data sets, based on the logistic models reported in Arkhipkin et al. (2000) and the simple linear and log-log models presented in Dawe and Beck (1997).

The r^2 for A cases ranged between 0.87–0.91, 0.77–0.89, and 0.95–0.97 for ML-at-age, BW-at-age, and $\log_e$WaL-at-age, respectively. Considering the E cases, the r^2 values ranged between 0.37–0.84, 0.37–0.90, and 0.35–0.90 for ML-at-age, $\log_e$BW-at-$\log_e$age, and $\log_e$WaL-at-age, respectively. Results indicate that similar (E1–E6) or even better (A1–A4) model fits were obtained by using WaL instead of ML and BW separately, with the advantage of using only one model to take into consideration two size indices. A possible explanation for that is that while length will increase with age under any conditions, weight may increase or decrease, but does not account for concurrent increases in length. However, weight-at-length does account for concurrent length increase, and so it is a better descriptor of growth performance.

Another very interesting point is the comparison of results obtained by applying the same models to the same data sets and using alternatively raw data from individuals and values computed from predicted lengths and weights. While both Models i and ii (i.e., the log-log and the specific logistic) do fit consistently well for both *I. coindetii* and *I. illecebrosus* datasets when using data based on predicted sizes, when raw individual data were used, Model ii fitted well *I. coindetii* datasets (Cases A), but did not fit *I. illecebrosus* datasets. Such differences in Model ii fits are likely the consequence of the "quality" of the available datasets. Raw individual age data in fact, are almost neither "balanced" (i.e., the same number of aged specimens for each age level) nor "optimal" (i.e., number of counts proportional to the variance of the estimates) because statolith processing is very time consuming and age classes are numerous. In cases of such high variability, few points could have a great influence on the estimates and the model may not fit properly. If the samples are larger, however, or if repeated blind counts are available (see, for example, Arkhipkin et al. 1998), then a statistical "weight" can be assigned to each point and a weighed regression performed to improve the fitting quality (Neter et al. 1985).

Comparisons Within and Among Species

WaL modeling showed some common patterns among species and groups, with the exception of the *I. coindetii* population of the Catalonian Sea. Regardless of the model applied to those exceptional cases, the parameters generated were quite different from those of all the other cases. To a certain extent, such differences may be related to the indirect method used in the present work to compute body weight-at-age, but it is also likely that age was originally overestimated for that group, as a consequence of the ageing method used (Arkhipkin personal communication; Dawe & Beck 1997, Gonzalez et al. 2000).

Results obtained for the other groups showed several common features of growth performance within the genus *Illex*, which are consistent with results of other studies (e.g., Dawe & Beck 1997, Haimovici et al. 1998, Arkhipkin et al. 2000). These features include superior growth performance of *I. coindetii* and *I. illecebrosus* specimens hatched in warm conditions relative to those hatched in colder conditions. Also consistent with previous studies, a superior growth performance in populations inhabiting warm waters (Strait of Sicily in the Central Mediterranean Sea, West Sahara, and Sierra Leone in the Eastern Atlantic) relative to that of con-specific or con-generic populations living in colder waters resulted. Last but not least, the previous conclusion that the Galician *I. coindetii* population appears to be more similar to the South Patagonian stock of *I. argentinus* than to any other *Illex* populations (Gonzalez et al. 1996) was confirmed.

One of the most interesting results of our study is the direct relationship found between the inflexion parameter C (from application of the specific logistic model) and the age-at-maturity. Size-at-maturity in *Illex* squid has rarely been objectively defined (e.g., as the size at which 50% of the specimens were classified as mature; cf. Jereb & Ragonese 1995). Usually, only ranges or mean size values of mature animals have been reported, although the onset of sexual maturation is considered to be a critical point in cephalopod life cycles (Mangold et al. 1993).

The C parameter may represent a useful maturity objective index for future studies of changes or trends in sexual maturation. For example, Pauly (1998) argued that principles of fish dynamics are also applicable to cephalopods. If this were true, then temperature would be directly related to growth rate and inversely related to maximum size- and age-at-maturity. Consequently, when comparing among populations, a shift of the inflection parameter to younger ages would be expected from colder to warmer habitats, related to an improvement in growth performance. This is consistent with results obtained in the present work.

Obviously further comparative studies are required especially within populations. Independent estimates of size- and age-at-maturity would be very useful in evaluating if a true relationship exists between the C parameter from the specific logistic model and the age at the onset of maturity.

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COMPARISON OF NUTRIENT COMPOSITION OF GONADS AND COELOMIC FLUID OF GREEN SEA URCHIN *STRONGYLOCENTROTUS DROEBACHIENSIS*

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ABSTRACT The compositional characteristics of sea urchin gonads and coelomic fluid from *Strongylocentrotus droebachiensis* harvested in the coasts of Newfoundland and thereafter reared in an aquaculture facility and fed on a *Laminaria* diet for a 3-week period, were assessed. Evaluations were performed on the basis of proximate composition, lipid class distribution, fatty acid composition, total and free amino acid composition, and contents of nucleic acids and carotenoids. Noticeable changes existed between proximate composition of sea urchin gonads and coelomic fluid. Moisture content was 74.7 ± 0.04 and 96.5 ± 0.03% in gonads and coelomic fluid, respectively. Gonads contained very high levels of lipids, proteins, and carbohydrates; whereas, these were present at very low levels in the coelomic fluid. Major nonpolar lipid classes were triacylglycerols (TAG), free fatty acids (FFA), and sterols (ST) while dominant polar lipid classes were phosphatidylcholine (PC), phosphatidylethanolamine (PE), sphingomyelin/ lysophosphatidylcholine (SM/LPC), and phosphatidylserine / phosphatidylinositol (PS/PI) in both the gonads and the coelomic fluid. Major saturated fatty acids (SFA) were 14:0 and 16:0; whereas, 20:1n-15 was the main monounsaturated fatty acid (MUFA) present. Furthermore, 20:5n-3 (eicosapentaenoic acid, EPA) was the dominant polyunsaturated fatty acid (PUFA) in the gonads and the coelomic fluid. The total amino acid (TAA) and free amino acid (FAA) profiles were dominated by glycine. The total FAA content was much higher in the gonads than in the coelomic fluid. In addition, the total carotenoid content of sea urchin gonads was approximately 6.4 times greater than that of coelomic fluid. Hence, most of the carotenoids were concentrated in the gonadal tissue. Echininone and fucoxanthin were the dominant carotenoids in the gonads and the coelomic fluid, respectively. The content of deoxyribonucleic acid (DNA) and ribonucleic acid (RNA) was much higher in the gonad than in the coelomic fluid, thus indicating greater biomass and protein synthetic activity in the former tissue. The present study demonstrates that sea urchin gonads have much in common with sea urchin coelomic fluid on a qualitative basis. However, there were marked quantitative differences between the two tissues.

KEY WORDS: amino acid composition, carotenoids, fatty acid composition, lipid class distribution, nucleic acids, *Strongylocentrotus droebachiensis*

INTRODUCTION

Sea urchins belong to the marine invertebrate phylum Echinodermata or spiny-skinned animals. These relatively small echinoderms have spherical bodies enclosed in a hard shell or "test" completely covered with numerous sharp spines. Sea urchins are omnivorous animals that live on the ocean floor, feeding on small crustaceans, fish offal, but mainly seaweed (Smith 1980). Thus, the eating quality of sea urchin gonads is dictated, to a certain degree, by the quality of kelp consumed. *Laminaria* kelps are the preferred source of feed for sea urchins. Kramer and Nordin (1979) reported that the green sea urchin *Strongylocentrotus droebachiensis* produces high-quality gonads when the availability of fresh kelp is adequate. The edible green sea urchin *S. droebachiensis* is abundantly distributed in the North Atlantic, Arctic, and North Pacific Oceans, but this species is currently exploited to a much lesser extent in the Northwest Atlantic and in the Northeast Pacific, and Northeast Atlantic (Walker & Lesser 1998). Furthermore, *S. droebachiensis* is a target species for the development of commercial echiniculture (Hagen 1996).

The edible portions of the sea urchin body are its reproductive organs; ovaries, and testes. Gonad yield from sea urchin may vary with the time and the site of harvest and generally ranges from 8–20% of the total body mass. When sea urchins are processed for gonads, the initial step is to break the shell and open it so that the five gonad sacs are exposed. The cracked shells are then allowed to drain for several minutes to dispose of coelomic fluid. Thus, during extraction of sea urchin gonads, large amounts of coelomic fluid are obtained. So far, there are no effective means of using sea urchin coelomic fluid in a useful manner. Furthermore, no information is available on the nutrient composition of sea urchin coelomic fluid. In fact, knowledge of nutrient composition may be useful to determine whether sea urchin coelomic fluid could serve as a potential source of a flavoring in fabricated seafood.

The objective of this study was to assess the nutrient composition of sea urchin coelomic fluid as compared with that of the gonads. Thus, proximate composition, lipid class distribution, fatty acid composition, amino acid composition, and contents of carotenoids and nucleic acids of gonads and coelomic fluid were determined. This may lead to potential commercial utilization of the processing byproducts from sea urchins, which would otherwise be discarded.

MATERIALS AND METHODS

Materials

One hundred twenty-five sea urchins were procured from the Sea Urchin Research Facility (SURF) at Bonavista Bay, Newfoundland and subsequently transported in aquarium coolers to our laboratory at Memorial University of Newfoundland. Urchins were captured from the wild (June 2000) and raised in raceways feeding on a *Laminaria* diet. Urchins were harvested for analysis after three weeks of feeding on a purely algal diet. Live urchins were stored at 4°C before the extraction of tissues. The gonads and coelomic fluid of sea urchins were separated after breaking the shell, using a specially devised sea urchin cracking tool. After extraction, sea urchin gonads were homogenized for 2 min using a cooled Waring blender (Dynamics Corporation, New Hartford, CT), and coelomic fluid was used for analysis as it is. In this study, sea urchin male and female gonads were pooled together for analysis. The tissues (both gonads and coelomic fluid) were flushed with liquid nitrogen and stored at −20°C until used for further analyses. All chemicals used were obtained from either Fisher Scientific (Fair Lawn, NJ) or Sigma Chemical Co. (St. Louis, MO). The solvents were of ACS-, pesticide-, or HPLC-grade.

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Determination of Proximate Composition

Moisture and ash contents of sea urchin tissues were determined according to the standard AOAC (1990) procedures. Crude protein content was obtained by Kjeldhal method (AOAC 1990), and total lipids were extracted and quantified by the Bligh and Dyer (1959) procedure. Carbohydrate content of each sample was determined by difference.

Analysis of Lipid Classes by Iatroscan

Instrumentation

The crude lipids obtained from Bligh and Dyer (1959) extraction were chromatographed on silica gel-coated Chromarods - S III and then analyzed using an Iatroscan MK-5 (Iatroscan Laboratories Inc., Tokyo) analyzer equipped with a flame ionization detector (FID) connected to a computer loaded with TSCAN software (Scientific Products and Equipment, Concord, ON) for data handling. A hydrogen flow rate of 160 mL per min and an airflow rate of 2,000 mL per min were used in operating the FID. The scanning speed of rods was 30 sec per rod.

Preparation of Chromarods

The Chromarods were soaked in concentrated nitric acid overnight followed by thorough washing with distilled water and acetone. The Chromarods were then impregnated by dipping in a 3% (w/v) boric acid solution for five minutes to improve separation. Finally, the cleaned Chromarods were scanned twice to burn any remaining impurities.

Standards and Calibration

A stock solution of each of the nonpolar lipids; namely, free fatty acid (FFA; oleic acid), cholesterol ester (CE), cholesterol (CHOL), monoacylglycerol (MAG; monoolein), diacylglycerol (DAG; diolein), and triacylglycerol (TAG; triolein), and the polar lipids; namely, phosphatidylcholine (PC), phosphatidylethanolamine (PE), phosphatidylinositol (PI), phosphatidylserine (PS), lysophosphatidylcholine (LPC), lysophosphatidylethanolamine (LPE), cardiolipin (CL), and sphingomyelin (SM) was prepared by dissolving each in a chloroform/methanol (2:1, v/v) solution and stored at −20°C. A range of dilutions of the stock solution, from 0.1 to 10 μg per μL, was prepared for use as working standards. Each compound was developed individually and run on the Iatroscan-FID to determine its purity and Rf value. For each compound peak, area was plotted against a series of known concentrations to obtain the calibration curve.

Iatroscan (TLC-FID) Analysis of Sea Urchin Lipids

The total lipids extracted were dissolved in chloroform/methanol (2:1, v/v) to obtain a concentration of 1 μg lipid per mL. A 1 μL aliquot of sample was spotted on silica gel-coated Chromarods - S III and conditioned in a humidity chamber containing saturated $CaCl_2$ for 20 min. The Chromarods were then developed in two solvent systems. The solvent system hexane/diethyl ether/acetic acid (80:20:2, v/v/v) was used for separation of nonpolar lipids (Christie 1982). Following their development, Chromarods were dried at 110°C for three minutes and scanned completely to reveal nonpolar lipids. For polar lipids, following the same procedure and drying, the Chromarods were scanned partially to a point just beyond the MAG peak to burn the nonpolar lipids. These partially scanned Chromarods were developed in a second solvent system of chloroform/methanol/water (80:35:2, v/v/v) for the separation of polar lipid classes (Christie 1982) followed by drying at 110°C for three minutes. Finally, the Chromarods were scanned completely to reveal polar lipids; the identity of each peak was determined by comparison with a chromatogram of standards acquired concurrently with the samples. The determination of weight percentages of individual lipid classes was achieved using the standard curves obtained for each authentic standard.

Analysis of Fatty Acid Composition of Lipids

Fatty acid composition of lipids was determined using gas chromatography (GC) as described by Wanasundara and Shahidi (1997). Fatty acid methyl esters (FAMEs) of total lipids of sea urchin gonads and coelomic fluid were prepared by transmethylating approximately 10 to 20 mg of each lipid sample in 2 mL of freshly prepared transmethylating reagent [6% (v/v) sulfuric acid in 99.9 mol% HPLC-grade methanol containing 15 mg of t-butylhydroquinone (TBHQ)] at 65°C for 15 h in a 6 mL Teflon-lined screw-capped conical vials. After incubation, the mixture was cooled, and 1 mL of distilled water was added to it. This was followed by extracting the FAMEs three times with 1.5 mL pesticide-grade hexane. A few crystals of TBHQ were added to each sample before extraction with hexane. The hexane layers were removed and combined in a clean test tube followed by washing twice with 1.5 mL of distilled water by vortexing. The aqueous layer was discarded after the first wash, while the hexane layer was removed and placed in a GC vial following the second wash. Hexane was evaporated under a stream of nitrogen in a fume hood. The dried FAMEs were then dissolved in 1 mL of carbon disulfide and used for GC analysis. FAMEs were separated using a gas chromatograph (Hewlett-Packard 5890 Series II, Hewlett-Packard, Mississuaga, ON) equipped with a fused silica capillary column (SUPELCOWAX-10, 0.25-mm diameter, 30-m length, 0.25-μm film thickness; Supelco Canada Ltd., Oakville, ON). The sample was injected into the GC analyzer using a Hewlett-Packard 7673 autoinjector (Hewlett-Packard, Toronto, ON). The temperature of the oven was programmed at 220°C for 10.25 min followed by ramping to 240°C at 20°C per min, where it was held for nine minutes. Helium at a flow rate of 2 mL per min was used as the carrier gas. The FAMEs were identified by comparing their retention times with those of authentic standard mixtures (GLC - 461, Nu-Check-Prep) and literature values (Takagi et al. 1980, Takagi et al. 1986). The relative content of fatty acids in the sample was determined using the peak areas of fatty acids.

Carotenoid Pigments

Extraction and Determination of Total and Individual Carotenoids

Carotenoids from each tissue were extracted three times with a total of 50 mL of acetone for two minutes. The homogenized samples were centrifuged (IEC Centra MP4 Centrifuge, International Equipment Co., Needham Heights, MA) at 4000 × g for five minutes. The supernatant was subsequently filtered through a

Whatman No. 1 filter paper. Carotenoid pigments in acetone were then transferred to 40 mL of n-hexane in a 250-mL separatory funnel. One hundred milliliters of a 0.5% sodium chloride solution were added to the mixture to maximize the transfer of carotenoids. The hexane layer was then transferred into a 50-mL volumetric flask and made up to volume. The absorption spectrum was then recorded (400–600 nm) using a Spectronic spectrophotometer (Spectronic Genesis, Toronto, ON). The total and individual carotenoid contents were determined by the method of McBeth (1972). The total content of carotenoids present per 100 g of tissue was calculated using the following equation.

$$\text{mg Carotenoid per 100 g tissue} = (A \times V \times 10^3)/\varepsilon \times W)$$

where, A = absorbance at λ_{max}; V = total volume of the sample (mL); ε = molar extinction coefficient, and W = weight of the tissue (g). Because the crude extracts usually contained a variety of carotenoids an average coefficient of 2,500 was used in the calculations.

The total pigment extracted was separated into individual carotenoids by means of thin-layer chromatography (TLC). The crude carotenoids were separated by preparative TLC on silica gel G (20 × 20 cm, 250 μm, Aldrich Chemical Co., Inc., Milwaukee, WI) using acetone/n-hexane (3:7, v/v) as the developing solvent.

Characterization of Fractions

Cochromatography on TLC provided the ultimate test for identification when authentic samples were available for comparison with unknown pigments. The unknown fraction and the authentic sample were spotted on either side in an equally proportionated mixture of the two pigments on silica gel G plates (20 × 20 cm, 250 μm, Aldrich Chemical Co., Inc., Milwaukee, WI); unknown fractions were considered to be identical to the authentic sample if the two did not separate upon subsequent development of the plate. When authentic samples were unavailable, the type of carotenoid in each fraction was tentatively identified according to its absorption maximum in n-hexane, ethanol, and chloroform (Goodwin 1955, Krinsky & Goldsmith 1960, Fox & Hopkins 1966, Britton 1995).

Determination of Total Amino Acids

The amino acid composition of sea urchin gonads and coelomic fluid was determined according to the procedure described by Blackburn (1968). Samples were lyophilized and then hydrolyzed for 24 h at 110°C with 6M HCl. Hydrochloric acid in the hydrolyzate was removed under vacuum, and the dried sample was reconstituted with a lithium citrate buffer (0.2 M, pH 2.2) for analysis. The amino acids in the hydrolyzate were separated, identified and quantified using a Beckman 121 MB amino acid analyzer (Beckman Instruments Inc., Palo Alto, CA). Sulfur-containing amino acids were determined by oxidizing the samples with performic acid before their hydrolysis in a 6M HCl solution (Blackburn 1968). Cysteine and methionine were measured as cysteic acid and methionine sulphone, respectively. To determine tryptophan, samples were hydrolyzed in 3M mercaptoethanesulfonic acid at 110°C for 22 h under nitrogen and then neutralized with lithium hydroxide and adjusted to pH 2.2 (Penke et al. 1974).

Determination of Free Amino Acids

Samples (10 g) were extracted with 20 mL of a 6% (v/v) perchloric acid (PCA) solution by homogenization using a Polytron homogenizer (Brinkmann Instruments, Rexdale, ON) at 10,000 rpm for two minutes in an ice bath. The homogenized samples were then incubated in an ice bath for 30 min. This was followed by centrifugation (IEC Centra MP4 Centrifuge, International Equipment Co., Needham Heights, MA) at 2,000 × g for 15 min. The residue was re-extracted with another 20 mL of 6% PCA. The supernatants were combined and filtered through a Whatman No. 4 filter paper. The pH of the filtrate was adjusted to 7.0 using a 33% KOH (w/v) solution. Precipitates of potassium perchlorate were removed by centrifugation at 2000 × g for 10 min. The supernatant was then acidified to pH 2.2 using a 10 M HCl solution, and the volume of the extract was brought to 50 mL with distilled water. Three milliliters of lithium citrate buffer (pH 2.2, 0.3M) were added to 1 mL of the extract, and the resultant solution was analyzed using a Beckman 121 MB amino acid analyzer (Beckman Instruments, Inc., Palo Alto, CA) for individual amino acids.

Determination of Nucleic Acids

The DNA and RNA contstituents of gonads and coelomic fluid of sea urchins were extracted according to the method of Schmidt and Thannhauser (1945) as modified by Munro and Fleck (1969). Five grams of each sample were homogenized in 80 mL ice-cold deionized water using a Polytron homogenizer (Brinkman Instruments, Rexdale, ON) at 10,000 rpm. Five milliliters of the homogenate were allowed to stand for ten minutes in ice and then centrifuged (IEC Centra MP4 Centrifuge, International Equipment Co., Needham Heights, MA) at 2000 × g for ten minutes. The residue was subsequently washed with 2.5 mL of ice-cold 0.2 M PCA and centrifuged at 2,000 × g for ten minutes followed by digestion of the residue in 4 mL of a 0.3 M KOH for one hour at 37°C in a water bath. The resultant solution was cooled in ice and mixed with 2.5 mL of 1.2 M PCA and allowed to stand for ten minutes, which finally resulted in the coagulation of proteins. The mixture was centrifuged at 2,000 × g for ten minutes, and the supernatant was recovered (Extract No.1). The precipitate was then washed twice with 2.5 mL of a 0.2 M PCA solution and centrifuged at 2,000 × g for five minutes. The supernatant was combined with extract No. 1 and 10 mL of a 0.6 M PCA were added to the mixture. This was used for RNA determination after diluting it up to 100 mL with distilled water. The residue was dissolved in 17 mL of a 0.3 M KOH solution at 37°C and diluted to the 50 mL mark in a volumetric flask with distilled water. The content of DNA in the samples was estimated by determining the deoxyribose content in the extract using the indole procedure of Ceriotti (1952), while RNA was determined by recording the absorbance of the nucleotide extracts at 260 nm using a Hewlett-Packard diode array spectrophotometer (Hewlett-Packard, Model 8452A, Hewlett-Packard [Canada] Ltd., Mississauga, ON). Protein interference at this wavelength was eliminated by employing a correction factor of 0.001 absorbance unit for each 1 μg per mL protein concentration in the extracts. The protein concentration of the extracts was measured using the Folin–phenol procedure of Lowry et al. (1951). Bovine serum albumin (BSA) was used as a standard. Calf thymus DNA (containing 82% single stranded DNA) and calf liver RNA (96% purity) were used as the standards for DNA and RNA determinations, respectively.

Statistical Analysis

Each experiment was replicated three times and mean values ± standard deviations reported for each sample. For statistical analyses, mean values of the experimental data were subjected to one way analysis of variance (ANOVA) using GraphPAD Instat Version 1.0. Significance was determined at 5% probability level.

RESULTS

Proximate Composition

Proximate composition of sea urchin gonads and coelomic fluid is shown in Table 1. The moisture and ash contents of sea urchin coelomic fluid were much higher than those of the gonads. On the other hand, the levels of protein, lipid, and carbohydrate in the coelomic fluid were much lower than those in the gonads on a fresh weight basis.

Lipid Class Distribution

The nonpolar and polar lipid classes of gonads and coelomic fluid of sea urchin *S. droebachiensis* are shown in Table 2. Major nonpolar lipid classes were TAG, FFA, and ST; whereas, main polar lipids classes were PC, PE, SM/LPC, and PS/PI in both gonads and coelomic fluid. Triacylglycerols constituted the main energy reserve in both tissues, contributing more than 65% to the total nonpolar lipids. On the other hand, PC was the dominant polar lipid, accounting for more than 60% in both gonads and coelomic fluid. The polar lipid classes SM and LPC as well as PS and PI did not show a clear chromatographic separation from each other during Iatroscan analysis.

Fatty Acid Composition

Fatty acid composition of sea urchin gonads and coelomic fluid is presented in Table 3. Qualitatively, the fatty acid compositions were the same in both tissues, while there were significant ($P < 0.05$) quantitative variations. In both tissues, 14:0 and 16:0 were the main saturated fatty acids. In addition, 18:0 and 20:0 were present in considerably high levels. The fatty acid 20:1n-15 was the dominant MUFA in both gonadal and coelomic fluid lipids. Furthermore, 16:1n-7, 16:1n-9, 18:1n-7, 20:1n-7, 20:1n-9, and 22:1n-11 were detected in noticeable amounts. Among PUFA, 20:5n-3 contributed the highest proportion to the total fatty acid content in both gonadal and coelomic fluid lipids.

Carotenoid Pigments

The total carotenoid content, on a dry weight basis, of sea urchin gonads and coelomic fluid was 23.2 ± 0.04 and 3.7 ± 0.1 mg per g tissue, respectively. Crude pigments from gonads and coelomic fluid were separated by TLC into eight and seven fractions, respectively. Crude pigments of both tissues, upon TLC separation, exhibited two major bands. In gonads, fractions I (Rf = 0.96) and II (Rf = 0.88) ran close to the solvent front, but they were adequately separated. For coelomic fluid, fraction I (Rf = 0.94) ran almost close to the solvent front; whereas, fraction IV (Rf = 0.42) ran well behind. The carotenoid fraction I of both gonads and coelomic fluid of sea urchin *S. droebachiensis* was confirmed to be β-carotene using an authentic β-carotene sample as established by cochromatography on silica gel TLC plates. Similarly, gonadal fractions III, IV, VI, and VII contained astaxanthin ester (Rf = 0.57), zeaxanthin (Rf = 0.51), canthaxanthin (Rf = 0.22), and free astaxanthin (Rf = 0.1), respectively. Furthermore, fraction II of gonadal crude pigments corresponded to echininone by means of absorption maxima in hexane, chloroform, and ethanol (Goodwin 1955, Krinsky & Goldsmith 1960, Fox & Hopkins 1966, Britton 1995). Thus, the observed λ_{max} values of echininone were 484/460, 466, and 475 nm in hexane, ethanol, and chloroform, respectively. In coelomic fluid, the pigment in fraction IV corresponded with fucoxanthin based on absorption maxima of 424/447/474, 423/446/472, and 454/488 nm in hexane, ethanol, and chloroform, respectively. Other minor carotenoids in the coelomic fluid were astaxanthin etser (Rf = 0.58), canthaxanthin (Rf = 0.23), and free astaxanthin (Rf = 0.1), which corresponded to fractions III, V, and VI, respectively. The other minor carotenoids were not analyzed because of their insufficient concentration to obtain absorption maxima and also lack of authentic samples.

TABLE 1.

Proximate composition of sea urchin gonads and coelomic fluid after feeding urchins on a *Laminaria* diet for three weeks.

Constituent	Gonads	Coelomic Fluid
Moisture	74.7 (0.04)	96.5 (0.03)
Ash	2.2 (0.2)	3.0 (0.02)
Protein	7.4 (0.2)	0.1 (0.02)
Lipid	4.7 (0.1)	0.1 (0.03)
Carbohydrate[a]	10.6 (0.2)	0.4 (0.1)

Results are mean values of three replicates (standard deviation). Values in each row with the same superscript are not different ($P > 0.05$) from one another.

[a] Determined by difference.

TABLE 2.

Quantification of non-polar and polar lipids (weight %) of sea urchin gonads and coelomic fluid after feeding the urchins on *Laminoria* diet for three weeks.

Lipid Classes	Gonad	Coelomic Fluid
Non polar lipids		
TAG	66.7 (0.8)	56.5 (0.7)
FFA	22.7 (1.1)	37.4 (1.0)
ST	10.6 (1.2)	6.1 (0.8)
MAG	tr	tr
DAG	tr	tr
Polar lipids		
PC	65.7 (1.4)	68.9 (0.8)
PE	17.3 (0.2)	27.3 (0.9)
SM/LPC	8.4 (0.7)	2.6 (0.2)
PS/PI	8.5 (0.6)	1.1 (0.5)

Results are mean values of three replicates (standard deviation). Values in each row with the same superscript are not different ($P > 0.05$) from one another. Abbreviations: TAG, triacylglycerol; FFA, free fatty acid; ST, sterol; MAG, monoacylglycerol; DAG, diacylglycerol; PC, phosphatidylcholine; PE, phosphatidylethanolamine; SM, sphingomyelin; LPC, lysiphosphatidylcholine; PS, phosphatidylserine and PI, phosphatidylinositol; and tr, trace.

Amino Acid Composition

The total and free amino acid compositions of sea urchin gonads and coelomic fluid are shown in Tables 4 and 5, respectively. Results so obtained did not show any clear variation on a qualitative basis for tissues examined. Thus, the spectrum of amino acids, both total and free, present was nearly the same for both gonads and coelomic fluid. Total amino acid profile indicated the

TABLE 3.

Fatty acid composition (weight %) of total lipids sea urchin gonads and coelomic fluid after feeding the urchins with *Laminaria* diet for three weeks.

Fatty Acid	Gonads	Coelomic Fluid
14:0	9.4 (0.1)^{a}	8.4 (0.1)^{a}
15:0	0.4 (0.02)^{a}	0.7 (0.02)^{a}
16:0	11.1 (0.1)^{a}	17.6 (0.3)^{a}
18:0	2.2 (0.04)^{a}	2.3 (0.1)^{a}
20:0	2.9 (0.1)^{a}	1.2 (0.1)^{a}
14:1n-7	0.8 (0.03)^{a}	0.3 (0.03)^{a}
16:1n-9	4.8 (0.1)^{ac}	1.6 (0.1)^{a}
16:1n-7	1.5 (0.1)^{a}	3.4 (0.2)^{a}
16:1n-5	0.3 (0.03)^{a}	0.6 (0.04)^{a}
18:1n-9	1.8 (0.1)^{a}	2.2 (0.1)^{a}
18:1n-7	3.6 (0.1)^{a}	4.2 (0.1)^{a}
18:1n-5	0.5 (0.02)^{a}	3.4 (0.2)^{a}
20:1n-15	7.5 (0.2)^{a}	5.6 (0.1)^{a}
20:1n-9	4.0 (0.03)^{a}	1.2 (0.03)^{a}
20:1n-7	2.2 (0.1)^{a}	1.9 (0.1)^{a}
22:1n-11	2.9 (0.04)^{a}	1.2 (0.1)^{a}
22:1n-9	0.4 (0.03)^{a}	1.1 (0.1)^{a}
16:2n-6	0.5 (0.03)^{a}	0.2 (0.02)^{a}
16:4n-6	1.9 (0.1)^{a}	ND
16:4n-3	ND	2.8 (0.1)^{a}
18:2n-9	ND	0.5 (0.02)^{a}
18:2n-6	1.1 (0.1)^{a}	0.9 (0.1)^{a}
18:3n-6	1.4 (0.03)^{a}	1.3 (0.1)^{a}
18:3n-3	1.3 (0.04)^{a}	0.5 (0.04)^{a}
18:4n-3	3.8 (0.03)^{a}	2.2 (0.03)^{a}
20:2Δ5, 11	1.8 (0.1)^{a}	1.7 (0.1)^{a}
20:2Δ5, 13	0.9 (0.1)^{a}	0.7 (0.03)^{a}
20:2n-6	1.7 (0.1)^{a}	1.7 (0.1)^{a}
20:4n-6	7.0 (0.1)^{a}	9.9 (0.1)^{a}
20:3n-3	1.7 (0.2)^{a}	0.9 (0.04)^{b}
20:4n-3	1.2 (0.2)^{a}	0.3 (0.02)^{a}
20:5n-3	16.3 (0.1)^{a}	16.5 (0.5)^{a}
22:5n-6	0.2 (0.1)^{a}	0.3 (0.03)^{a}
22:5n-3	0.6 (0.1)^{a}	0.7 (0.04)^{a}
22:6n-3	1.4 (0.1)^{a}	0.6 (0.1)^{a}

Results are mean values of three replicates (standard deviation). Values in each row with the same superscript are not significantly different ($P > 0.05$) from one another. ND, not detected.

TABLE 4.

Total amino acid content (mg/g protein) of sea urchin gonads and coelomic fluid after feeding urchins on a *Laminaria* diet for three weeks.

Amino Acid	Gonads	Coelomic Fluid
Alanine	42.5 (0.4)^{a}	63.1 (0.5)^{b}
Arginine	80.7 (0.5)^{a}	74.2 (0.6)^{b}
Cysteine	7.2 (0.1)^{a}	5.7 (0.2)^{b}
Glutamic acid	87.2 (0.2)^{a}	90.6 (0.6)^{a}
Glycine	118.0 (0.7)^{a}	143.6 (0.7)^{b}
Histidine	35.5 (0.3)^{a}	27.0 (0.8)^{b}
Hydroxyproline	3.3 (0.2)^{a}	2.6 (0.2)^{a}
Isoleucine	77.1 (0.1)^{a}	60.9 (0.9)^{b}
Leucine	65.9 (1.0)^{a}	62.6 (0.3)^{b}
Lysine	81.1 (0.9)^{a}	77.1 (0.9)^{a}
Methionine	1.2 (0.3)^{a}	0.9 (0.1)^{b}
Phenylalanine	53.2 (0.9)^{a}	42.1 (0.6)^{b}
Proline	40.2 (0.5)^{a}	48.4 (0.1)^{b}
Serine	47.1 (0.1)^{a}	46.5 (0.7)^{b}
Threonine	47.8 (0.4)^{a}	47.2 (0.5)^{b}
Tryptophan	1.4 (0.1)^{a}	1.1 (0.2)^{b}
Tyrosine	36.3 (0.8)^{a}	38.6 (0.4)^{b}
Valine	78.3 (0.4)^{a}	75.1 (1.0)^{b}
Total (mg/g protein)	989.4 (2.2)	985.4 (1.3)

Results are mean values of three replicates (standard deviation). Values in each row with the same superscript are not different ($P > 0.05$) from one another.

dominance of glycine in both tissues analyzed. However, on a dry weight basis, the content of glycine in sea urchin coelomic fluid was significantly ($P < 0.05$) higher than that of gonads. Almost all the essential amino acids were present in both gonads and coelomic fluid of *S. droebachiensis*. With respect to FAA content of sea urchin tissues, the total FAA content was much higher in sea urchin gonads than that in the coelomic fluid. However, in both tissues glycine was the dominant FAA contributing 57.1 and 56.3% to the total amount in the gonads and coelomic fluid, respectively.

Content of Nucleic Acids

The content of nucleic acids in sea urchin gonads and coelomic fluid, on a dry weight basis, was different. The content of DNA of sea urchin gonads and coelomic fluid was 3.93 ± 0.1 and 1.02 ± 0.07 μg per g tissue, respectively; whereas, corresponding values for the content of RNA were 2.63 ± 0.06 and 0.49 ± 0.03 μg per g tissue, respectively. Thus, the content of DNA was higher than that of RNA in both tissues analyzed. Furthermore, the ratio of RNA/DNA was 0.7 ± 0.1 and 0.5 ± 0.03 for sea urchin gonads and coelomic fluid, respectively.

DISCUSSION

Reproductive State of Sea Urchins

For feeding experiments, sea urchins were obtained from the wild in the month of June, representing the spring season. In general, gonad development in sea urchins may include five different stages of resting, growing, premature, mature, and spawning (de Jong-Westman et al. 1996). In resting, gonad size is at a minimum, which usually occurs after spawning. *S. droebachiensis* has an annual reproductive cycle with major spawning period in the late winter or early spring (Keats et al. 1984). Therefore, at this stage, urchins were presumably in the state of resting; hence, they have undergone a large drop in gonad size following spawning.

In general, gonadal yield is strongly affected by the seasonal reproductive cycle of sea urchins. During spawning, a high proportion of the gonad mass is released as gametes (Thompson 1984). Once spawning occurred, this may exert a significant effect on the biochemical composition of gonads. Because urchins were fed on a *Laminaria* diet after harvesting and consequently subject to intense feeding, this may have a significant effect on the nutrient composition of sea urchin tissues. Generally, both food quality and quantity affect sea urchin growth (Lawrence & Lane 1982), thus excessive feeding resulting in the accumulation of nutrients in the tissues.

Proximate Composition

The major nutrients of sea urchin *S. droebachiensis* gonads were polysaccharides, proteins, and lipids, similar to that reported

TABLE 5.

Free amino acid content (μ/g dry weight) of sea urchin gonads and coelomic fluid after feeding urchins on *Laminaria* diet for three weeks.

Amino Acid	Gonads		Coelomic Fluid	
	μg/g	%	μg/g	%
Alanine	2,872 (77)^{a}	14	899 (17)^{b}	7.5
α-aminoadipic acid	68 (5)^{a}	0.3	——	
Arginine	180 (3)^{a}	0.9	507 (19)^{b}	4.2
Asparagine	13 (1)^{a}	0.1	——	——
Aspartic acid	73 (8)^{a}	0.4	80 (3)^{b}	0.7
Cystathionine	115 (2)^{a}	0.6	76 (39)^{b}	0.6
Cysteine	214 (12)^{a}	1.0	90 (7)^{b}	0.7
Glutamic acid	874 (12)^{a}	4.2	478 (14)^{b}	4.0
Glutamine	647 (34)^{a}	3.1	184 (8)^{b}	1.5
Glycine	11,751 (223)^{a}	57.1	6,771 (430)^{b}	56.3
Histidine	100 (6)^{a}	0.5	111 (10)^{b}	0.9
Hydroxyproline	124 (11)^{a}	0.6	88 (6)^{b}	0.7
Isoleucine	313 (11)^{a}	1.5	175 (6)^{b}	1.5
Leucine	370 (23)^{a}	1.8	293 (7)^{b}	2.4
Lysine	356 (7)^{a}	1.7	312 (11)^{b}	2.6
Methionine	68 (4)^{a}	0.3	134 (8)^{b}	1.1
Phenylalanine	164 (2)^{a}	0.8	164 (12)^{b}	1.4
Proline	140 (10)^{a}	0.7	4,909 (147)^{b}	2.7
Sarcosine	332 (14)	1.6	——	——
Serine	316 (5)^{a}	1.5	163 (10)^{b}	1.4
Threonine	521 (27)^{a}	2.5	189 (12)^{b}	1.6
Tryptophan	264 (11)^{a}	1.3	140 (3)^{b}	1.2
Tyrosine	217 (16)^{a}	1.1	212 (5)^{b}	1.8
Valine	273 (16)^{a}	1.3	272 (9)^{b}	2.3
Total (mg/g)	21 (1.0)^{a}		12.0 (0.8)	

Results are mean values of three replicates (standard deviation). Values in each row with the same superscript are not different ($P > 0.05$) from one another.

by Fernandez et al. (1995). However, coelomic fluid contained 96.5 ± 0.03% moisture; thus, its contents of lipid, protein, and polysaccharide were extremely low. On the other hand, the ash content of sea urchin coelomic fluid was much higher than that of the gonads. In general, sea urchin gonads are known to contain high levels of protein. They also have considerably high lipid levels; whereas, carbohydrate levels are low (McClintock & Pearse 1987). However, in our study, the carbohydrate content was high and accounted for approximately 10% of the total amount, on a fresh weight basis. Furthermore, in coelomic fluid, although the relative proportion of carbohydrate was only 0.4 ± 0.1%, this was four times more than that of its protein and lipid contents. From this study, it is apparent that content of lipid, protein and carbohydrate in the coelomic fluid was much less than that of the gonads. The urchins in this work were given a diet purely comprised of *Laminaria* kelp to resemble the urchin's preferred natural diet. In general, the diet plays a very important role in the compositional characteristics of these animals (Nishikiori 1989, Fernandez et al. 1995, Agatsuma 1998) and almost all studies have only determined compositional characteristics of the gonads. Thus, Agatsuma (1998) showed that a diet of fishmeal increased moisture levels, and Nishikiori (1989) observed that moisture content in the gonads of *S. nudus* was below 70% when the urchins were fed *Laminaria japonica* to satiation. None of these studies reported compositional characteristics of sea urchins' coelomic fluid. In our study, when urchins were fed on a *Laminaria* diet, gonads contained 74.7 ± 0.04% moisture, which was significantly ($P < 0.05$) lower than that of the coelomic fluid.

Lipid Class Composition

The lipid composition of marine invertebrates is influenced by several factors, including pattern of feeding, gametogenesis, and possibly environmental conditions (Jezierska et al. 1982). Wax esters have been reported to constitute energy reserves in various marine invertebrates (Sargent 1976), but this was not the case for sea urchin *S. droebachiensis*. TAG formed the main energy reserve in these animals, and their gonads and coelomic fluid were qualitatively composed of similar lipid classes. The same nonpolar lipid class distribution has been observed in *S. droebachiensis* gonads and coelomic fluid collected form Nova Scotia (Takagi et al. 1980). The nonpolar lipids of gonads and coelomic fluid consisted mainly of TAG, FFA, and ST. Triacylglycerols are usually considered to serve as storage lipids in eukaryotic cells (Sul et al. 2000). Thus, sea urchin lipids contained much larger amounts of storage lipids, principally TAG, which constituted more than 60% of the total nonpolar lipids of gonads and coelomic fluid.

Although, qualitative composition of nonpolar lipids of gonads and coelomic fluid of *S. droebachiensis* was similar, relative content of individual classes differed. Hence, both sea urchin gonads and coelomic fluid were composed of the same major lipid classes, both nonpolar and polar, but their relative contents were markedly different. Thus, relative content of TAG in sea urchin gonads was much higher than that of coelomic fluid; whereas, that of FFA in gonads was much less than that of coelomic fluid. It was apparent in the preliminary experiments that both gonads and coelomic fluid contained high levels of FFA. Therefore, it was thought that partial hydrolysis of TAG may lead to an underestimation of TAG content. However, the impact of this on a nutritional value of the fatty acids involved is inconsequential. To verify the above fact, fatty acid content was determined in a set of freshly harvested sea urchins. The gonads were extracted as quickly as possible at 0°C immediately after homogenization. It was assumed that hydrolysis of lipids because of the activity of endogenous enzymes is minimized under these conditions. The FFA content was 15.5 ± 1.7% of the total nonpolar lipids upon Iatroscan analysis. Hence, the high levels of FFA observed for stored sea urchin tissues following homogenization could be attributed to the hydrolysis of TAG during storage of samples at −20°C.

In general, the energy supplied to the animal by the breakdown of lipid reserves comes primarily from oxidation of fatty acids. Farkas (1979) has shown that the production of FFA can be induced by stress. Thus, the environmental temperature and diet can be specified as factors exerting a major impact on the content and metabolism of fatty acids in animals (Farkas et al. 1978).

There were noticeable differences in the relative content of sterols in sea urchin tissues. In general, cholesterol level may depend on dietary level and stage of sexual development (Love 1970). In fact, diet and nutritional status are known to be the main factors that influence cholesterol levels (Dave et al. 1975). On the other hand, during gametogenesis, a redistribution of cholesterol takes place that may lead to high levels of cholesterol in the gonads (Idler & Tsuyuki 1985). Therefore, all relevant factors must be considered to explain the content of cholesterol in different tissues.

In the present study, the relative content of ST in the gonads was significantly ($P < 0.05$) higher than that in the coelomic fluid.

Vaskovsky and Kostetsky (1969) have performed TLC on polar lipids of sea urchin *S. nudus* and *S. intermedius*. The polar lipid fraction was separated into five components of which PC, PE, and SM constituted the major polar lipid classes present. Furthermore, lipid extracts of different organs of the same animal had a similar qualitative polar lipid composition (Vaskovsky & Kostetsky 1969). In this study, both gonads and coelomic fluid showed qualitative similarities in their polar lipid fractions. Thus, PC, PE, SM/LPC, and PS/PI constituted the polar lipids of *S. droebachiensis* gonads and coelomic fluid, and PC was dominant in both tissues, with a contribution of more than 65% to the total content of polar lipids. Similarly, Floreto et al. (1996) demonstrated that sea urchin *Tripneustes gratila* fed on a seaweed diet had PC and PE as the major lipid constituents, and PC contributed a greater proportion than PE.

Fatty Acid Composition

The fatty acids of total lipids of sea urchin gonads and coelomic fluid were typically similar to those of other marine species with a dominance of 16:0 and 20:5n-3 (Wanasundara 1996). Although, 22:6n-3 is a typical fatty acid in marine lipids, it contributed only 1.4 ± 0.1 and 0.6 ± 0.1% to the total fatty acids in the lipids of sea urchin gonads and coelomic fluid, respectively. Holland (1978) reported that the predominance of 20:5n-3 and 22:6n-3 in typical marine fatty acids is a result of low- temperature adaptation. This helps in the maintenance of cell membrane fluidity in organisms living in the cold environments.

Considerable data are available on the fatty acid composition of sea urchins (Takagi et al. 1980, Kaneniwa and Takagi 1986). The fatty acid 16:0 was the major SFA in the sea urchin *S. droebachiensis* harvested from Herring Cove, Nova Scotia (Takagi et al. 1980). Fujino et al. (1970) analyzed fatty acid composition of sea urchins *Anthocidaris crassispina*, *S. pulcherrimus*, *S. franciscanus*, *S. intermedius*, and *Echinus esculentus*. In all these samples, 16:0 was the prominent SFA followed by 14:0. The fatty acid 18:0 was found to occur in considerable amounts. Similarly, in the present study, the predominant SFA were 16:0 and 14:0 in the lipids of both gonads and coelomic fluid of *S. droebachiensis*.

Among MUFA 20:1n-15 was present up to 11% in the total fatty acids of urchins (Takagi et al. 1980). Ackman and Hooper (1973) reported that such marine animals as periwinkle (*Littorine littorea*), moon snail (*Lunata triseriata*), and sand shrimp (*Crangon septemspinosus*) contain 20:1n-15, but at much lower levels of up to 0.2% of the total fatty acids. However, this has not been commonly reported as being typical of marine lipids. In our study, 20:1n-15 was also the major MUFA in both tissues analyzed. On the other hand, seaweeds, the natural diet of sea urchins, have not been reported to contain 20:1n-15 (Ackman & McLachlan 1977); hence, the formation of 20:1n-15 in sea urchin tissues may be biosynthetic in origin, because this was not dependent on the diet.

The occurrence of such unusual 5-olefinic fatty acids as 18:1n-13, 20:1n-15, 20:2Δ5,11, 20:2Δ5,13, 20:3Δ5,11,14, and 20:3Δ5,11,14,17 has been noticeable in lipids of sea urchins accounting for as much as 6–21% of the fatty acids of total lipids (Takagi et al. 1980, Kaneniwa & Takagi 1986). In this study, the amount of 5-olefinic acids found in the lipids of both gonads and coelomic fluid was in the range of 7–10%. The presence of 5-olefinic fatty acids has been reported in 12 species of Echinoidea collected in Japan (Takagi et al.1986); thus, they serve a common and characteristic feature of sea urchin lipids.

The amount of eicosapentaenoic acid (20:5n-3) was quite high in sea urchin lipids (Takagi et al. 1980). Pohl and Zurheide (1979) reported that urchins that consumed *Laminaria* had a high content of 16:4n-3, 18:4n-3, 20:4n-6, and 20:5n-3. Similarly, sea urchin *S. droebachiensis* in our study consumed *Laminaria* for only a three week period, and their gonadal and coelomic fluid lipids contained quite high levels of these fatty acids. Thus, the fatty acid profiles of sea urchin tissues somewhat reflect that of their diets as was also observed by Floreto et al. (1996). However, certain fatty acids, such as 16:4n-3, 20:4n-6, 20:5n-3, and 20:1n-11, which constitute the major fatty acids of sea urchin tissues, were not detected in their diets; therefore, suggesting that sea urchins are capable of synthesizing them from lower fatty acid precursors. Similarly, in the present study 16:4n-3, 20:1n-11, 20:4n-6, and 20:5n-3, among others, may have been formed by chain elongation of precursors. In general, the sea urchin fatty acids; namely, 16:4n-3, 20:4n-6, and 20:5n-3, may possibly confer some structural function and, hence, are purposely synthesized by the animal (Floreto et al. 1996).

Carotenoid Pigments

In the sea urchin *S. droebachiensis*, carotenoids were mainly concentrated in the gonadal tissue. Hence, the total content of carotenoids in the gonads was about 6.3 times more than that of the coelomic fluid. However, the content of carotenoids in different tissues may vary with the reproductive stage of urchins. Hence, during gametogenesis most of the carotenoids in other tissues may be transferred into gonads, consequently increasing their carotenoid content (Griffiths & Perrott 1976).

Echininone and fucoxanthin were characterized as the major carotenoids present in the gonads and the coelomic fluid, respectively. In addition, β-carotene was identified in both tissues. Echininone was found to be the main pigment with a lesser amount of β-carotene in the gonads of *S. purpuratus* (Griffiths 1966), *S. droebachiensis* (Griffiths & Perrott 1976) and *Tripneustes gratila* (Shina et al. 1978). Tsushima et al. (1995) found that β-echininone and β-carotene were the major carotenoids in the gonads of 19 out of 20 sea urchin species examined. Meanwhile, the major carotenoids of brown algae, the natural preferred diet of sea urchins, consist of β-carotene, violaxanthin, and fucoxanthin (Matsuno & Hirao 1989). Furthermore, there is bioconversion of β-carotene to β-echininone via β-isocryptoxanthin in sea urchins; which takes place mainly in the gut wall, and the resultant β-echininone is incorporated into the gonads (Tsushima et al. 1993). Kawakami et al. (1998) showed that fucoxanthin, the major carotenoid in brown algae, did not accumulate in the gonads. In fact, in the present study on *S. droebachiensis*, fucoxanthin did not occur in the gonads. On the contrary, coelomic fluid had fucoxanthin as its major carotenoid.

Amino Acid Composition

Although marine invertebrates characteristically contain a high intracellular concentration of FAA, the composition of the FAA pool may vary among species (Gilles 1979). In the present study, glycine was the dominant amino acid in both TAA and FAA profiles in both sea urchin gonads and coelomic fluid. Komata et al. (1962) reported that glycine was dominant in the gonads of sea urchin *S. pulcherrimus*, and its content ranged from 35–41% of total FAA. Lee and Haard (1982) reported that glycine constituted 18–60% of the FAA in the gonads of sea urchin *S. droebachiensis*. The gonads and coelomic fluid of sea urchin *S. droebachiensis* in this study contained 11.9–14.6% glycine in the TAA profile, respectively. However, glycine was not the dominant amino acid in the gonads of the sea urchin *Paracentrotus lividus*, although it contributed a considerable amount to the TAA pool (Cruz-Garcia et al. 2000). Other than glycine, alanine, arginine, glutamic acid, lysine, and methionine are considered important for taste, even though some of them were present in small quantities (Lee & Haard 1982). These amino acids were present in considerable amounts in both gonads and coelomic fluid of sea urchins in this study.

It has been found that different combinations of taste-active components (substances that influence the taste of any food) as well as their relative amounts are of paramount importance in producing the characteristic flavor of each seafood (Fuke 1994). In general, glutamine and glycine, which were present in higher amounts in the gonads than coelomic fluid, are known to be taste-active in sea urchins and other seafoods, regardless of their quantity. Sea urchin gonads seemed to be sweeter when little or no glutamine was present, and alanine was found in considerably high levels. Alanine is a taste-active component in sea urchin tissues, contributing noticeably to both TAA and FAA contents. Furthermore, valine and methionine are known to be taste-active only in sea urchins; whereas, arginine was also taste-active in sea urchins because of its high content (Fuke 1994). Both methionine and arginine were present at a higher proportion in the coelomic fluid than in the gonads. Similarly, the contents of aspartic acid, histidine, and especially proline were much higher in the coelomic fluid than those in the gonads. Thus, amino acids play a major role in the taste of sea urchin gonads. In our study, various amino acids contributed differently to both the TAA and FAA of sea urchin gonads and coelomic fluid.

Contents of Nucleic Acids

In general, quantitative analysis of nucleic acid provides a relatively simple means of estimating recent growth rate of sea urchins. The processes of cellular growth and division require the synthesis of nucleic acids and proteins. The fact that RNA is a precursor to protein synthesis led to its use as an indicator of growth rate (Church & Robertson 1966). The primary function of RNA involves protein synthesis; whereas, DNA is the primary carrier of genetic information. Because the majority of cellular DNA is chromosomal, the quantity of DNA per cell is quasiconstant in somatic tissues; the tissue DNA concentration reflects cell numbers (Sulkin et al. 1975; Bulow 1987). Therefore, DNA content has usually been used as an index of cell numbers or biomass (Regnault & Luquet 1974). In this study, the DNA content in the gonads was approximately four times higher than that in the coelomic fluid. Although the gonad is a tissue with a higher biomass as compared with coelomic fluid, the latter contains mostly coelomic fluid with a lower biomass. On the other hand, the RNA/DNA ratio has been used as an estimate of growth for a variety of invertebrates (Sulkin et al. 1975). Thus, the RNA/DNA ratio is an index of protein synthetic activity per cell and reflects the protein synthesizing capacity for estimating recent *in situ* protein increase (Bulow 1987, Hovenkamp & Witte 1991). In fact, correlation between RNA concentration or RNA/DNA ratio and growth rate has been observed for a wide variety of organisms (Sutcliffe 1970). Furthermore, the gonadal RNA content was about 5.4 times higher than that in the coelomic fluid, thus demonstrating higher protein synthetic activity in the gonads. In general, gonad is the site of gametogenesis, which involves much protein synthesis. Furthermore, the RNA/DNA ratio was much lower in coelomic fluid than that in gonads, indicating greater protein synthetic activity per cell in the gonads. This is an indication that gonad is a tissue with greater *in situ* protein growth as compared with coelomic fluid.

CONCLUSIONS

The present study demonstrated that sea urchin gonadal and coelomic fluid tissues had many common compositional characteristics. Most of the parameters analyzed did not show qualitative differences; whereas, there were quantitative differences. In fact, gonads of sea urchins are a site of nutrient storage in addition to being the reproductive organs. The accumulation of nutrient reserves contributes to the growth and development of the commercially important sea urchin gonads. Although sea urchin coelomic fluid has not yet been exploited commercially, evaluation of its composition may lead to its potential use as a flavoring source.

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THE DEVELOPMENT OF A POSITIVE NON-INFECTIOUS CONTROL FOR THE DETECTION OF *PERKINSUS* USING THE RAY TEST

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ABSTRACT To establish a noncontagious control for the Ray thioglycollate test for the detection of *Perkinsus* in mollusks we evaluated nonviable stages of *P. olseni* for enlargement of hypnospores and blue/black iodine stain. Trophozoites made nonviable with formalin, irradiation or colchicine failed to swell in thioglycollate. They remained small and did not differentially stain in iodine. Trophozoites that had already developed into hypnospores in thioglycollate were rendered inactive by freezing, ethanol or formalin immersion. They retained their iodinophilic properties and thus could provide a partial control for the Ray Test.

KEY WORDS: *Perkinsus*, abalone, fluid thioglycollate medium

INTRODUCTION

Members of the genus *Perkinsus* are protozoan parasites found exclusively in mollusks. *Perkinsus* parasites have long been recognized as a cause of mortality in commercially important mollusks (Ray & Chandler 1955). *Perkinsus marinus* has been demonstrated to have profound effects on the oyster *Crassostrea virginica*, leading to reduced growth, reduced fecundity, and increased mortality (Menzel & Hopkins 1955, Mackin 1962). In Australia, *P. olseni* infections result in deep abscesses and soft yellow pustules in commercially important abalone, *Haliotis rubra* and *H. laevigata*. This is of great concern to the endemic abalone industry as infected individuals are unacceptable for processing (Lester & Davis 1981). Transmission appears to occur through zoospores that develop via hypnospores (Goggin et al. 1989). Hypnospores can be found within the abscesses (Goggin & Lester 1995) and may also be produced by culturing trophozoites in fluid thioglycollate medium (FTM) (Ray 1952). Other species of *Perkinsus* produce hypnospores though the role of zoospores in transmission between mollusks is not clear (Perkins 1996).

Diagnosis of *Perkinsus* infections is commonly accomplished by the FTM assay (Ray 1952). Trophozoites in infected tissue develop into usually much larger hypnospores that stain blue-black with Lugol's iodine (Ray 1952). The reaction with iodine relies on the development of hypnospores; trophozoites remain brown (Ray 1952, Stein & Mackin 1957). Prior to the development of this technique, diagnosis relied on fresh tissue smears or histologic sections. These methods are labor intensive, use expensive materials, rely on a high degree of expertise, and lack sensitivity to detect low levels of infection (Ray 1954).

Infected hosts frequently show no overt sign of disease, and as such, infected tissue can easily reach processing plants undetected (Goggin et al. 1990). Here they may be examined for the presence of *Perkinsus* with the FTM assay, however, due to inexperience in recognizing the parasite or through errors in the formulation of the culture media, investigators may fail to detect infections.

The development of positive reference material to assist *Perkinsus* diagnosis in fisheries laboratories would be an invaluable tool to help minimize misidentifications and false negatives. The material needs to be noncontagious to prevent cross-infection into local mollusk populations during shipping or in disposal after use, while maintaining the key attributes of the FTM assay: enlargement of hypnospores in FTM and uptake of the iodine stain (Ray 1952).

Fisher and Oliver (1996) stated that dead trophozoites of *P. marinus* fail to enlarge in FTM. We sought to make *P. olseni* material nonviable to see if swelling would still occur. We also sought to determine whether hypnospores, following enlargement in FTM, could be rendered inactive while retaining their iodinophilic properties.

MATERIALS AND METHODS

Trophozoite Inactivation

Pieces of infected mantle tissue from three blood cockles, *Anadara trapezia*, infected with *P. olseni* (Murrell et al. 2002) were exposed to either dilute formalin, irradiation, and colchicine to attenuate the viability of hypnospores while retaining the characteristics of the Ray Test. Several tests were run on tissues from one animal to enable the results from different dose levels to be compared. Tissue from animal 1 was immersed in one of four dilute formalin solutions [1:4 × 10^3, 1:4 × 10^4; 1:4 × 10^5; 1:4 × 10^6 formalin:seawater (approximately 34‰)]. For a period of 30 minutes tissue from animal 2 was treated with gamma irradiation. The self-contained gamma radiation source of ^{60}Co had a dose rate of 670 Gy h^{-1}. Duplicate wet tissue samples were placed in glass petri dishes and irradiated to a maximum absorbed dose rate of 600 Gy in increments of 200 Gy. Variations in absorbed dose were minimized by placing thin tissue samples within a uniform portion of the radiated field. Two tissue samples from animal 3 were placed in FTM to which was added colchicine, at one of two concentrations: 10^{-4} M and 10^{-6} M for six hours, after which they were rinsed in seawater.

After treatment all tissues were placed in FTM, supplemented with 200 mg chloromycetin and 200 units of mycostatin to reduce fungal and bacterial contamination (Ray 1966), and incubated at 25°C for five days. A second pair of samples from animal 3 was left in the colchicine-supplemented FTM for the full period of incubation (5 days).

Following incubation, a portion of infected tissue was examined and hypnospore abundance counted, using a compound microscope at ×40 magnification in five fields of view. To facilitate easy enumeration, part of the tissue sample was stained with iodine prior to counting. Hypnospores were teased out of the unstained tissue and transferred to a glass petri dish containing seawater. The seawater in the dish was replaced twice daily. Hypnospores that adhered to the dish were allowed to develop; a process that typi-

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TABLE 1.

Effects of formalin, irradiation and colchicine treatment on trophozoite enlargement, resulting hypnospore viability, and iodinophilia.

Treatment	Duration of Treatment	Swelling Evident After FTM Culture (Y/N)	No. Enlarged Hypnospores Present (5 Fields 40×)	% Hypnospores Viable	Presence of Iodinophilia (Y/N)
Control	—	Y	480	80%	Y
1:4 × 10^3 formalin:seawater	1 h	N	0	–	–
1:4 × 10^4 formalin:seawater	1 h	N	0	–	–
1:4 × 10^5 formalin:seawater	1 h	N	0	–	–
1:4 × 10^6 formalin:seawater	1 h	Y	510	90%	Y
Control (no treatment)	—	Y	520	90%	Y
Gamma 200 Gy	—	Y	400	100%	Y
Gamma 400 Gy	—	Y	30	85.7%	Y
Gamma 600 Gy	—	Y	4	100%	Y
Control	—	Y	90	100%	Y
10^{-4} M Colchicine	6 h	Y	80	83.3%	Y
10^{-6} M Colchicine	6 h	Y	70	100%	Y
10^{-4} M Colchicine	entire incubation	Y	170	93.3%	Y
10^{-6} M Colchicine	entire incubation	Y	200	100%	Y

cally took 1–5 days. Parasites were inspected under a dissecting microscope and were deemed viable only if cell division occurred within five days. To assess whether treated cells retained their iodinophilic properties irrespective of viability, individual hypnospores were isolated from the treated tissue and transferred to a separate petri dish, where they were stained with 3–4 drops of Lugol's iodine. Iodinophilia was based qualitatively on the uptake of stain by the hypnospore and was assessed approximately three minutes after application (Quick 1972). Control tissues from all three mollusks were placed directly into FTM and incubated for the equivalent period of time to confirm that they were infected with *Perkinsus*.

Hypnospore Inactivation

To obtain hypnospores, pieces of mantle, foot, digestive gland, and gill from *A. trapezia*, from Wynnum, Queensland and from *H. rubra* and *H. laevigata* collected from South Australia, were incubated in 20 ml FTM at room temperature (approximately 24°C) for 4–6 days. The medium was supplemented with 200 mg chloromycetin and 200 units of mycostatin (Ray 1966). The incubated tissues containing resulting hypnospores were then subjected to various treatments: freezing at −20°C; immersion in 10% formalin; and immersion in 70% ethanol. The effectiveness of each treatment on hypnospore viability and iodinophilia was assessed after 24, 48, and 72 h. The viability of hypnospores prior to treatment was confirmed by viability testing of a random subset of enlarged cells.

RESULTS

Trophozoite Inactivation

In all treatments, trophozoites that developed into hypnospores in FTM were capable of further development and were evidently

TABLE 2.

Effects of various treatments on hypnospore viability and iodinophilia.

Host	Treatment	Duration of Treatment	% Hypnospores Viable	Presence of Iodinophilia (Y/N)
A. trapezia	Control	—	80%	Y
H. rubra	Control	—	80%	Y
H. laevigata	Control	—	100%	Y
A. trapezia	10% formalin	24 h	0%	Y
A. trapezia	10% formalin	48 h	0%	Y
A. trapezia	10% formalin	72 h	0%	Y
H. rubra	10% formalin	72 h	–	–
H. laevigata	10% formalin	72 h	0%	Y
A. trapezia	70% ethanol	24 h	0%	Y
A. trapezia	70% ethanol	48 h	0%	Y
A. trapezia	70% ethanol	72 h	0%	Y
H. rubra	70% ethanol	72 h	0%	Y
H. laevigata	70% ethanol	72 h	0%	Y
A. trapezia	Freezing (−20°C)	24 h	0%	Y
A. trapezia	Freezing (−20°C)	48 h	0%	Y
A. trapezia	Freezing (−20°C)	72 h	0%	Y
H. rubra	Freezing (−20°C)	72 h	0%	Y
H. laevigata	Freezing (−20°C)	72 h	0%	Y

viable (Table 1). Treatment had little effect on the parasitic stages that had enlarged. No hypnospores were evident in tissues exposed to formalin concentrations of 1:4 × 10^3, 1:4 × 10^4; 1:4 × 10^5. Tissues exposed to 1:4 × 10^6 formalin:seawater provided similar hypnospore numbers to those of the control (Table 1). Trophozoites exposed to gamma irradiation showed a progressive decline in hypnospore numbers with increasing radiation exposure (200 Gy, 400 Gy, and 600 Gy) (Table 1). Colchicine treatment had little effect on hypnospore enlargement and viability, irrespective of treatment concentration. The majority of hypnospores retained from incubation appeared viable, with motile zoospores evident after five days examination (Table 1).

Hypnospore inactivation

All treatments killed hypnospores within 24 h of treatment. Nevertheless, all the dead hypnospores exhibited clear iodinophilia, staining a deep blue on application of Lugol's iodine (Table 2).

DISCUSSION

The negative correlation between treatment intensity (or duration) and hypnospore abundance, particularly for the irradiated tissue, followed by the successful development of recovered hypnospores indicate that nonviable trophozoite stages fail to enlarge in FTM. This is in agreement with Fisher and Oliver (1996), who stated that enlargement of *P. marinus* trophozoites in FTM only occurs if the parasite is viable. Formalin treatment at concentrations ≥1:4 × 10^5 formalin:seawater appeared to prevent hypnospore enlargement, with no hypnospores recovered from these concentrations. The extremely weak formalin (1:4 × 10^6 formalin:seawater) appeared to have little effect on trophozoite viability, with treated tissues exhibiting a similar enlarged hypnospore abundance to that of the control. Similarly, colchicine concentrations of 10^{-4} M or 10^{-6} M had little effect on parasite viability. Colchicine has been demonstrated to be a potent microtubule inhibitor at such concentrations (Wiest et al. 1993), thus preventing cell division. The fact that parasites treated with colchicine remained viable and underwent division once out of the treatment is consistent with microscopic observations that no cell division occurs during trophozoite differentiation to hypnospores and cell enlargement. Swelling of *Perkinsus* cells in FTM had been suggested to occur as a result of the direct uptake of the media (Ray 1952), although the mechanisms of this process are not yet described.

As nonviable trophozoites failed to enlarge in FTM, and thus failed to become iodinophilic, we tested whether hypnospore stages could be killed and still retain their iodinophilic properties. All treatments tested killed hypnospores. The sensitivity of hypnospores to low temperature is in accordance with Chu and Greene (1989), who observed that hypnospores of *P. marinus* exhibited 100% mortality at 0°C for 24 h. Goggin et al. (1990) observed motile zoospores from cultured tissues previously chilled at 4°C, 0°C and frozen at −20°C, showing that trophozoites are more tolerant to low temperatures than hypnospores.

In all cases, nonviable hypnospores were iodinophilic. Thus, such tissue could be transported risk-free to processing plants to serve as a positive reference for sample comparison. Although this is not a control for FTM development, it still supplies users with a positive control that is safe, noncontagious, and simple to use.

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EVALUATION OF SUBSTITUTE DIETS FOR LIVE ALGAE IN THE CAPTIVE MAINTENANCE OF ADULT AND SUBADULT UNIONIDAE

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ABSTRACT Ten nonlive algal diets were evaluated as potential broodstock diets for adult and subadult unionids. These diets varied significantly in their ability to support growth, reproduction and survival. Growth, increase in glycogen stores, and limited glochidial formation were seen in most unionid species on two of the diets. However, long-term survival (>3 y) remained problematic, and the cause of mortality in these animals could not be determined. While two of the diets tested are potentially useful for supplemental feeding of adult unionids to increase glycogen levels during quarantine, or during short-term captive maintenance in the laboratory, none can be recommended without reservation for long-term maintenance because of the lack of survival after three years during this study.

KEY WORDS: unionidae diets

INTRODUCTION

Nearly 70% of the freshwater mussels (Unionacea) in North America are currently facing extinction (Williams et al. 1993). Conservation efforts have focused on relocation of endangered populations, and aquaculture of recently transformed larvae. Captive maintenance of endangered animals is a common technique used to enhance and preserve species-at-risk, however unionids have proved difficult to maintain in captivity or to relocate into new habitats (Cope & Waller 1995). Most aquaculture efforts have concentrated on developing live algal diets that will support the growth and survival of larvae (≤1 year of age). A tri-algal diet has recently been produced that appears to support survival of a few species for about one year after larval transformation (see Gatenby et al. 1994, Gatenby et al. 1996, Gatenby et al. 1997). Adult unionids have rarely been kept alive for more than three years even in hatchery ponds or raceways. One problem in maintaining adult unionids is the lack of information regarding actual nutritional requirements. Recent studies have indicated that while live algae may be supplying certain key nutrients, detritus and bacteria are an important part of unionid diets (Nichols & Garling 2000).

Marine aquaculturists have experienced similar difficulties in long-term feeding of adult oysters and clams, and usually rely on natural food supplies found in offshore grow-out areas. Hatchery production of live algae is often used to rear seed marine bivalves to planting size (1–2 mm), but is rarely used as a sole food source for adult animals due to the difficulties and costs associated with maintaining sufficient year-round supplies and lack of knowledge of long-term nutritional needs (Knauer & Southgate 1999, Heasman et al. 2000). In recent years, attempts have been made to replace live algae with artificial diets to reduce the need for expensive algal production (Coutteau & Sorgeloos 1993). These artificial feeds have been successful, but this success varies with both feed type and farm operator. The objective of our study is to determine if similar types of non-live algal feeds could be developed to support adult unionid survival, growth, and reproduction under captive conditions.

MATERIALS AND METHODS

We tested commercial and laboratory-prepared (experimental) feeds from 1994 to 1998 on eight unionid species *Amblema plicata* (Say, 1817), *Cyclonais tuberculata* (Rafinesque, 1820), *Lampsilis fasciola* (Rafinesque, 1820), *Lampsilis ventricosa* (Say, 1817), *Lampsilis siliquoidea* (Barnes, 1823), *Leptodea fragilis* (Rafinesque, 1820), *Pyganodon grandis* (Say, 1829), and *Quadrula quadrula* (Rafinesque, 1820). The size and age of the animals varied and both adult and subadult animals were tested. Adult animals in our experiments were at least five years of age according to external annuli. Subadults animals were less than three years but younger than one year based on external annuli. Shell morphometrics for all animals were measured upon arrival in the laboratory and individual tracking numbers etched onto one of the shell valves.

Unionids were held at the Great Lakes Science Center in a flow-through system of 8-L rectangular aquaria, containing 10 cm of coarse gravel, with a water replacement rate of 4L/h. Baseline water quality parameters were: $CaCO_3$ of ~100 mg/L (EDTA titrimetric method, APHA 1989); dissolved oxygen 8.0 ppm (Winkler method, APHA 1989); dissolved ammonia of <0.5 ppm (phenate method, APHA 1989); and a pH of 7.8 (Fisher Scientific Accumet pH meter model #AB15). Water quality parameters were measured weekly and during any die-off of unionids. Water temperature averaged 15°C, and the light regimen was on a 12 light/12 dark cycle.

Diet Formulations

We tested 10 diet formulas in this study (Table 1). Five were commercially available (treatments #1–5), and 5 were experimental mixtures (treatments #6–10). The diets were chosen on availability and/or prior successful use in culturing marine bivalves or zebra mussels *Dreissena polymorpha* (Pallas, 1771).

The five commercial diets were: treatment (TR) #1-dried *Chlorella* sp. (Earthrise Co., California); TR#2-marine algal paste *Thalassiosira pseudonana* (Hust.) (Hasle and Heimdal) (from Coastal Oyster Inc.); TR#3-Hatchfry Encapsulon (30 μ size particles, Argent Co. Washington); TR#4-fish flake food (tropical, various retailers), and TR#5-a manipulated yeast diet (Artemia Reference Center, Ghent Belgium).

The five experimental diets were a combination of bacterial/ciliate cultures grown in the laboratory, commercially available invertebrate enrichment feeds, and animal feed supplements incorporated for protein, dextrose, and other nutrients. The first experimental diet (TR #6) was a microencapsulated feed with food particles embedded in a gel matrix prepared according to Langdon's

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TABLE 1.

Diets fed to adult and subadult unioids.

	TR#1. Dried *Chlorella*	TR#2. Marine algal paste	TR#3. Hatchfry encapsulon	TR#4. Fish Flake Food	TR#5. Yeast	TR#6. Encapsulated feed	TR#7. Egg chow	TR#8. Bacterial slurry #A	TR#9. Bacterial slurry #B	TR#10. Bacterial slurry #C.
Adults	+	+	+	+	+	+	+	+	+	+
Subadults							+			+

work on marine bivalves (Langdon & Levine 1983, Langdon & Bolton 1984; Buchal & Langdon 1995). Diet Tr#7, was based on the analysis of the gross biochemical composition of freshwater bivalves (Secor et al. 1993). This formula, (called egg chow) was a mixture of 60% dried powdered chicken egg (ICN Biomedicals Inc.), 30% powdered dextrin (ICN Biomedicals Inc.), 9% liquid safflower oil, and 1% vitamin supplement (Rep-Cal Herptivite, Los Gatos, California) mixed and finely ground prior to feeding.

The other three experimental diets were formulated from bacterial/ciliate slurry based on Stuart's (1982) work on the marine bivalve *Aulacomya ater* (Molina, 1782). These were prepared by soaking finely ground vegetation in water for three days to encourage the growth of bacteria and ciliates and the breakdown of cellulose. Stuart used kelp as a base; we used freshwater marsh grass (*Phalaris* spp.). This basic bacterial slurry formula is TR#8 (bacterial/slurry #A). Treatment #9 (bacterial slurry #B) was a mixture of 50% TR#9 and 50% dried *Chlorella* spp. Treatment #10 (bacterial slurry #C) was a mixture of 30% TR#8, 30% dried *Chlorella*, 10% Rich Advanced (a liquid mixture of lipids and algal growth enhancers from Sanders Corp. Ogden, Utah) and 10% Sanders Black Gold (a flake similar in composition to Rich Advanced).

The ration level of all diets was maintained at 5–8 mg diet dry weight/L of aquarium water for at least 15 h out of the day. This ration was based on the average total organic particulate matter values found in the Huron River near a large free-living unionid bed as described in Nichols and Garling (2000).

Measuring Success of Diet Formulations

Growth and survival were the critical criteria for assessing diet success for subadult unionids; reproduction (glochidia formation) and survival were the criteria used for adult unionids. Changes in maximum shell length were also recorded, but not further statistically tested. There was a wide variation in age of adult animals used and even a nutritionally ideal diet, older animals could not be expected to show the same potential for shell growth as younger adults. In tests using adult unionids, 10 randomly selected individuals from each of the following six species for six species—A. plicata (size range measured across longest anterior/posterior shell plane 45–110 mm), *L. siliquoidea* (56–119 mm), *L. ventricosa* (71–98 mm), *L. fragilis* (61–167 mm), *Pyganodon grandis* (78–149 mm), and *Quadrula quadrula* (48–61 mm)—were assigned to all ten diet treatments. Fewer *C. tuberculata* (51–77 mm) and *L. fasciola* (36–45 mm) were available (15 individuals each) thus, only 5 adults from these two species were randomly assigned to three diets (TR#7, TR#9 & TR#10).

The number of subadult unionids available varied by species; *A. plicata* (n = 75), *C. tuberculata* (n = 15), *L. fasciola* (n = 2), *L. ventricosa* (n = 10), *L. siliquoidea* (n = 2), *L. fragilis* (n = 100), *P. grandis* (n = 100), and *Q. quadrula* (n = 50). Within each species, ages and sizes of animals were similar to minimize variability. Due to the unequal sample size, the number of individuals and number of diets tested were limited. Two diets (TR#7 & TR#10) were tested for a period of 280 days. The distribution of mussels in these treatments was as follows: in TR#7 *A. plicata* (n = 27), *C. tuberculata* (n = 7), *L. fasciola* (n = 1), *L. ventricosa* (n = 5), *L. siliquoidea* (n = 1), *L. fragilis* (n = 50), *P. grandis* (n = 50), and *Q. quadrula* (n = 18); in TR#8 and TR#9, *A. plicata* (n = 5) and *Q. quadrula* (n = 3). The remaining subadults were fed TR#10.

Adult unionids were measured monthly (maximum shell length to the nearest mm) and survival checked daily. Reproductive efforts were monitored by the development of glochidia in the marsupium, mantle lure behavior, and glochidial release in the test aquaria. Marsupia were examined by gently prying open the animal and visually examining the gills for obvious swelling on a monthly basis. Each individual subadult unionid was measured every two weeks (maximum shell length to the nearest mm) and survival checked daily. Autopsies were performed on any animals that died and tissues dissected and examined for flukes, fungus, or bacteria, or gross structural changes in appearance.

Changes in glycogen content were measured in an additional group of animals from January to December 1998. Only one species, *P. grandis*, was readily available and used for the test. Twenty adult *P. grandis* were placed on TR#7 and TR#10 (10 animals each diet) to determine the glycogen status of all soft tissues as a measure of fitness. Glycogen content based on wet soft tissue weights was obtained using the homogenized tissue and the phenol-sulfuric acid method used by Haag et al. (1993) to assess clam fitness and reported as mg/g wet tissue. This was a whole body analysis, using 3 randomly selected animals from each of the two diets at the beginning of the (January 2) and at the end (December 28) of the 1997 test year. All ten animals were from the same age class based on external age lines (10 y old) and had a shell length of 135–140 mm.

Two of the nonproprietary diets were analyzed for biochemical content. The diets, TR#7 and TR#10 were prepared in the laboratory and then whole samples frozen to −40 C. Analyses were performed on an HPLC using standard techniques as cited in Association of Official Analytical Chemists (AOAC 1995). Protein (total), lipids (cholesterols, phytosterols, and total lipids), and carbohydrates (total) and fiber content were measured.

The statistical relationship between differences in growth rates, survival rates and glycogen concentrations of adult and subadult unionids on the various diets was tested. Differences in growth rates between subadults of within each species over time, on different diets, were analyzed using a linear regression of the monthly measurements of all individuals. Since the test group within each species was identical in number and similar in length and age, no data transformations were performed. Analysis of covariance (ANCOVA) in a sequential analysis of the slopes was used to determine growth differences between species in the same diet treatment. Growth statistics were based on changes in length, not in length at T = 0. Percent survival data was analyzed using a χ^2 test. Treatments without survivors were not included in any of the analyses. Results were considered significantly different at the $P < 0.05$ level.

RESULTS

Adults

Of the ten diets tested on adult unionids, none can be recommended without reservation. At least two, however, do show potential for use in long-term captive maintenance. Initially, all of the ten diet formulations were cleared from the water column, and directly ingested (based on fecal production) by all species of unionids. However, two of the diets caused apparent stress in the animals and failed to support growth or survival for more than 30 days; six diets supported growth and survival for at least one year; three diets supported glochidia formation, and; one supported growth, reproduction, and survival but the animals died after the third year (Table 2). All diet formulations caused problems with water quality and some mortality was more directly related to rapid changes in water quality than to diet.

Diet TR#2 caused all adult animals tested to extrude extensive mucous strands outside of the shell that proved detrimental to water quality. Within 24 h after feeding long strands of mucus were drifting through the water column, the water was cloudy, and ammonia levels spiked from <1 to 8 ppm. Twenty-five percent of the unionids on this feed died within the first month, probably due to water quality problems; thus this diet was eliminated from the experiment after 30 days.

A second commercial diet, TR#3, was dropped from the tests after eight months although this commercial rotifer-replacement feed was initially very successful. All adult unionid species fed well on it, and growth was seen in the first three months on the diet in some species. Five of the ten adult *Q. quadrula* grew an average of 2 mm in the first three months, with one individual adding 4 mm of shell in that time period. Two of the ten adult *L. siliquoidea* grew 2 mm each and seven of the ten *Leptodea fragilis* grew an average of 3 mm each in the first three months. No growth was seen in any animal after this period or in other species during the test. After the fifth month of testing, when we began using feed from a different batch, the unionids refused to eat the new feed. The feed was not ingested (no fecal matter produced). We reordered the feed to see if the problem was batch related, but the mussels did not feed on the next batch either. Since the unionids continued to refuse this diet, it was eliminated from the experiment after an additional sixty days.

Of the three remaining commercial diets, TR#4 and TR#5 could not support survival of any adult unionid species for >13 mo (Table 3 and Table 4). None of the unionids showed any shell growth while on these diets. Percent survival and growth did occur on the last commercial feed TR#1. Individuals of all species survived at least 15 mo and two species, *P. grandis* and *L. fragilis*, showed shell deposition during the first four months, but not afterwards. Four out of 10 adult *P. grandis* and 3 out of 10 adult *L. fragilis* grew 1–2 mm over the first four months on these dried green algae, but shell growth then ceased. Mortality rates of 100% occurred within 13 mo.

Four of the five experimental diets (TR #7–10) were successful in supporting initial limited growth and survival, but long-term survival (>3.5 y) was still problematic (Table 2 and Table 4). The exception was the encapsulated feed, TR#6. This encapsulated feed was ingested by the unionids, but within 12 h of feeding all the animals were gaping and non-responsive to touch on soft body parts. We dropped this feed from the diet tests after 30 days and 60% mortality in all species.

The best diet for supporting survival, growth and reproduction of adults of most species, at least up to year 3, was the high-protein egg chow (TR#7). The exception was *L. fasciola*, all of who died regardless of diet treatment. At the beginning of year 3, the survival rates of the adult unionids feeding on TR#7 were: *A plicata* 81%, *C. tuberculata* 80%, *L. ventricosa* 72%, *L. siliquoidea* 65%, *Leptodea fragilis* 64%, *P. grandis* 71%, and *Q. quadrula* 69%.

TABLE 2.

Test response of adult and subadult unionids to treatment diets.

	Ingested	Ingested but caused stress	Survived at least 6 months	Survived ~1 year	Survived ~2 years	Survived ~3 years	Showed shell growth	Initiated glochidia
TR#1. Dried *Chlorella*	+		+	+			+	
TR#2. Marine algal paste	+	+ (1)						
TR#3. Hatchfry encapsulon	+		+				+	
TR#4. Fish Flake Food	+		+	+				
TR#5. Yeast	+		+					
TR#6. Encapsulated feed	+	+ (1)						
TR#7. Egg chow	+		+	+	+	+	+	+ (5)
TR#8. Bacterial slurry #A	+		+ (2)	+	+	(4)	+	
TR#9. Bacterial slurry #B	+		+	+	(4)	(4)	+	
TR#10. Bacterial slurry #C	+		+	+	(3)	(3)	+	+

(1). Stopped testing after one month

(2). Kills *Pyganodon grandis* almost immediately and *Leptodea fragilis* within a couple a weeks

(3). Tested only for one year

(4). Tested only two years.

(5). 20% of females died after glochidial release

TABLE 3.

Percent survival of adult unionid at 12 months on treatment diets. N = 10 for each species, each diet, except for *C. tuberculata* and *L. fasciola* where N = 5/diet tested.

	Unfed	TR#1. Dried *Chlorella*	TR#3. Hatchfry Encapsulon	TR#4. Fish flake food	TR#5. Yeast	TR#7. Egg show.	TR#8. Bacterial slurry #A	TR#9. Bacterial slurry #B	TR#10. Bacterial slurry #A
A. plicata	45%	100%	51%	25%	100%	100%	100%	100%	100%
C. tuberculata	*	*	*	*	*	100%	*	100%	100%
L. fasciola	*	*	*	*	*	0%	*	0%	0%
L. ventricosa	21%	100%	14%	5%	2%	100%	3%	100%	100%
L. siliquoidea	15%	100%	11%	10%	1%	100%	4%	100%	100%
L. fragilis	12%	100%	6%	0%	0%	100%	0%	0%	100%
P. grandis	32%	100%	37%	0%	0%	100%	0%	0%	100%
Q. quadrula	0%	100%	54%	0%	79%	100%	100%	100%	100%

* Indicates diet not fed to that species

Twenty-one percent of the *P. grandis* and 15% of the *L. fragilis* females formed glochidia during year 2, on this diet. However, during the third year adult unionids began to die, and by the beginning of year four all had perished. The body weight and glycogen levels (discussed below) of these animals were high, indicating that starvation was not a factor. At times soft tissue growth was so rapid that the mussels could not completely close their shells. Autopsies showed no signs of parasitism or other disease factor, but all of these animals had greatly enlarged kidneys. The proximate analysis indicates that TR#7 is a high protein (~65%)/low carbohydrate (22%)/high lipid (13%) feed that is naturally high in cholesterol (78% of total lipid), but contains no phytosterols.

The series of bacterial/ciliate slurries, TR#s 8–10, differed in their ability to support adult unionid growth and survival. Treatment #8 proved an acceptable feed for species such as *A. plicata* and *Q. quadrula*, but killed all of the *P. grandis* within a day or two of the initial feeding and 99% of the *Lampsilis* species within a few weeks. Most of the other species died within the 13-month period. On the other hand, *Amblema plicata* and *Q. quadrula* adults survived and grew well on this diet during the two years of testing. The greatest increase in shell growth was seen in *A. plicata*. The average increase was 6 mm; maximum was 11 mm and minimum 3 mm over the 48-mo period. The amount of growth in *Q. quadrula* was about half that seen in the *A. plicata* adults. No reproductive effort was seen in any species (Table 4).

TR#9 did not improve survival and growth when compared with TR#8. Neither adult *P. grandis* nor *L. fragilis* could tolerate this feed, but once again, 82% of the *A. plicata* and 77% of the *Q. quadrula* survived for 2 y. *Amblema plicata* grew more than *Q. quadrula*, averaging 9 mm, with a maximum of 17 mm, and a minimum 4 mm over the 48-mo period. The amount of growth in *Q. quadrula* averaged 9 mm, with a maximum of 12, and a minimum of 2. No reproductive effort was seen in any species (Table 4).

Treatment #10, which combined the basic bacterial/ciliate slurry, dried algae, with various micronutrients and lipids proved acceptable to adults of all species. *Pyganodon grandis* and *L. fragilis* did well on this diet, as did all the other unionid species tested. Survival of the adults of all species was 100% after one year, with the exception of *L. fasciola*, which we were not successful at handling regardless of what they were fed or handled. Note that this diet was only tested for a one-year period and long-term data is not available. TR#10 is a low protein (~8%), low lipid (5%), high carbohydrate (87%) feed. Cholesterol comprises 75% of total lipid, algal sterols at 20% and miscellaneous lipids at 5%.

Pyganodon grandis fed TR#7 and TR #10 showed a consistent increase in glycogen levels from January 1998 to December 1998. The glycogen levels of animals on TR#10 rose from an average of 7.2 ± 1.9 mg/g in January 1998, to an average of 9.7 ± 2.4 mg/g by December 1998 but this increase was not significantly different (t-test, $n = 10$, $P = 0.059$). During the same period, animals on the TR#7 showed a statistically significant increase ($P < 0.05$) in glycogen from an average of 7.6 ± 1.1 mg/g to 11.2 ± 0.5 mg/g (t-test, $n = 10$, $P = 0.036$). Glycogen concentrations of *P. grandis* that had been feeding on TR#7 for at least 36 mo (36–45 mo) were significantly higher than *P. grandis* that had been on TR#7 for only 12 mo, averaging 14.1 ± 2.6 mg/g as compared with the 11.2 ± 0.5 mg/g (t-test, $n = 20$, $P = 0.042$). Initial glycogen concentrations are not available for the animals kept on TR#7 for the 36–mo period.

Subadults

The growth rates of subadult unionids fed on TR#7 and onTR#10 differed more by species than by diet. *Pyganadon grandis* and *L. fragilis* grew significantly faster on TR#7 and on the TR#10 than did the other species over a 350-day period, with *P. grandis* showing an average increase in shell length of 8.7 mm and *L. fragilis*, 8.1 mm. There was no significant difference in growth rates between these species on either diet. The thick-shelled species (*A. plicata*, *C. tuberculata*, and *Q. quadrula*) grew significantly less, averaging 6 mm. *Lampsilis siliquoidea* and *L. ventricosa* grew even less, averaging only 3 mm during the test period. Survival during the test period was 100% for all species except *L. fasciola*. Mortality rates for *L. fasciola* were 100%. The growth equations, r^2, and P values are presented in Figure 1. Significant differences in growth rates are as follows, with species sharing a line not significantly different (Arcsine transformed data, ANCOVA, $P \leq 0.05$): A = *A. plicata*, C = *C. tuberculata*, L = *L. fragilis*, P = *P. grandis*, Q = *Q. quadrula*, E = TR#7, B = TR#10.

Within diet

LB PB AB CB QB LE PE AE CE QE

Between diets

AB AE CB CE LB LE PB PE QB QE

TABLE 4.

Adult unionid % survival at 24 months on various diets. N = 10 for each species, each diet, except for *C. tuberculata* and *L. fasciola* where N = 5/diet tested. The bacterial slurry #C was not tested for longer than one year.

	Unfed	TR#1. Dried *Chlorella*	TR#3. Hatchfry Encapsulon	TR#4. Fish flake food	TR#5. Yeast	TR#7. Egg chow	TR#8. Bacterial slurry #A	TR#9. Bacterial slurry #B
A. plicata	0%	0%	0%	0%	0%	87%	78%	82%
C. tuberculata	*	*	*	*	*	89%	*	0%
L. fasciola	*	*	*	*	*	0%	*	0%
L. ventricosa	0%	0%	0%	0%	0%	78%	0%	0%
L. siliquoidea	0%	0%	0%	0%	0%	71%	0%	0%
L. fragilis	0%	0%	0%	0%	0%	70%	0%	0%
P. grandis	0%	0%	0%	0%	0%	72%	0%	0%
Q. quadrula	0%	0%	0%	0%	0%	75%	80%	77%

* indicates diet not fed to that species.

Water Quality

Water quality was difficult to maintain in the test chambers, particularly when feeding the bacterial slurries. There were 22 episodes of water quality problems during the four years of tests. During these events, dissolved oxygen levels would plummet to <1 ppm and ammonia levels rise to >3 ppm often in less than 12 h. Mortality was seen in adult mussels of all ten species being fed TR#2 and TR#6 during these events (25% and 60% respectively within 24 h). None of the other test animals died during these events, but gaping and lack of response to touch on soft tissue body parts were noted. No other diet treatments lead to mortality events affecting all ten species of unionids within 12 h of feeding. Rapid mortality seen in TR#8 and 9 were limited to two species (*P. grandis* and *L. fragilis*), not all ten.

DISCUSSION

One problem in evaluating these diets is that none of the unionids survived for longer than 3.5 y, although a couple of feeds supported growth and short-term survival (≤3.5 y). Our data indicate that unionids are capable of feeding on a wide variety of materials, and can survive and grow for months on non-live algae diets. Long-term survival as would be needed for broodstock maintenance remains problematic. Other than survival, the criteria we selected for measuring diet success, such as growth, reproduction

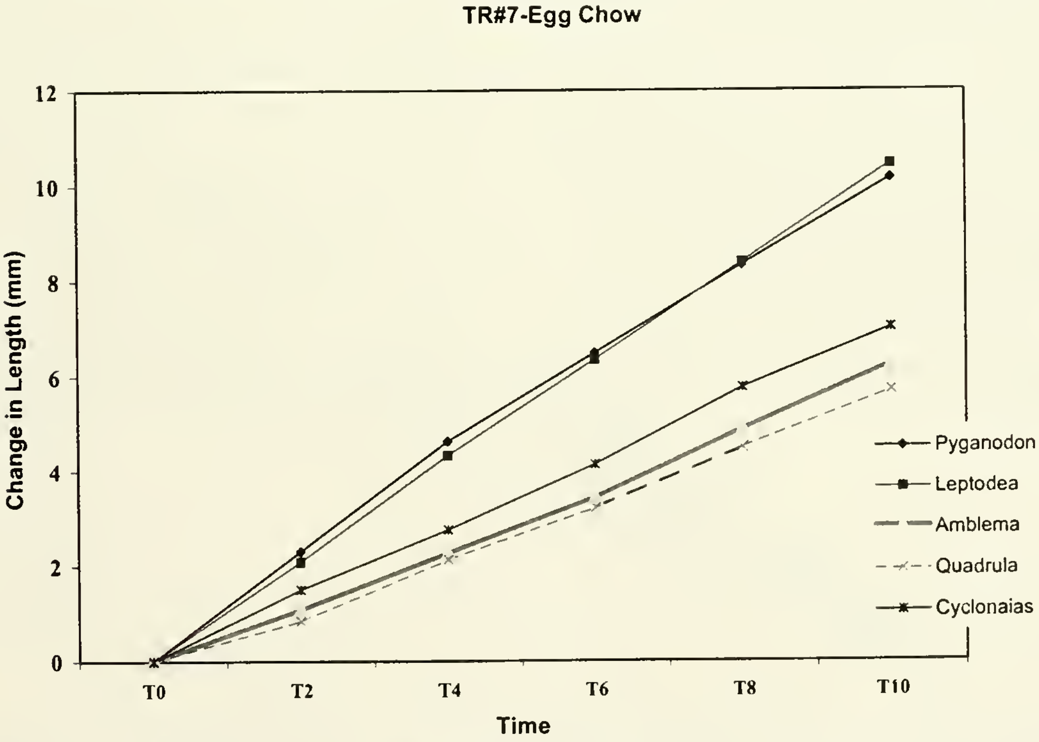

Figure 1. Comparison of growth equations of various unionid subadults fed the diet TR#7 for 350 days. ***Ambelma plicata*** **$y = 3.33677\mathrm{Ln}(x) - 0.7147$ $R^2 = 0.9095$;** ***Cyclonais tuberculata*** **$y = 3.8123\mathrm{Ln}(x) - 0.6746$ $R^2 = 0.935$;** ***Leptodea fragilis*** **$y = 5.7222\mathrm{Ln}(x) - 1.0147$ $R^2 = 0.941$;** ***Pygandon grandis*** **$y = 5.5916\mathrm{Ln}(x) - 0.8214$ $R^2 = 0.9602$;** ***Quadrula quadrula*** **$y = 3.1152\mathrm{Ln}(x) - 0.6959$ $R^2 = 0.9112$.**

and glycogen concentration were not capable of predicting the gradual die-off of all test animals after 36 mo. Additional criteria relating to physiologic mechanisms and underlying digestive capabilities are needed so that changes in diet and environmental conditions can be implemented before mortality occurs.

The two diets that were most effective in our tests, TR#7 and TR#10, differed substantially in protein, lipid, carbohydrate, and phytosterol composition, but did not differ in their ability to support subadult unionid growth or adult survival. TR#10 is a low protein (~8%), low lipid (5%), high carbohydrate (87%) feed with added algal sterols. In contrast, TR#7 is a high protein (~65%), low carbohydrate (22%), higher lipid (13%) feed that is naturally high in cholesterol, but contains no phytosterols. Our hypothesis is that TR#10 is the closest in nutritive content and physical structure to the food resources used by wild unionids in shallow temperate rivers (see Nichols & Garling 2000). This diet proved acceptable to all species tested in our study, supported significantly higher growth rates in subadults and kept 100% of the test animals alive for the entire year of the study. However, while promising results were obtained, the problem in whole-heartedly recommending this diet is that it was tested for only one year. It is possible that this diet, like TR#7 cannot support long-term (>3 y) survival.

Up until year three of our study, we thought TR#7 was a successful diet formula. It was easy to make in the laboratory, was readily ingested by all species, and supported growth, survival, glycogen storage, and limited reproduction, at least until year three. The question remains unanswered as to why long-term survival was not supported. One problem might relate to protein levels. This TR#7 is a high protein feed (~65% protein), and high protein may not be a dietary requirement of the age class of mussels used in this study. Subadults grew as well on the low protein (8%) levels found in TR#10 as they did on the high protein TR#7 (Fig. 1 and Fig. 2). Our work on a unionid community in a small temperate river indicate that they preferentially use a lower protein food (the <28 μ fine particulate organic matter,~6.7% protein) as their main food source (Nichols & Garling 2000).

Feeding a high-protein feed such as the TR#7 as a sole diet at the amount used in this study may have overfed the animals. Glycogen levels in animals fed on egg chow were far higher than those recorded by Naimo et al. (1998), Naimo and Monroe (1999), and Patterson et al. (1999) for newly caught unionids. However, food quantity may not have been the only problem. Feeding excessive dietary protein to vertebrates that normally use low protein feeds will support rapid growth and reproduction initially, but can cause high mortality due to excess nitrogen excretion causing kidney failure after a few years and alter mineral metabolism. This supposition is not a direct cause-and-effect relationship in unionids. While all the dying unionids on this feed did show greatly enlarged kidneys, unionid kidneys do not excrete excess nitrogen, as do vertebrate kidneys; the gills perform that function. However, the primary function of unionid kidneys is to control ionic balance, including mineral balance, of bodily fluids. Theoretically, excess protein can affect ionic balance due to alteration of blood pH through excess amine production and stress the metabolic balance of the unionid; we could not determine cause and effect from our study. We cannot recommend the long-term use of high protein feeds for unionids. However, short-term or supplemental feeding at a lower ration than used in this study might be acceptable to prevent glycogen loss during quarantine as reported by Patterson et al. (1999).

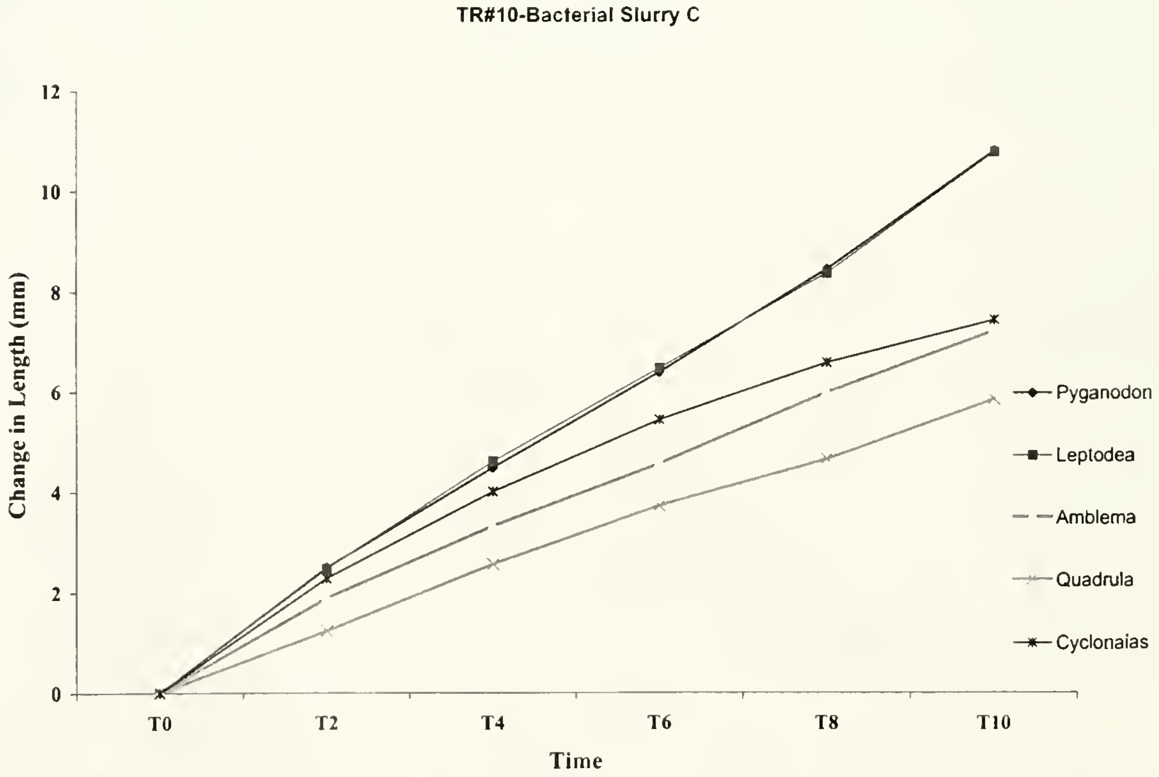

Figure 2. **Comparison of growth equations of various unionid subadults fed diet TR#10 over 350 days.** ***Ambelma plicata*** **$y = 3.9346Ln(x) - 0.4899$ $R^2 = 0.9649$;** ***Cyclonais tuberculata*** **$y = 4.1877Ln(x) - 0.3063$ $R^2 = 0.9909$;** ***Leptodea fragilis*** **$y = 5.5873Ln(x) - 0.7751$ $R^2 = 0.9608$;** ***Pygandon grandis*** **$y = 5.7785Ln(x) - 0.903$ $R^2 = 0.9426$;** ***Quadrula quadrula*** **$y = 3.1988Ln(x) - 0.5076$ $R^2 = 0.9533$.**

There is certainly the possibility that unionid mortality after three years in captivity relates more to environment than nutrition. Even aquaculture efforts that feed their unionids live algae report incidents of poor water quality that at times does not lead to immediate mortality (Gatenby et al. 1994, Gatenby et al. 1996). However, such events may produce sublethal stress that over a period of time and after a number of incidents may eventually kill the adult unionids. One of the greatest problems in using the types of feeds we tested is that of maintaining water quality, especially with bacterial/ciliate slurries (Tr#s 8–10).

There are a number of other environmental factors that may produce sublethal stress, including altered flow, light, and temperature regimes. Such environmental factors need further study before we can establish captive management protocols for the various unionid species, as there will be variability in environmental tolerances. Survival was to some degree species-specific. *L. fasciola* could not be kept alive under any type of environmental condition, even though we were able to keep other *Lampsilis* species alive for several years.

Unionids can survive, grow, and even reproduce for about 3–3.5 y on non-live algal diets, but long-term survival remains problematic. Health monitoring criteria using glycogen concentrations, shell growth rates, reproduction, and survival did not provide enough warning to prevent mortality. Additional criteria to judge success or failure of captive management protocols, other than death, need to be developed. These types of non-live algal diets may function for supplemental feeding, but at this time, maintaining adult unionid populations in captivity will require access to natural foods and water supplies from water systems that support native unionid fauna to increase the likelihood of long-term survival.

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ABSTRACTS OF TECHNICAL PAPERS

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CONTENTS

S. K. Allen, Jr.
Brood stock sources for hatchery-based stock enhancement of oyster reefs: Essential questions and recommendations.. 889
T. D. Alphin and M. H. Posey
Ecological function of oysters in southeastern North Carolina 889
D. Bishop
Simplification of shellfish restoration methods 889
M. Bowen, K. Groves, C. Heinig, and A. Frick
Expanding and sustaining shellfisheries of Casco Bay 889
M. F. Frana, E. A. Venso, K. Brohawn, W. Beatty, M. Ellwanger, R. McKay, B. Evans, and M. Phipps-Dickerson
DNA fingerprinting of nonpoint source *Escherichia coli* contamination in a Chesapeake Bay watershed 890
D. W. Brown
A national strategy for coastal habitat restoration 890
R. J. Bruckner and R. L. Takacs
Beyond the project: Values of community-based habitat restoration 890
R. D. Brunbaugh, W. J. Goldsborough, L. A. Sorabella, and J. A. Wesson
Oyster broodstock enhancement in Virginia and application of a new monitoring technique 891
D. Bushek, R. F. Dame, D. M. Allen, A. J. Lewitus, E. T. Koepfler, and D. Edwards
Nutrient cycling in intertidal creeks along the southeast US: Are oysters in control? 891
G. W. Calvo, L. M. Ragone Calvo, and E. M. Burreson
Disease resistance in a selectively bred *Crassostrea virginica* strain 891
D. Cheney, R. Elston, B. MacDonald, K. Kinnan, A. Suhrbier, G. Cherr, C. Friedman, F. Griffin, A. Hamdoun, J. Mitchell, L. Righetti, and L. Burnett
Summer mortality of the Pacific oyster, *Crassostrea gigas:* Influences of culture methods, site conditions, and stock selection 892
F.-L. E. Chu, V. G. Encomio, S. Stickler, S. Allen, and J. La Peyre
Physiological condition and defense-related activities among eastern oyster populations 892
P. Comar, L. Kracker, P. Bauersfeld, and M. Meaburn
A unified information system for shellfish restoration 892
M. Davidson, L. Holst, H. Bokuniewicz, C. Smith, and K. Tetrault
Effects of pearl net stocking density on survival, growth, and gonadal maturation of bay scallops 893
R. A. Elston, R. M. Estes, A. Gee, R. P. Herwig, K. Kinnan, and S. Rensel
Probiotic approach to enhance health of hatchery produced shellfish seed 893
E. Fahy
Management by size limit of the Whelk *Buccinum undatum* Fishery in the south west Irish Sea 893
L. D. Coen and A. Fischer
Managing the future of South Carolina's oysters: An experimental approach evaluating current harvesting practices and boat wake impacts 894
P. M. Gaffney, K. S. Reece, and J. C. Pierce
Genomic approaches to marker development and mapping in the Eastern oyster, *Crassostrea virginica* 894
W. J. Goldsborough, R. D. Brumbaugh, D. W. Meritt, and J. A. Wesson
Community-based oyster restoration: Case studies from Chesapeake Bay 894
M. Gomez-Chiarri and P. Muñoz
Molecular immune responses of the Eastern oyster to the parasite *Perkinsus marinus* 895
G. C. Cherr, C. S. Friedman, F. J. Griffin, A. Hamdoun, J. Mitchell, L. Righetti, D. P. Cheney, R. A. Elston, and B. McDonald
Factors affecting the stress response in oysters on the west coast: Implications for summer mortality 895
N. H. Hadley and L. D. Coen
Community-based oyster habitat restoration and enhancement in South Carolina 895
J. M. Harding and R. Mann
Essential or just opportunistic fish habitat? Utilization of restored complex shellfish habitat by fish species 896
E. E. Hofmann, J. M. Klinck, E. N. Powell, S. E. Ford, S. Jordan, and E. Burreson
Effects of climate variability on the prevalence and intensity of Dermo and MSX diseases in Eastern oyster populations 896

C. Smith, S. Dumais, L. K. Holst, and M. Davidson
Field trial of a Bay scallop (*Argopecten irradians*) spawner sanctuary 896
A. C. Ibe and P. O. Abohweyere
Restoring critical habitats in the Niger Delta for shellfish production 896
M. Janowicz
Stakeholder and concensus – How do we make these elements work for effective action? 897
S. J. Jordan, K. N. Greenhawk, C. B. McCollough, and M. L. Homer
Oyster biomass and abundance in northern Chesapeake Bay: Trends in relationship to harvest, recruitment, parasitic diseases, and environmental variation 897
C. J. Judy and E. Campbell
Public and private oyster restoration in Maryland's Chesapeake Bay 897
P. Kamermans and A. C. Smaal
Mussel culture and cockle fisheries in The Netherlands: Finding a balance between economy and ecology 898
R. J. Dugas, W. R. Keithly, M. Bourgeois, P. Meier, D. Lavergne, and A. Diagne
An economic analysis of public ground oyster reef restoration in central Louisiana damaged by hurricane Andrew 898
K. E. Landrum, K. M. St. Pé, B. Ache, and F. Kopfler
The rerouting of stormwater discharges for wetlands enhancement, levee protection, and oyster habitat protection and restoration 899
K. E. Landrum
The shellfish challenge initiative: A cooperative success story in the Barataria-Terrebonne National Estuary 899
T. Landry, M. Ouellette, and P. Cormier
Oyster population restoration in Caraquet, N.B.; Phase I, population assessment 899
K. L. LeBlanc
Community-based initiatives for improving water quality in Southwestern New Brunswick, Canada—an update on success 899
A. T. Leggett, R. Brumbaugh, W. Goldsborough, and A. McDonald
Application of commercial-scale oyster aquaculture to reef restoration 899
M. Luckenbach, F. O'Beirn, P. Ross, J. Nestlerode, and L. Sorabella
Dollars and sense of oyster restoration: An examination of nitrogen removal by a restored oyster reef 900
R. B. Luftig and W. Pelon
Reduction in the *Vibrio vulnificus* load of oysters by a novel short-term combination biodepuration treatment 900
R. Mann and J. M. Harding
Coming soon to a restoration site near you: The invading predatory oriental gastropod *Rapana venosa* 900
R. Mann
Up close and personal: A suggested quantitative approach to broodstock enhancement on shellfish restoration sites 901
D. W. Webster and D. W. Meritt
Linking public and private partners for restoration aquaculture in Maryland's seaside bays 901
D. W. Meritt and S. Tobash
Production of disease-free oyster seed using shallow water nurseries in the Mid-Chesapeake Bay 901
K. A. Nelson and G. I. Scott
A multifactorial approach for describing the relationship between the classification of shellfish harvesting waters and adjacent land use in Murrells and North Inlet, South Carolina 901
J. A. Nestlerode, M. W. Luckenbach, P. G. Ross, and F. X. O'Beirn
Influence of oyster reef structure on fish assemblages: Does the placement of artificial substrate enhance fish populations? 902
M. Norman
Shellfish restoration in Ireland: The need for novel partnerships 902
M. Ouellette, M. Hardy, T. Landry, N. G. MacNair, and A. Boghen
Reproductive biology of the northern quahaug, *Mercenaria mercenaria*, in Prince Edward Island, Canada 902
K. T. Paynter, Jr.
Considerations for oyster restoration in Maryland: Disease genetics, density, reproduction, and habitat creation 903

P. J. Pazo
Culture techniques applied to wild bivalve beds in Galicia, NW Spain 903
A. L Pereira, F. A. Ruano, L. Chicharo, and D. Matias
The influence of environmental factors in Japanese oysters health condition cultivated in the South of Portugal 903
P. Perina and D. Perina
The "EEEOHM" (Eastfields' Environment Enhancing Oyster Holding Module) 903
E. A. Bochenek, E. N. Powell, J. M. Klinck, and E. E. Hofmann
A biochemically-based model of the growth and development of *Crassostrea gigas* larvae 904
J. M. Klinck, E. N. Powell, J. N. Kraeuter, and S. E. Ford
A fisheries model for managing the oyster fishery during times of disease 904
J. Power and D. B. Walker
Shellfish data management and reporting system (SDMRS) 904
S. P. Powers, C. H. Peterson, and J. H. Grabowski
Restoring oyster reefs for fish: Estimating enhanced secondary production of restored oyster reefs 905
B. RaLonde
Restoring the littleneck clam resource for Native American subsistence use in the Prince William Sound, Alaska 905
K. M. Reece, W. L. Ribciro, K. L. Hudson, and S. K. Allen, Jr.
Development of *Crassostrea virginica* microsatellite markers for a genetic linkage map and genetic monitoring of restoration projects 905
D. B. Rouse, R. K. Wallace, and F. S. Rikard
Oyster reef restoration research in Mobile Bay, Alabama 905
D. C. Sherwood
Transplanting broodstock oysters, *Crassostrea virginica*, onto reconstructed oyster reefs to increase spat recruitment in the Piankatank River 906
S. E. Shumway
Harmful algal blooms and shellfish restoration: Can they co-exist? 906
L. A. Sorabella, M. W. Luckenbach, and F. X. O'Beirn
A comparison of two oyster (*Crassostrea virginica*) strains for productivity and suitability for use in oyster reef restoration efforts 906
S. M. Tobash and D. W. Meritt
Oyster restoration and the University of Maryland: Interactions between research, industry, and the public 907
A. K. Volety, M. Savarese, and S. G. Tolley
Effects of watershed alterations on oyster populations in Southwest Florida estuaries: An ecological approach 907
J. A. Wesson and L. B. McKay
The Virginia Oyster Heritage Program 907
J. J. Whitten
Enhancing and sustaining North Shore shellfishing through aquaculture 908
A. Willner
Community-based oyster restoration in an urban estuary: Developing an oyster culture and reef restoration program in the Hudson-Raritan Estuary 908

BROOD STOCK SOURCES FOR HATCHERY-BASED STOCK ENHANCEMENT OF OYSTER REEFS: ESSENTIAL QUESTIONS AND RECOMMENDATIONS.

S. K. **Allen, Jr.**, Aquaculture Genetics and Breeding Technology Center, Virginia Institute of Marine Science, College of William And Mary, Gloucester Point, VA 23062.

Oyster populations are subdivided into genetically distinct units with major divisions occurring over large geographic scale because of larval dispersal. Populations resist local adaptation because of population mixing through migration. However, over the course of the last 50 years, populations of Chesapeake Bay oysters may have lost alleles for disease resistance from the combination of disease mortality followed by heavy harvesting of survivors. Artificial breeding can increase the frequency of disease-resistant alleles, and several varieties of disease-resistant oysters are available as brood stock today. Some of these strains have been used to populate reefs and are likely to survive, grow, and breed on the reefs. Reproduction of the disease-resistant strains will produce disease-resistant spat over surrounding areas. There may also be natural stocks of oysters that resist disease, such as those from the Gulf of Mexico, where Dermo historically occurs. However, using "artificial" or genetically distinct oysters from the hatchery for reef restoration could also entail some risk to natural genetic diversity. For example, artificially selected populations have (by definition) reduced genetic variation over wild stocks. Interbreeding of the two may alter wild populations. At present, the risk (or benefit) of using alternative stocks is unknown. The results and recommendations from a workshop on genetic considerations for hatchery-based reef restoration will be presented.

ECOLOGICAL FUNCTION OF OYSTERS IN SOUTHEASTERN NORTH CAROLINA.

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Oysters serve a variety of functions within the estuarine systems of Southeastern North Carolina. A number of juvenile fish and decapods use oyster habitat for refuge/forage during some portion of their lives. Here, we present results from several studies evaluating the use of oyster habitat by juvenile fish and decapods compared with alternate habitats. An abundance of fish and decapods were examined in isolated and mixed oyster habitats using Breder traps in small tidal creek estuarine systems whereas net sampling was used to compare faunal abundances in oyster reefs along larger spatial scales in the presence and absence of sea grass beds. In both cases, abundance in oyster reefs were compared with vegetated or unvegetated marsh edge habitats. On the larger scale, use of oyster reefs was also compared with abundance patterns within seagrass beds. The mixed results of these studies indicate that the importance of oyster reefs as a refuge/forage habitat varies seasonally within a given system as well as among small estuarine systems based on the presence of alternate habitats. We also present preliminary information on more indirect effects of oysters as modifiers of water quality using transplant/removal studies.

SIMPLIFICATION OF SHELLFISH RESTORATION METHODS.

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As the shellfish aquaculture industry continues to grow, methods of husbandry to reduce labor, increase yield, and produce higher quality products are in a constant evolution. Based on the simple fact that smart people learn from their mistakes and really smart people learn from other's mistakes, there is a lot that can be learned from the aquaculture industry to transfer to restoration projects. Although all the answers for husbandry are not in place, many dynamics and protocols positively affect restoration projects by moving them forward at a faster pace. Attitudes towards labor efficiency using different equipment ideas and management techniques will be discussed. This will give attendees references that, used as-is or with slight modification, could benefit their efforts significantly. An interactive audiovisual presentation with examples of methods from around the world used today will enhance the presentation.

EXPANDING AND SUSTAINING SHELLFISHERIES OF CASCO BAY.

M. Bowen,[1] **K. Groves,**[2] **C. Heinig,**[3] and **A. Frick,**[4] [1]Normandeau Associates, Normandeau Associates Inc., 251 Main Street, Yarmouth, ME 04096; [2]Casco Bay Estuary Project, University of Southern Maine, Law School Building, Portland, ME 04104; [3]Mer Assessment Corporation, 14 Industrial Parkway, Brunswick, ME 04011; and [4]Albert Frick Associates, Inc., 95a County Road, Gorham ME 04038.

One of the missions of the Casco Bay Estuary Project is to ensure communities around Casco Bay in Maine have a healthily shellfish harvest that sustains commercial and recreational shellfishing for generations to come. A "clam team" of stakeholders, including the US Environmental Protection Agency, the Friends of Casco Bay, Maine Department of Marine Resources, individual cities and towns, and the Maine Department of Environmental Protection was formed to find the most productive shellfish areas currently closed to harvest, determine sources of contamination, and find ways to remediate. A field review of the 57 clam flats—800 acres—of soft-shell clam habitat that are currently closed to harvest targeted 22 of these, totaling 370 acres of highly productive clam flats. Review of water quality data pinpointed sources of contamination. Many of the flats are closed simply because of the presence of an overboard discharge system that treats household waste. The project is currently supporting an intensive effort to design and construct replacement systems, a collaborative effort between the towns, state, and individual homeowners. Additional water sampling efforts are in progress to determine other nonpoint sources of contamination, including farm runoff, leaking septic systems, and wildlife. A third element of the project is investigating the sustainability aspect, investigating the effectiveness of regulatory options, including licensing, harvest limits and techniques, and conservation closures.

DNA FINGERPRINTING OF NONPOINT SOURCE *ESCHERICHIA COLI* CONTAMINATION IN A CHESAPEAKE BAY WATERSHED. **M. F. Frana,[1] E. A. Venso,[2] K. Brohawn,[3] W. Beatty,[3] M. Ellwanger,[3] R. McKay,[3] B. Evans,[3]** and **M. Phipps-Dickerson,[4]**

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Fecal coliform contamination has closed shellfish harvesting areas and public beaches and threatened recreational areas in the Chesapeake Bay watershed. Bacteriological water quality testing currently performed in these watersheds does not identify the sources of contamination. Therefore, no pollution control or mitigation efforts have been undertaken, despite the large economic impact for this area of the Midatlantic. Possible sources include runoff from crop fields, wildlife, discharge from boats, and runoff from >1,300 animal production farms on the Eastern Shore of the Chesapeake Bay. Although municipal waste water plant effluent and on-site waste water treatment (septic) systems could contribute, shoreline surveys conducted by the Shellfish Sanitation Program of the Maryland Department of the Environment indicate that nonpoint sources are responsible for the elevated levels of coliform bacteria in this watershed. It is understood that these sources would contribute not only bacteria but also excess nutrients and possibly other water contaminants that can negatively impact public health as well as the sensitive plant and animal species that dwell in the watershed. The methodologies used to determine the specific sources of *Escherichia coli* contamination are described, including choice of sample locations and environmental variables, sampling techniques, DNA analysis of strain-specific *E. coli*, and interpretation of the data. Preliminary data are presented, including selected DNA fingerprints and relationships among and between total coliforms and *E. coli* MPNs and six environmental and water chemistry variables. Ultimately, Geographic Information Systems mapping will be used for spatial analysis as a key to the understanding needed for pollution control and mitigation.

A NATIONAL STRATEGY FOR COASTAL HABITAT RESTORATION. **D. W. Brown,**

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Shellfish habitats make up a significant portion of the important aquatic habitats in our coastal waters that provide the living space for marine and estuarine fish and shellfish. Unfortunately, in many areas along our coastline, many habitats, including shellfish habitats, are being destroyed and the natural systems they support are failing. The National Oceanic and Atmospheric Administration recently joined with Restore America's Estuaries and the Pew Charitable Trusts to launch a major partnership initiative to restore important habitats in our coastal estuaries. A major element of this initiative is to develop a national strategy for coastal habitat restoration, including important shellfish habitats. The purpose of the strategy is to identify specific habitat problems in each coastal region and to determine the most viable restoration approaches to address degraded areas for these regions. The National Strategy will (1) actively promote the increased protection of existing habitats; (2) establish specific regional and national restoration goals and objectives; (3) provide a framework for setting restoration priorities; (4) identify and integrate the science and new technologies needed for effective restoration; and (5) energize cooperative partnerships among private and public stakeholders. This presentation will review ongoing and planned actions by the NGO community, federal agencies, and the private sector to develop a national strategy for coastal habitat restoration, including shellfish habitats, by the fall of 2001.

BEYOND THE PROJECT: VALUES OF COMMUNITY-BASED HABITAT RESTORATION. **R. J. Bruckner[1]** and **R. L. Takacs,[2]**

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The NOAA Community-based Restoration Program (CRP) began in 1996 to inspire local efforts to conduct meaningful, on-the-ground restoration of marine, estuarine, and riparian habitat. The CRP is a systematic effort to catalyze partnerships at the national and local level to contribute funding, technical assistance, land, volunteer support, or other in-kind services to help citizens implement restoration projects that promote stewardship and a conservation ethic for living marine resources. The CRP links funding and technical expertise to citizen-driven restoration projects and emphasizes collaborative strategies built around improving NOAA trust resources and the quality of the communities they sustain. Oyster restoration projects, although not all explicitly off-limits to harvesting, have emphasized the habitat benefits of reef restoration, from three-dimensional habitat conducive to spat settlement, to the benthic organisms that make up the ecological diversity of oyster reefs themselves, to the fish and openwater communities that aggregate around hard-bottom reef habitat. In addition to implementing projects, this innovative funding/partnership source has provided the mechanism to "field test" new restoration strategies, such as reef design and construction, unique management approaches like sanctuaries, reserves, and satellite bars, and often has served as the springboard for larger-scale, river-wide restoration efforts. The availability of technical expertise and matching funds and the positive results achieved by community-based shellfish restoration efforts have catalyzed other federal, state, and local entities to participate, effectively broadening the partnering and stewardship opportunities, increasing the areas available for shellfish restoration, and leveraging the amount of funds available for habitat restoration efforts.

OYSTER BROODSTOCK ENHANCEMENT IN VIRGINIA AND APPLICATION OF A NEW MONITORING TECHNIQUE.

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The transplanting of both wild and hatchery-produced oysters onto oyster sanctuary reefs is increasingly frequent as a component of oyster restoration efforts in the Chesapeake Bay. Since 1996, oysters have been added to more than a dozen state or privately managed sanctuary reefs in Virginia in an effort to enhance localized oyster spawning success. Wild oysters, purchased with both state and private funds, have accounted for approximately 70% of the total number of oysters added to reefs. Increasingly, however, the oysters added to reefs are hatchery produced, grown by citizens and students volunteering through programs such as the Chesapeake Bay Foundation's Oyster Corps. To date, more than 800,000 oysters grown by volunteers have been added to Virginia's system of reefs. Although definitive data are scarce, there appears to be good empirical evidence that these stocking efforts have enhanced spat settlement rates on and around sanctuary reefs. Dive surveys and patent-tong data show substantial increases in localized spat settlement in tributaries where oysters have been added to reefs in recent years. To better understand spat settlement dynamics around the reefs, spat cages, small cages filled with a known volume of shell, are now being used to monitor oyster settlement around selected reefs. A strong correlation exists between spat cage data and diver-surveys on nearby reefs ($r = 0.95$, $P < 0.01$), suggesting that spat cages may be a low-cost means of both involving the public in restoration and of evaluating results of broodstock enhancement and reef restoration projects.

NUTRIENT CYCLING IN INTERTIDAL CREEKS ALONG THE SOUTHEAST US: ARE OYSTERS IN CONTROL?

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Ecologically, oyster reefs provide habitat, filter water, and facilitate nutrient cycling. We experimentally removed oyster reefs to examine their role in the structure and function of intertidal creek ecosystems. Surprisingly, the removal of oyster reefs did not significantly alter nutrient concentrations, nekton usage, or phytoplankton production. Our calculations show that oysters do not produce enough ammonium to satisfy phytoplankton productivity, but nekton, water column remineralization, and sediments more than account for the deficit. These observations were interpreted as an indication of functional redundancy in the system. Flagellates, which are preferred over diatoms as food by the oysters, dominated the phytoplankton during summer when ammonium concentrations were high. Diatoms dominated during the colder months. Shifts in phytoplankton dominance corresponded to the seasonal arrival and departure of nekton in the creeks. Because nekton comprised more than double the biomass of oysters during summer, fishes and macrocrustaceans may play a greater role in nutrient remineralization than has been previously considered. At the meso-scale ecosystem level, the loss of nutrient remineralization activities attributable to the removal of oyster reefs was compensated by other components within the system, but phytoplankton communities changed, apparently in response to changes in grazing. Oysters clearly play important roles, but defining the importance of shellfish restoration in the management of coastal ecosystems requires an understanding of the ecosystem science, a consideration of scale, and the realization that tidal creek systems exhibit complex responses.

DISEASE RESISTANCE IN A SELECTIVELY BRED *CRASSOSTREA VIRGINICA* STRAIN.

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During 1997 to 1999, DEBY oysters, a VIMS stock that was selectively bred for four generations at a disease endemic site in the lower York River, Virginia, were evaluated for survival, growth, and disease susceptibility in comparison with progeny from wild Mobjack Bay (MB) and Tangier Sound (TS) brood stocks. MB and TS stocks are relevant to rehabilitation of Chesapeake Bay oysters because the former have been routinely used for aquaculture and the latter have been recently used for reef restoration because of their putative disease resistance. Oysters ($n = 1500$ of each group, mean shell height = 15–17 mm) were deployed in floating mesh cages at a low salinity (<15 ppt) site and a moderate salinity (15–25 ppt) site in the lower Chesapeake Bay, and at a high salinity (>25 ppt) site on the Atlantic Coast of Virginia. Twenty-eight months after deployment, cumulative mortality in MB and TS was 84–100%. In contrast, cumulative mortality in DEBY at low, moderate, and high salinity sites was, respectively, 21, 51, and 36%. By November 1999, mean shell height in MB and TS at low-, moderate-, and high-salinity sites was, respectively, 77, 88–90, and 57–59 mm. In comparison, mean shell height in DEBY was 92, 101, and 72 mm. Although similar low levels of MSX were observed in all groups, *P. marinus* infections in MB and TS were more intense than in DEBY throughout the study. This promising oyster strain has potential to facilitate commercial aquaculture and reef restoration efforts in Chesapeake Bay.

SUMMER MORTALITY OF THE PACIFIC OYSTER, *CRASSOSTREA GIGAS:* INFLUENCES OF CULTURE METHODS, SITE CONDITIONS, AND STOCK SELECTION.

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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 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. Recent studies in Puget Sound, Washington and Tomales Bay, California, 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 growout location to increase survival of Pacific oysters. Field observations indicate oysters are subject to extreme variations in a number of parameters during intertidal cycles. An increased rate of oyster mortality and modified physiological response appear to be strongly correlated with both elevated temperatures and extended periods of depressed DO. The DO reductions are sometimes coupled with heavy macroalgae blooms and high phytoplankton densities. This and other works indicate oyster summer mortality rates are also 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 which retain desirable characteristics of good growth and meat yield. This research was supported by grant numbers NA86RGOOl5 and NA96RG0488 from the National Sea Grant College Oyster Disease Research Program and matching contributions from West Coast shellfish farmers.

PHYSIOLOGICAL CONDITION AND DEFENSE–RELATED ACTIVITIES AMONG EASTERN OYSTER POPULATIONS.

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The goal of our study is to identify oyster stocks that are resistant/tolerant to the disease caused by the parasite, *Perkinsus marinus* (Dermo). We are comparing the physiological condition and defense factors of putative "Dermo resistant" and "nonresistant" oysters (*Crasssostrea virginica*) deployed in the fall of 1999 at two sites in the Chesapeake Bay (Port Kinsale, Yeocomico River; Regent Point, Rappahannock River), where Dermo disease is known to occur, but not MSX (disease caused by *Haplosporidium nelsoni*). These oysters are F1 progenies from presumably genetically distinct oyster populations (three Gulf of Mexico and three Chesapeake Bay populations, and one hatchery strain) and represent geographical disparity. Oysters have been sampled monthly since May 2000. Initial analysis showed that all the stocks have grown significantly since deployment and the Rappahannock River Stock has the fastest growth. Tissue dry weights of this stock increased significantly over time at both sites. Contents of glycogen, protein, and lipid increased with growth. All stocks sampled from May to July had low *P. marinus* infection and prevalence. Mortality was low in all stocks and lower in the Gulf of Mexico than Chesapeake Bay populations. No significant differences were noted in levels of plasma protein and lysozyme among stocks. Currently we are analyzing oysters sampled in August and September. Correlation between growth, physiological, biochemical, and defense condition and *P. marinus* infection among oyster stocks will be discussed. This research was funded by the NOAA-Virginia Sea Grant-Oyster Disease Research Program.

A UNIFIED INFORMATION SYSTEM FOR SHELLFISH RESTORATION.

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The Shellfish Information Management System (SIMS) is an intergovernmental data system designed to provide a current central source of information on shellfish safety, resource, and habitat useful to multiple users at local, state, regional, and national levels. SIMS is being developed as a GIS-enabled, web-accessible relational database of shellfish harvest water survey, classification, and resource information. Most of the data in SIMS is provided by state agencies, and SIMS will allow more extensive access to and integrative analysis of that information. In 1999, the National Ocean Service, Center for Coastal Environmental Health and Biomolecular Research in Charleston, South Carolina, began partnering with a growing number of coastal states in the design and applications for SIMS. This spatially enabled, Oracle database is designed with extensive query functionality, visualization, and analytical capabilities for a wide range of shellfish safety, water quality, resource, and restoration concerns. Shellfish restoration is a new component being developed for incorporation into SIMS so that trends in restoration can be quantified and visualized. Water quality, benthic and habitat suitability, shellfish resource, presence of disease agents, social and economic factors, and other influences impact shellfish restoration decisions and actions. Discussion will include the rationale and means for collecting and integrating such restoration data layers into SIMS.

EFFECTS OF PEARL NET STOCKING DENSITY ON SURVIVAL, GROWTH, AND GONADAL MATURATION OF BAY SCALLOPS.

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The stocking densities under which bay scallops are reared can have long-term effects on survival. growth, and spawning success that may not be evident while the scallops are in culture. To investigate the influence of stocking density on scallop production, hatchery reared bay scallops were stocked in pearl nets at three different densities during the summer. In the fall, bay scallop survival and shell heights were recorded. The animals were transferred to lantern nets and stocked at two different densities, grouped by their initial densities in the pearl nets, and over wintered. Bay scallops raised at high densities exhibited lower survival and slower growth than those raised at lower densities. Regardless of density in the lantern nets, growth and survival still showed the negative effects of initial crowding in the pearl nets. Two-way analyses of variance revealed significant differences among the pearl net and lantern net treatments in scallop survival and growth. Gonadal indices show that all the bay scallops, regardless of treatment, spawned at the same time. At the time of spawning there were no significant effects of density on gonadal index. Bay scallop restoration efforts should ensure that scallops are reared under conditions that maximize survival and growth.

PROBIOTIC APPROACH TO ENHANCE HEALTH OF HATCHERY PRODUCED SHELLFISH SEED.

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Bacterial diseases of intensively cultured larval and juvenile shellfish cause significant losses in hatcheries and nurseries. In addition, chronic bacterial infections are a significant cause of bivalve seed losses postplanting. From commercial hatchery case histories, a number of virulent juvenile oyster bacterial pathogens have been isolated, characterized, and pathogenicity confirmed by challenge procedures. Prevention and control strategies for bacterial pathogens in hatcheries and nurseries must include routine sanitation of system surfaces, water filtration, brood stock sanitation, and maintenance of low dissolved organic levels. Antibiotics have been used in experimental settings but are not routinely used on production scale systems because of cost as well as risk of producing resistant strains. A program to select and test probiotic strains of bacteria, as an alternative to antibiotic use, is underway. and results to date will be presented. Bacterial pathogens were first screened by comparing whole cell fatty acid profiles. Based on this evaluation, most pathogens were consistent or close to the *Vibrio* genus, but probiotic candidates represented a variety of bacterial genera. Selected representative isolates were further characterized using biochemical criteria and 16s rDNA sequencing. Candidate probiotic bacteria are first tested in agar plate inhibition tests. Strains showing inhibition to isolated pathogens are tested for haemolytic activity and pathogenicity to shellfish seed. Candidates passing these tests are then tested for inhibition of mortality and morbidity response in laboratory pathogen challenges. This research was supported in part by Saltonstall-Kennedy program (National Marine Fisheries Service, U.S. Department of Commerce) grant to Pacific Shellfish Institute, Olympia, Washington.

MANAGEMENT BY SIZE LIMIT OF THE WHELK *BUCCINUM UNDATUM* FISHERY IN THE SOUTH WEST IRISH SEA.

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Whelk landings in the south west Irish Sea increased from 56 t in 1990 to 6,575 t in 1996 after which they stabilized between 3,600 and 4,600 t annually. At its peak, the fishery supported approximately 80 vessels but this number has halved since this time. In 1994, a size limit of 50 mm was introduced for conservation purposes. Age-based assessments of the landings were conducted in 1994, 1996. 1997, and 1999, for which purpose the fishery, ranging from 52°10′ to 53°30′, is divided into four sectors. Landings to the four sectors display biological characteristics that indicate the occurrence of a number of stocklets rather than a single stock unit. Compliance with the size limit has been poor. From 20 to 33% of total landings (by number) in any of the assessed years have been less than the legal limit. Trends in cpue have been monitored since 1990. Some fishermen in the center sectors improved their yield between 1994 and 1998. Whelk have responded to a reduction in fishing effort since 1996, immediately after which averaged mortality coefficients (Z) were highest (0.79); they declined to 0.61 in 1999. The survival of the whelk fishery in the south west Irish Sea is attributed to the instability of the market which is dominated by a single customer, South Korea. A more effective size limit for this fishery would be 68 mm (83 mm in the northern sector), and this is considered unrealistic. suggesting that alternative management measures will have to be introduced.

MANAGING THE FUTURE OF SOUTH CAROLINA'S OYSTERS: AN EXPERIMENTAL APPROACH EVALUATING CURRENT HARVESTING PRACTICES AND BOAT WAKE IMPACTS. L. D. Coen and **A. Fischer,** Marine Resources Research Institute, SCDNR, Charleston, SC 29412.

Oyster reefs provide an important intertidal habitat to the Southeastern United States. However, harvesting and recreational boating invariably impact these critical habitats and their associated functions. In 1998, we began to experimentally evaluate the direct impacts of four harvesting practices (complete harvest, cull-in-place, clamming, and rake down) on intertidal oyster resources. Initially, 26 sites were sampled by quadrat to establish baseline assessments. Initial mean oyster size (SH) across sites ranged from 23–33 mm, with initial densities ranging from 1,700–7,500 oysters/m^2. Then, the above harvesting practices were simulated at replicated sites, each paired with an adjacent control site. Water quality (temperature, DO, salinity, chl *a*) was measured during the study period. Trays of shell were deployed at each site to evaluate oyster recruitment and growth. After approximately 1 year, more than 133,000 oysters recruited to the 130 deployed trays. This recruitment, a surrogate for larval supply/habitat quality, and the baseline assessments are analyzed and discussed. In 1999, we conducted experiments to understand how boat wakes compromise shell (cultch) deployments for oyster restoration and marsh erosion control. For this, we deployed stabilized (mesh) and unstabilized shell treatments, monitoring cultch retention after controlled boat wakes. In the first pilot experiment, unstabilized treatments lost 33.6% (7.7 cm) more shell than stabilized treatments after exposure to 32-controlled boat passes. In a second experiment, 22.4% (5.17 cm) more was lost after only 24 passes. Both harvesting practices and recreational boating wakes can potentially impact the growth, recruitment, and recovery of intertidal oyster resources. Additionally, oyster reefs that fringe marshes can serve as moderators of both marsh and bank erosion. Further studies with remote sensing technologies should be employed to monitor the oyster-marsh interaction.

GENOMIC APPROACHES TO MARKER DEVELOPMENT AND MAPPING N THE EASTERN OYSTER, *CRASSOSTREA VIRGINICA*. P. M. Gaffney,[1] K. S. Reece,[2] and **J. C. Pierce,[3]** [1]College of Marine Studies, Lewes, DE 19958; [2]Virginia Institute of Marine Science, Rt. 1208, Gloucester Point, VA 23062; and [3]University of the Sciences in Philadelphia, 600 S. 43d St., Philadelphia, PA 19104.

In response to the dramatic decline in the Atlantic oyster fishery, efforts are underway to expand hatchery production of the Eastern oyster, for both commercial farming and for replenishment of disease-challenged natural populations. In particular, there is a strong demand for genetically improved oyster strains resistant to two common protozoan parasites, Dermo and MSX. The genetic improvement process will be enhanced by the development of molecular markers and a genetic linkage map. To facilitate future marker development in *C. virginica,* we obtained 0.7 MB of random genomic sequence data from a small-insert (1 kb) pGEM library. A modest number of significant BLASTX hits may prove valuable for designing type I markers for comparative mapping with the Pacific oyster. In addition, we the searched the sequence database for repetitive sequences. Several satellite DNA sequences were identified and compared with putative satellite sequences obtained by traditional cloning methods. Our database yielded useful information on the distribution of microsatellite loci. Dinucleotide microsatellites were dominated by the AG motif (66%). Trinucleotide microsatellites included all possible motifs in apparently equal frequencies. Tetranucleotide microsatellites were more common than trinucleotides and, unlike the other microsatellite classes, were frequently associated with repetitive sequences, with a strong tendency for certain tetranucleotide motifs to be associated with particular repetitive sequences. This information will be useful for tetranucleotide microsatellite marker design, as well as interpretation of linkage mapping data. The repetitive sequence database will be used as an adjunct for designing new primers, to reduce the frequency of non-target amplification.

COMMUNITY-BASED OYSTER RESTORATION: CASE STUDIES FROM CHESAPEAKE BAY. W. J. Goldsborough,[1] R. D. Brumbaugh,[1] D. W. Meritt,[2] and **J. A. Wesson,[3]** [1]Chesapeake Bay Foundation, 162 Prince George Street, Annapolis, MD 21401; [2]University of Maryland, Center For Environmental Science, P.O. Box 775, Cambridge, MD 21613; and [3]Virginia Marine Resources Commission, P.O. Box 756, 2600 Washington Avenue, Newport News, VA 23607.

Public support for oyster restoration in the Chesapeake Bay region has increased in recent years, largely because of expanded opportunities for direct citizen involvement in restoration work The commercial value of oyster restoration is the most easily appreciated aspect of restoration, whereas associated benefits such as improved fish habitat and water quality are only recently being more widely recognized. As opportunities for public participation have expanded, the support for restoration has increasingly been based on these associated ecosystem benefits, particularly in developed areas where water quality may preclude commercial or recreational harvest of bivalves. One of the principal ways that the public now participates in oyster restoration is by growing hatchery-produced oysters using small-scale aquaculture techniques (i.e., "oyster gardening") for eventual transplanting onto broodstock sanctuary reefs. Analyses of four local examples of citizen involvement in oyster gardening/restoration in the Chesapeake reveal a general pattern of roles and responsibilities for successful community-based restoration. Local leadership, sources for shell and seed, education, technical guidance, amenable government rules and regulations, media exposure, and funding emerge as key factors. In the Chesapeake, a partnership approach based on cooperation between various combinations of citizens, schools, local businesses, local service organizations, watershed associations, academic institutions, state and federal agencies, conservation organizations, and private foundations has successfully addressed these needs. It is becoming increasingly clear that the groundswell of public support for restoration resulting from community-based approaches is a key factor in generating increased public funding for restoration.

MOLECULAR IMMUNE RESPONSES OF THE EASTERN OYSTER TO THE PARASITE *PERKINSUS MARINUS*.

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Microbial pathogens and parasites like *Perkinsus marinus* and *Haplosporidium nelsoni* place a large economic burden on oyster fisheries and aquaculture. Although there has been a steady increase in our knowledge on the pathology and epizootiology of the diseases caused by these parasites, relatively little is known about the molecular mechanisms involved in the response of oysters to infection. The goal of this project funded by the ODRP is to monitor systematically the induced expression of genes involved in the response of *Crassostrea virginica* to infection by the parasite *Perkinsus marinus*. An mRNA differential display technique coupled with stringent verification assays (reverse Northern blot) will be used to isolate oyster and parasite sequences expressed in a differential manner after challenge of oysters with cultured *P. marinus*. Genes will be cloned and sequenced using standard molecular techniques. The temporal and tissue patterns of expression of the candidate genes in oysters will be studied using Northern blot. Preliminary results from the challenge experiments will be presented at this meeting.

FACTORS AFFECTING THE STRESS RESPONSE IN OYSTERS ON THE WEST COAST: IMPLICATIONS FOR SUMMER MORTALITY.

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Summer mortality of *Crassostrea gigas* on the West Coast of the United States is an unpredictable phenomenon of unknown etiology but one that is hypothesized to be caused by multiple stressors. Previous research has identified a dinoflagellate *(Gymnodinium sanguineum)*, temperature, and low dissolved oxygen as possible contributors. We have attempted to delineate the independent effects of two of these suspected factors, phytoplankton and temperature, while conducting parallel field studies in California and Washington to determine the effects of seed stock lineage and seed planting times. Laboratory challenges confirmed that G. *sanguineum* can produce stress/mortality in the absence of other insults. Phytoplankton bloom events have coincided with field mortality; however, the species present were a *Pseudonitzchia*-like species and *Prorocentrum spp.*, not *G. sanguineum*. Previous research on temperature effects showed that the ability of *C. gigas* to tolerate otherwise lethal temperatures occurred after sublethal thermal shock and induction of the heat shock protein 70 (HSP70) family. This is termed the heat shock response (HSR). We have examined the abilities of *C. gigas* from three different habitats (Toten Inlet, WA; Mud Bay, WA; Tomales Bay, CA) to mount a HSR and compared this ability with environmental and summer mortality data. Our current findings suggest that chronic sublethal environmental stressors such as heat and immersion can induce HSP70 expression and acquisition of thermal tolerance in *C. gigas*. However, these chronically stressed animals exhibit a compromised HSR; they do not tolerate post-heat shock temperatures as high as nonchronically stressed counterparts. This research was funded by National Sea Grant College Program Office: Oyster Disease Research Program.

COMMUNITY-BASED OYSTER HABITAT RESTORATION AND ENHANCEMENT IN SOUTH CAROLINA.

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Oyster reefs provide important habitat for finfish, crabs, and shrimp; improve water quality; and, when located adjacent to *Spartina* marsh, form a natural bulwark to reduce erosion. Oyster habitats nationwide are threatened by adverse effects of coastal development. The majority of oysters in South Carolina occur intertidally, where they may be exposed for as much as 6 h because of the ~2 meter tidal range. This makes them especially vulnerable to physical disturbances such as boat wakes. Substrates in South Carolina are typically soft mud and oyster shell provides one of the few hard surfaces for larval oyster attachment. Oysters readily recruit to shell placed in areas which otherwise may have no recruitment because of lack of suitable substrate. At sites with appropriate characteristics functional oyster reefs may be established in 3 to 5 years, with some attributes beginning earlier. This program will use community volunteers to establish multiple small-scale oyster habitats by planting oyster shell and covering it with stabilizing mesh. We will also develop a volunteer-based monitoring program to evaluate restoration success. Community partners with existing volunteer contacts have been enlisted to assist in this program. An oyster shell-recycling program is being established to generate shell for future restoration projects. Schoolchildren will be involved through collaboration with the Charleston Math and Science Hub to develop classroom and field activities directly related to oyster habitats. Materials (pamphlets, a website, CD) will be developed to educate the public about oyster habitats and shell recycling.

ESSENTIAL OR JUST OPPORTUNISTIC FISH HABITAT? UTILIZATION OF RESTORED COMPLEX SHELLFISH HABITAT BY FISH SPECIES.

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Shellfish restoration typically creates complex habitat in regions where such habitat is limited or absent. Observations to date suggest that increasing habitat complexity supports more diverse representation in other trophic levels. Such observations have been used to argue for shellfish restoration sites in the wider context of essential fish habitat. We present temporal and spatial data on fish utilization of a cline of habitats from a complex, "restored" site, through a two-dimensional but spatially complex site, to a monotonous sand bottom, and pose the question as to whether fish utilization of this cline suggests "essential" or simply opportunistic utilization of the varying resource.

EFFECTS OF CLIMATE VARIABILITY ON THE PREVALENCE AND INTENSITY OF DERMO AND MSX DISEASES IN EASTERN OYSTER POPULATIONS.

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With previous funding from the National Sea Grant Oyster Disease Research Program, we developed numerical models that simulate the annual cycles of intensity and prevalence of the diseases, Dermo, caused by *Perkinsus marinus,* and MSX, caused by *Haplospordium nelsoni,* in Eastern oyster *(Crassostrea virginica)* populations in Delaware Bay and the upper Chesapeake Bay. The host-parasite models consist of models for the growth of the parasites, as well as a model for the growth and development of the oyster. The external forcing for the models is from time series of temperature, salinity, food supply, and total suspended solids. Our recent project has focused on combining the two disease models with the oyster growth model. Simulations with the combined two disease-oyster model provide insight into the effect of variability in environmental conditions in initiating and controlling epizootics of Dermo and MSX in Chesapeake and Delaware Bays. The combined model also provides a mechanism for investigating possible interactions between Dermo and MSX diseases that modulate the level of intensity and overall prevalence of the two diseases in oyster populations. Initial results suggest that there is only limited interaction between the two diseases in the host and that environmental conditions are the primary determinant of which disease is dominant at a given location, as long as the oysters are susceptible to both disease agents and that the dose of infective particles does not vary.

FIELD TRIAL OF A BAY SCALLOP *(ARGOPECTEN IRRADIANS)* SPAWNER SANCTUARY.

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The populations of Bay scallop in New York waters have experienced critical decline over the past two decades, caused in large part by the occurrence of Brown Tide algal blooms and its concomitant effects on habitat and shellfish health. Management efforts in the past have focused heavily on hatchery-produced stock, which has been free planted into the estuary. Frequently, follow-up investigations show no juvenile scallops at the release sites, and the ultimate fate of the seed stock is unknown, except through extrapolation of harvest data. New York State Department of Environmental Conservation, working with Cornell Cooperative Extension's Marine Program, set out to evaluate "spawner sanctuaries" as a management method to ensure that reproducing scallops are present in the system in densities sufficient to increase the population. Mature scallops ($n = 15{,}000$) that had been over wintered in a creek adjacent to Cornell's hatchery were stocked into lantern nets at a density of 100 animals per tier and deployed into Cutchogue Harbor in Peconic Bay, New York. A larval drift model and diver transects were used to calculate relative contribution of the sanctuaries to the 1999 year class set in Cutchogue Harbor. Results were further normalized to reflect differences in gonad weight between wild stock and hatchery-reared scallops.

RESTORING CRITICAL HABITATS IN THE NIGER DELTA FOR SHELLFISH PRODUCTION.

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The Niger Delta is a fan-shaped piece of land located between 504'.00 and 7040'.00 longitude and stretching from the Benin River in the west to the Bonny River in the east, covering an area of about 16,340 km^2. This low lying region, riddled with intricate water channels through which the river Niger empties into the sea, consists of three broad ecological zones: freshwater, mangrove, and the coastal sandridges. The mangrove ecosystems are prime areas for production, constituting spawning and nursery grounds for near shore, demersal and pelagic fish species including shellfishes. Shellfish of economic importance found in the Niger Delta include the oyster *Crassostrea gasar* that settles as spat on mangrove aerial roots at intertidal levels, the periwinkles *Tympanotonous fuscatus* and *Pachymelia quadriserata,* and Penaeid shrimps that generate over US $195,977.26 from 10,664 MT annually in foreign exchange. Anthropogenic activities in the form of deforestation, sand mining and nourishment, channelization, dredging, oil and gas exploitation, and rapid urbanization are presently reducing the aerial extent of the mangroves as well as degrading the mangrove environment. This has an impairing effect on the shellfish production potential of critical habitats of the Niger Delta. This work thus advocates rational use of the mangrove ecosystem and reforestation of the mangrove swamp where possible such that one of the goals of the shellfish restoration effort of "restoration or enhancement of populations of commercially exploited shellfish depressed by over-harvesting and or reduced environmental quality" could be achieved in the Niger Delta.

STAKEHOLDER AND CONSENSUS—HOW DO WE MAKE THESE ELEMENTS WORK FOR EFFECTIVE ACTION? **M. Janowicz,** New Brunswick Department of Environment and Local Government, 364 Argyle Street, Fredericton, New Brunswick, E3B 1T9 Canada.

One model for effective stakeholder involvement requires implementation of a consensus decision-making process. This allows each stakeholder to understand the context within which each participant is working and requires the development of respect among the participants. This is an age-old concept but in reality, few in 21st century North America can easily accept and work within it. This article will examine why stakeholder involvement is a necessary means to formulate approaches to shellfish restoration or any other local economic development and planning program. It will explore the foundations of consensus decision-making including examination of concepts of democracy, discussion on Native North American decision-making processes and other decision-making models. And, finally, it will identify some methodology for achieving an effective stakeholder, consensus decision-making process with the emphasis on developing a common ground of understanding.

OYSTER BIOMASS AND ABUNDANCE IN NORTHERN CHESAPEAKE BAY: TRENDS IN RELATIONSHIP TO HARVEST, RECRUITMENT, PARASITIC DISEASES, AND ENVIRONMENTAL VARIATION. **S. J. Jordan, K. N. Greenhawk, C. B. McCollough,** and **M. L. Homer,** Maryland Department of Natural Resources, Sarbanes Cooperative Oxford Laboratory, 9045 S. Morris St., Oxford, MD 21654.

The Chesapeake Bay Program has committed to a 10-fold increase in the Bay's oyster population. Oysters are patchily distributed over about 1,500 km^2 of the Bay floor. Therefore, it is impractical to assess their absolute numbers by direct means. Traditionally, landings data, with their inherent inaccuracies and biases, have been the only means of estimating trends in the population. Maryland's monitoring program records relative numbers and size distributions of oysters annually at 43 fixed sites. By applying a length:weight equation to size-frequency data from this fishery-independent survey, we computed an index of relative biomass that varied from year to year in response to the relative abundance and size distribution of the oyster populations. The index reflects interannual variations in recruitment and growth, as well as mortality caused by the oyster parasites *Haplosporidium nelsoni* and *Perkinsus marinus*. An index of market oyster (>72 mm shell height) biomass had a strong predictive relationship with annual harvests, but an index of sub-market oysters (<72 mm) was not a good predictor of harvests in subsequent years, probably because of high and variable rates of natural mortality due to parasitic diseases. Relative biomass is a sound indicator for measuring progress towards the oyster restoration goal, and has promising applications in fisheries-related stock assessment.

PUBLIC AND PRIVATE OYSTER RESTORATION IN MARYLAND'S CHESAPEAKE BAY. **C. J. Judy** and **E. Campbell,** Maryland Department of Natural Resources, Shellfish Division, 580 Taylor Avenue, Annapolis, MD 21401.

Oyster restoration is a shared venture between government and the private sector. Maryland oyster projects through the 1990s have been a collaboration between state, federal, and private groups. Projects have focused on the creation of oyster sanctuaries to protect broodstock and enhance benthic community diversity, restoration of habitat; and planting of seed oysters, primarily from hatcheries. In round numbers, the acreages for a cooperative project range from a few to over 10 and the number of oysters planted in a year range from about 10 million to over 50 million total. A wide range of participants constitutes the private sector: environmental groups, non-profit oyster restoration groups, community groups, private citizens, watermen and school groups. The number of projects by such groups has risen dramatically since the early 1990s and encompasses types of projects not normally conducted by State agencies alone. Other projects are more uniquely governmental. The long standing Maryland Department of Natural Resource's seed and shell programs plant about 400 acres of seed and 800 acres of shell per year. The number of oysters planted as seed range between 120 million to over 800 million per year. These projects mostly produce market oysters, but environmental and broodstock benefits accrue from such mass plantings. The Federally funded Reef Program conducted by the State restores oyster populations in sanctuaries using shell and seed resources. Together, public and private entities are working toward improving oyster habitat and oyster populations to improve the industry and the ecological role of oysters.

MUSSEL CULTURE AND COCKLE FISHERIES IN THE NETHERLANDS: FINDING A BALANCE BETWEEN ECONOMY AND ECOLOGY. **P. Kamermans** and **A. C. Smaal,** Netherlands Institute For Fisheries Research, Centre for Shellfish Research, P.O. Box 77, 4400 Ab Yerseke, The Netherlands.

In the Netherlands, mussel seed is fished in a coastal sea in the North of the country (Wadden Sea) and cultured in an estuary the

South (Oosterschelde). Dredging for cockles takes place in the Wadden Sea, and two estuaries in the South (Oosterschelde and Western Scheldt). The Wadden Sea and the Oosterschelde are nature reserves where human activities are possible only when they do not cause negative effects. In 1993, a policy was formulated to ensure preservation of bird populations and restoration of mussel banks and seagrass meadows. As a result of this policy, fishing for mussel seed and cockles is not allowed in areas with a high potential for the development of mussel banks and seagrass fields. The location of these areas is based on GIS models. All vessels are equipped with a black box to control the closed areas. There is some debate about the closure because fishermen have the impression that fishing improves the sediment for settling of mussel larvae. Consumption-sized cockles and mussels are also the preferred prey of oystercatchers and eider ducks. Therefore, the policy makes use of a quotum system in the cockle fisheries. Each year, basin-wide surveys take place to determine the total amount of cockles present. In years when cockle stocks are low an amount is reserved for the birds. Both fishermen and environmentalist question the calculated amounts needed by the birds. An overview of the viewpoints of the interest groups and the role of policy makers and scientists is given.

AN ECONOMIC ANALYSIS OF PUBLIC GROUND OYSTER REEF RESTORATION IN CENTRAL LOUISIANA DAMAGED BY HURRICANE ANDREW.

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In August 1992, Hurricane Andrew heavily damaged Louisiana coastal environments, particularly oyster, *Crassostrea virginica,* reef communities. The transport and transfer of tremendous amounts of sediment and vegetative matter resulted in massive oyster mortalities and extensive reef damage. The Louisiana Department of Wildlife and Fisheries received $5.1 million of federal funds for restoration of oyster habitats on both Louisiana public and private oyster grounds. Of these funds, $3.2 million were used in Terrebonne Parish, the area most severely impacted. Restoration efforts were comprised of sweeping buried reefs and depositing cultch material for oyster reef construction. Some 1,780 acres of waterbottoms were swept by commercial oyster harvesters with bag-less oyster dredges. Mined oyster shells/clam, *Rangia,* shell mixture were deposited (at a rate of approximately 132 cubic yards per acre) on 306 acres of waterbottoms in 1994 (42,576 cubic yards) and 553 acres in 1995 (70,902 cubic yards). Economic benefits associated with a restoration effort of this nature accrue to both oyster consumers and oyster producers. To consumers, the benefits reflect a reduction in price paid for the harvested product, which in turn translates to an increase in willingness to pay relative to what was paid (i.e., consumer surplus). To producers, the benefits reflect an increase in returns to the scarce resource, oyster population, used in the production process (i.e., producer surplus). This study provides an estimate of benefits derived from the restoration efforts and compares these benefits to costs. Overall, the results indicate a favorable benefit to cost ratio.

THE REROUTING OF STORMWATER DISCHARGES FOR WETLANDS ENHANCEMENT, LEVEE PROTECTION, AND OYSTER HABITAT PROTECTION AND RESTORATION.

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The Barataria-Terrebonne estuary is losing over 22 square miles of emergent wetlands each year because of erosion, saltwater intrusion, and natural and anthropogenically-induced subsidence. An extensive levee system has successfully halted overbank flooding of the Mississippi River, eliminating sustaining inputs of sediments and freshwater to the Barataria-Terrebonne estuary. This situation represents not only the imminent loss of a nationally significant wetland resource but also threatens a unique culture, local infrastructure, and the region's significant contribution to the national economy. Runoff from rural and agricultural areas is collected in a borrow canal inside the back levee and then pumped into adjacent wetland areas by a series of stormwater pump stations. Over 250 pump stations currently discharge stormwater, draining approximately 500,000 acres, in the Barataria-Terrebonne estuary. These pump discharges are generally directed into large, human-made canals to ensure that stormwater is quickly evacuated from the leveed area and they often flow directly to high-salinity bays through some of Louisiana's prime oyster growing waters. Redirecting discharges so that they are retained in adjacent wetlands may maintain lower local salinities, provide a sediment source to subsiding wetland areas, and support plant growth, directly benefiting the degrading wetland systems, especially those directly seaward of levees that protect property from storm surges and flooding. Retention of storm water may also produce corollary water quality benefits, such as nutrient uptake and pathogen die-off prior to encountering oyster-growing areas. The Barataria-Terrebonne National Estuary Program is leading an effort to monitor changes at pump station sites in the estuary to demonstrate the benefits of this unique process.

THE SHELLFISH CHALLENGE INITIATIVE: A COOPERATIVE SUCCESS STORY IN THE BARATARIA-TERREBONNE NATIONAL ESTUARY. **K. E. Landrum,** Barataria-Terrebonne National Estuary Program, P.O. Box 2663, Nicholls State University, Thibodaux, LA 70310.

The Shellfish Challenge Initiative is an interagency and interstate effort undertaken to establish progress on the Environmental Protection Agency's Gulf of Mexico Program Shellfish Challenge. With an overall goal of increasing Gulf shellfish beds available for safe harvest by ten percent, more than 200 experts in shellfish management, habitat restoration, and pollution control helped develop 32 shellfish restoration strategies targeting 24 watersheds in the Gulf of Mexico. A watershed implementation initiative was developed within the Barataria-Terrebonne National Estuary resulting in the identification of 61 oyster restoration opportunities, including geographically targeted projects to reduce inputs of fecal coliform bacteria, enhance shellfish habitat, revise shellfish management procedures, and collect and analyze additional needed information to better assess project feasibility. The 61 candidate restoration projects were ranked by members of the Barataria-Terrebonne National Estuary Program Management Conference, and detailed implementation plans were developed for the four selected priority projects. The priority projects included the following: the installation and improved use of marina pumpouts and dump stations; connecting poorly operating individual wastewater treatment systems to community level treatment systems; rerouting stormwater runoff to suitable wetlands; and revising the shellfish relay system. Implementation of the four projects is underway with active educational and interactive workshop components designed for state and local officials and the general public. Funding allocations by local and state government attest to their involvement and acceptance of the implementation process and their agreement to promote active stewardship of an economically important resource and conservation principal.

OYSTER POPULATION RESTORATION IN CARAQUET, N.B.; PHASE I, POPULATION ASSESSMENT. **T. Landry, M. Ouellette,** and **P. Cormier,** Department of Fisheries and Oceans, GFC, P.O. Box 5030, Moncton N.B., E1A 4Y1 and Department of Agriculture, Fisheries and Aquaculture, 22 Boul. Saint-Pierre, Caraquet, N.B. E1W 1B6, Canada.

A decrease in the productivity of oysters in Caraquet Bay, N.B. is generating some interest in restoration projects. The first phase of this initiative is to conduct a quantitative assessment of the distribution, abundance and population structure of the natural beds in this bay, which represents the most northern location with a sustainable oyster *(Crassostrea virginica)* population. The results from 1999 assessment is the fifth of a series of similar exercises conducted in 1974, 1979,1987, and 1991 but the first to use a geostatistical approach to data analysis. A comparison between the two assessment methods reveals that the geostatistical approach is more accurate and of greater use for the next phase of this project, which will look at identifying and characterizing the suitable oyster habitat of this bay for restoration efforts. The comparison among the five assessments over the past three decades is showing that the status of this population is approaching a critical state in terms of recruitment and habitat quality and quantity. The restoration of this oyster population is of great socio-economical and ecological importance to this area.

COMMUNITY-BASED INITIATIVES FOR IMPROVING WATER QUALITY IN SOUTHWESTERN NEW BRUNSWICK, CANADA—AN UPDATE ON SUCCESS. **K. L. LeBlanc,** Eastern Charlotte Waterway Inc., 17 Main Street, St. George, New Brunswick, E5c 3h9, Canada.

The Southwestern New Brunswick Clam Resource Committee (CRC) was formed in 1997 to better understand the importance of the soft-shell clam *(Mya arenaria)* fishery and to prevent further loss of shellfish growing areas due to monitoring cutbacks in Southwestern New Brunswick (NB). Soft-shell clams are the main molluscan shellfish harvested in the region. The committee has over 20 stakeholders that include nonprofit groups, industry and government and is chaired by Eastern Charlotte Waterways Inc. (ECW, a nonprofit group). The mandate of the committee is to preserve the clam fishery in Southwestern NB because of its long-term socio-economic importance to the region. In 1998, the CRC developed the Cooperative Bacterial Monitoring Program (CBMP), which allows industry and community-based contributions for the maintenance and improvement of the classification of growing areas. Under this community-based initiative coastal water samples are collected over a 1,500-km length of shoreline over a two-year period. Presently, 67% of the growing areas are available for the harvest of clams in Southwestern NB during all or selected times of the year, an increase of 32% over 3 years. However, it is important to note that clean-up efforts in growing areas coupled with the CBMP are responsible for the improvements of water quality that have permitted an increase in soft-shell clam harvest. The CRC coordinates clean-up efforts through the Fundy Flats Remediations plan, a program managed under ECW.

APPLICATION OF COMMERCIAL-SCALE OYSTER AQUACULTURE TO REEF RESTORATION. **A. T. Leggett, R. Brumbaugh, W. Goldsborough,** and **A. McDonald,** Chesapeake Bay Foundation, 142 W. York Skeet, Suite 318, Norfolk, VA 23510.

Oyster reef restoration projects in the Chesapeake Bay increasingly involve the addition of broodstock to enhance localized oyster spawning activity. Since 1996, more than 4 million adult oysters have been transplanted onto sanctuary reefs in Maryland and Virginia waters. Volunteers and school students have grown and transplanted a significant number of hatchery-produced oysters, in collaboration with state management agencies. In an effort to increase the numbers of hatchery-produced oysters being transplanted onto sanctuary reefs, the Chesapeake Bay Foundation has initiated a commercial-scale grow out operation in the lower York River with a annual production goal of 1 million adult oysters. Oysters produced by this program will approximately double the number of oysters available for transplanting onto sanctuary reefs in the lower Chesapeake Bay each year. A new cage system was developed in accordance with existing regulations in Virginia governing shellfish aquaculture, and was used to maximize the number of oysters produced over a relatively small acreage of leased oyster grounds. Data on growth and mortality as well as water quality parameters are collected as a routine part of the operation. Early monitoring results showed a 78% increase in size (measured volumetrically) of 4–8 mm seed, and a 63% increase in 8–12 mm seed one week after deployment in mid-July. The oysters produced by the operation will not only be used to enhance broodstock populations on sanctuary reefs, but will also be used as "natural capital" to enticing further public and private investment in oyster restoration.

DOLLARS AND SENSE OF OYSTER RESTORATION: AN EXAMINATION OF NITROGEN REMOVAL BY A RESTORED OYSTER REEF.

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Arguments for the conservation and restoration of oyster reefs, often at the expense of fisheries exploitation, include water quality benefits derived from feeding activities of the oysters and reef-associated fauna. Yet, there has been limited basis for directly evaluating the water quality improvements associated with conservation or restoration of oyster reefs and for comparing those benefits to the economic value derived from oyster fishery production. Using data from oyster populations developing on experimental reefs near the mouth of Chesapeake Bay, we model the nitrogen uptake and release attributable to the oysters and develop nitrogen budgets for the reefs on an area-normalized basis. We then explore the potential effects of fisheries exploitation of these reefs by modeling the harvest of market-sized oysters and examining the effects on nitrogen removal. Finally, we consider the economic returns from oyster harvesting in relation to the costs associated with alternative nitrogen removal. The results give context to water quality benefits to be derived from oyster reef sanctuaries and should help to guide fisheries management decisions related to balancing conservation and exploitation.

REDUCTION IN THE *VIBRIO VULNIFICUS* LOAD OF OYSTERS BY A NOVEL SHORT-TERM COMBINATION BIODEPURATION TREATMENT.

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Based upon an improved method, *Vibrio vulnificus* phage can be maintained and stored at high titer. Further, mass spectroscopic analysis of Gulf coast oysters, clams and shrimp has shown stimulation of a unique anti-molluscan protein that varies in MW 4 to 22 kd and has anti-*Vibrio vulnificus* activity. When the protein and phage are used together, eradication of *Vibrio vulnificus* occurs to more than 8 logs. Analysis by Edman degradation of the 22-kd oyster protein revealed a unique N-terminal 16 amino acid fragment, as did analysis of two cyanogen bromide gel purified fragments. The proteins were not detected in Japanese or Olympia (Washington) oysters (kindly provided by Dr. C. Kaysner, FDA). Finally, a new rapid assay to study the effect of temperature and brief bacterial exposure has been developed, suggesting the possibility that *Vibrio vulnificus* could undergo a non-culturable state under certain conditions (K. Johnston, pers. comm.). Our intention is to isolate the genes expressing the 4- and 22-kd proteins; then express them in large amounts to use with the specific phage in a biodepuration procedure. This work was supported by SK Grant #NA97FD0062 to RBL from NOAA.

COMING SOON TO A RESTORATION SITE NEAR YOU: THE INVADING, PREDATORY ORIENTAL GASTROPOD *RAPANA VENOSA*.

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Rapana venosa Valenciennes 1846 (Neogastropoda, formerly Muricidae, currently Thaididae) is a predatory gastropod native to the Sea of Japan, Yellow Sea, East China Sea, Bohai Sea, and Taiwan. The species has been introduced to the Black Sea, Adriatic Sea, and Aegean Sea, where it is generally considered to be responsible for decimation of local commercially valuable mollusc species. It was first reported in the Chesapeake Bay in 1998. Ballast water transport of larval stages from the eastern Mediterranean or Black Sea is the suspected vector of introduction. To date over 1200 specimens of adult *Rapana* have been collected from Hampton Roads and a limited region of the Southern Chesapeake Bay. Population demographics, records of *Rapana* egg cases in the field and our ability to culture early life history stages at prevailing temperature and salinity strongly suggest active breeding in this receptor location. Temperature and salinity tolerance data for *Rapana* suggest that it can both invade the higher salinity regions of most East Coast estuaries and survive on exposed shorelines from Cape Cod, MA to Charleston, SC. Dispersal is facilitated by pelagic development, and may be exacerbated by ballast water transport of larval stages originating in Hampton Roads. Hard substrate habitat, typical of many current shellfish restoration efforts, appears optimal for post settlement stages, but larger adults may invade soft sediments. Predation has been demonstrated on a range of commercially valuable shellfish species including *Mercenaria mercenaria, Crassostrea virginica, Mya arenaria* and *Mytilus edulis.*

UP CLOSE AND PERSONAL: A SUGGESTED QUANTITATIVE APPROACH TO BROODSTOCK ENHANCEMENT ON SHELLFISH RESTORATION SITES. R. Mann, School of Marine Science, Virginia Institute of Marine Science, College of William and Mary, Gloucester Point, VA 23062.

Shellfish broodstock are typically added to restored habitat to facilitate rapid recruitment by aggregating spawning adults and thus increasing fertilization efficiency. Although this is conceptually attractive there exist few data on which to build quantitative guidelines to optimize the practice. For example, published size versus fecundity relationships for oysters are based on data that has both methodological and size limitations. Similarly, fertilization models are based on sea urchin studies from flow regimes that are arguably quite different from shellfish restoration sites. A quantitative approach is proposed wherein a variety of size-fecundity and fertilization models are proposed for examination in building guidelines to optimize both size and density of placement of shellfish used in broodstock enhancement. The biological and economic aspects of these alternatives are compared.

LINKING PUBLIC AND PRIVATE PARTNERS FOR RESTORATION AQUACULTURE IN MARYLAND'S SEASIDE BAYS. D. W. Webster[1] and D. W. Meritt,[2] [1]University of Maryland, Wye Research & Education Center, PO Box 169, Queenstown MD 21658 and [2]Shellfish Aquaculture Specialist, University of Maryland Center for Environmental Science, Horn Point Lab, Po Box 775, Cambridge, MD 21613.

Traditional harvest of the bard clam (*Mercenaria mercenaria*) in Maryland's seaside bays utilizes the hydraulic escalator dredge. This method has raised concern among environmental groups due to its perceived impact upon eel grass (*Zostera spp.*) in shallow estuarine waters. Meanwhile, an increase in demand for hard clams and strong wholesale prices have caused many clammers who normally harvest softshell clams (*Mya arenaria*) in the Chesapeake Bay to shift harvest to the seaside bays, placing increasing pressure on that area. Hard clam aquaculture is well known and may provide an alternative to harvesters, who will likely come under increasingly restrictive regulations. Differences in culture methods for the clam have evolved in many states to take advantage of local conditions. In Maryland, the clam aquaculture industry has only recently begun, with few persons currently investing in the technology to produce these animals through husbandry. During 1998, investigations were begun with cooperation from university and private companies to develop seed production and evaluate grow out techniques. The Maryland Industrial Partnership (MIPS) program has funded development of a hard clam nursery/grow out operation. University of Maryland Sea Grant Program (UMSG) funded a survey of the hard clam disease QPX to assess background levels of this potential problem. University of Maryland Cooperative Extension (UMCE) funded an extension project to assess grow out techniques, as well as conduct outreach educational programs designed to bring the technology to those who can use it. These cooperative studies are described as well as future directions for the project.

PRODUCTION OF DISEASE-FREE OYSTER SEED USING SHALLOW WATER NURSERIES IN THE MID-CHESAPEAKE BAY. D. W. Meritt and S. Tobash, University of Maryland, Center for Environmental Science, Horn Point Laboratory, Po Box 775, Cambridge, MD 21613.

Recently in Maryland, there has been an emphasis on the production of disease-free oyster spat for use in oyster restoration. Spat produced using traditional methods utilize sites where oyster parasites are common and are typically infected at the time of relay to the grow-out site. As part of the Action Plan for Oyster Recovery in Maryland, the Maryland Oyster Roundtable established oyster recovery zones in several major tributaries of Chesapeake Bay into which only disease-free oysters can be introduced. Given the problem with producing disease-free oyster seed using natural methods, hatcheries have been employed for disease-free seed production. Since 1994, the University of Maryland's Horn Point hatchery has produced over 90 broods of spat using in-water nursery systems. Dermo, the disease caused by the parasite *Perkinsus marinus,* is of greatest concern in these systems due to low salinities. Only one brood of spat has tested positive for Dermo since 1994. Based on trials conducted over the past six years, we have demonstrated that it is not only possible but also likely that uninfected oyster seed can be produced using hatcheries and shallow-water nursery systems. Disease-free seed are being used to test the idea that by prohibiting the movement of parasites into upstream portions of the oyster producing rivers, dermo will be naturally purged from oyster populations in those regions. Early data suggest that there is some validity to this concept.

A MULTIFACTORIAL APPROACH FOR DESCRIBING THE RELATIONSHIP BETWEEN THE CLASSIFICATION OF SHELLFISH HARVESTING WATERS AND ADJACENT LAND USE IN MURRELLS AND NORTH INLET, SOUTH CAROLINA. K. A. Nelson and G. I. Scott, National Ocean Service, 218 Fort Johnson Road, Charleston, SC 29412.

Urbanization poses a particular threat to the coastal areas of the southeastern United State, where the lands surrounding the wetlands are still relatively undeveloped compared with other regions. Fecal coliforms, including *Escherichia coli,* are important indicators of public health since human and/or animal feces may come in contact with and contaminate drinking water supplies or filter-feeding shellfish. The measurement of the concentration of fecal coliforms is the current criterion for deciding when and if shellfish harvesting should be approved. Predictive models that would correlate information on land use change and development would be useful so that downgrades in water quality can be predicted before they occur. The approach used for this study involved an historical comparison of land use change and fecal coliform bacterial densities on Murrells Inlet (MI) (urbanized site) and North Inlet (NI) (pristine site). Both MI and NI are bar-built estuaries are located on the northern coast of South Carolina near Myrtle Beach. The microbiological and water quality data used in this research covers the period of 1967–1995 and the following parameters were utilized: date of sampling, most probable number of fecal coliform bacteria, salinity, rainfall, and water temperature. The regressions models utilized the above parameters and a change in trend term that accounted for both instantaneous and gradual changes in water quality that may arise from a particular intervention. For MI, the 1980 intervention consisted of both the construction of a jetty and the conversion from septic tanks to a main sewer line. For NI, the 1973 intervention was the construction of Baruch Laboratory. For MI, the intervention, controlling for other parameters, was found to be significant at the alpha = 0.05 level. This means that there was a significant decrease in the increasing trend of bacteria for MI and that the conversion to the sewage line had a beneficial effect on water quality and probably dominated the jetty effect. For NI, the laboratory construction had no impact on water quality so background natural sources of bacteria probably dominated the small increase from human sources. These findings indicate that the use of Intervention Analysis may provide coastal managers with an effective process to evaluate landscape changes on bacterial water quality in shellfish harvesting areas.

INFLUENCE OF OYSTER REEF STRUCTURE ON FISH ASSEMBLAGES: DOES THE PLACEMENT OF ARTIFICIAL SUBSTRATE ENHANCE FISH POPULATIONS? **J. A. Nestlerode,[1] M. W. Luckenbach,[2] P. G. Ross,[2]** and **F. X. O'Beirn,[2]** [1]Virginia Institute of Marine Science, College of William & Mary, Gloucester Point, VA 23062, [2]Virginia Institute of Marine Science, College of William & Mary, Wachapreague, VA 23480.

The objectives of oyster reef habitat restoration within Chesapeake Bay are not only the enhancement of oyster stocks but also to restore the physical structure and ecological function of these systems. We revisit the artificial reef fisheries attraction vs. production issue by comparing transient nekton community structure among reef structures constructed of different substrates. The substrate materials (oyster shell, surf clam shell, and pelletized coal ash) used in this study offer the opportunity to examine how habitats with various degrees of structural complexity contribute to differences in habitat use in terms of microhabitat availability, predation risk, and diversity and abundance of prey. Results indicate that oyster shell reefs, which have best supported the development of an oyster population, offer the highest degree of structural complexity and support a more diverse community of both resident and transient nekton. Furthermore, increased availability of nesting sites (empty articulated oyster shells) and a high abundance of benthic prey items support the thesis of increased fish production rather than simply attracting fish to the reef structure from nearby habitats. The patterns observed here provide evidence that proper reef architecture and subsequent reef community development lead to increased finfish production and should give context to the importance of substrate selection in similar restoration activities.

SHELLFISH RESTORATION IN IRELAND: THE NEED FOR NOVEL PARTNERSHIPS. **M. Norman,** Taighde Mara Teoranta (Marine Research Limited), Carna Co., Galway Ireland.

Historically shellfish restoration projects in Ireland have been undertaken for a singular reason and driven by a single user group. Frequently, the goal has been increased commercial production of shellfish, and the restoration has been pursued by a grouping of fishermen or aquaculturists. These projects have a narrowly defined objective, profitability, and frequently "fail" when they do not achieve this. However, some recent restoration attempts have been performed by partnerships. These projects are driven by multiple user groups and thus have a range of goals. It is postulated that this partnership approach has more chance of success as the partners support each other in "staying the course" and as the goals are broader, making success more achievable.

REPRODUCTIVE BIOLOGY OF THE NORTHERN QUAHAUG, *MERCENARIA MERCENARIA*, IN PRINCE EDWARD ISLAND, CANADA. **M. Ouellette, M. Hardy, T. Landry, N. G. MacNair,** and **A. Boghen,** Department of Fisheries and Oceans, Science Branch, Gulf Fisheries Centre, P.O. Box 5030, Moncton, New Brunswick, E1C 9B6; Department De Biologie, Universite De Moncton, Moncton, Nouveau-Brunswick, E1A 3E9; and Department of Fisheries and Tourism, PO Box 2000, Charlottetown, Prince Edward Island, C1A 7N8, Canada.

The northern quahaug, *Mercenaria mercenaria*, is an important species for both the commercial and recreational fisheries as well as for aquaculture purposes in Prince Edward Island. The management strategy of the quahaug resource is largely based on the minimum legal size of 50 mm. At the same time, there is a growing concern regarding the sustainability of the clam industry and hence, an evolving interest in stock enhancement. Effective broodstock management, however, requires basic information about the animal's reproductive biology. Sexual maturity, ovocyte size, gonado-somatic ratios, and time of spawning were established for quahaugs sampled from two sites in West River, PEI. Histological methods and physiological condition indices are used to determine of the spawning activities. Findings revealed that the minimum size at sexual maturity was 25 mm and 30 mm (shell length) for males and females respectively. Furthermore, there was a positive correlation between ovocyte size and shell length. Seasonal variation coincided with spawning predictions based on conventional physiological condition indices. As well, the gonado-somatic contribution increased as a function of length. Both histological and condition index data support the likelihood that a major spawn occurs in mid-June. The study provides useful information on the reproductive biology of *M. mercenaria* and could contribute towards a reassessment of existing management and grow-out strategies. Establishment of reproductive sanctuaries is also being investigated as a method to increase the annual recruitment success in this study bay.

CONSIDERATIONS FOR OYSTER RESTORATION IN MARYLAND: DISEASE, GENETICS, DENSITY, REPRODUCTION, AND HABITAT CREATION. **K. R. Paynter, Jr.,** Department of Biology, University of Maryland, College Park, MD 20742.

Over the last 2 years, we have conducted numerous experiments and monitored several State and Federally funded restoration projects in the Maryland portion of Chesapeake Bay. A summary of the results of these activities will be presented. Field experiments have revealed that oyster seed cohorts from different broodstocks appear to have differing resistances to disease. Videographic observations from the field have shown that high-density oyster plantings result in significant community enhancement leading to diverse benthic ecosystems. Laboratory studies have shown that benthic fishes such as gobies and blennies prefer natural clumps of oyster shell compared to equal volumes of loose oyster shell. In addition, other laboratory studies have shown that eggs introduced into the water column more than a few centimeters from introduced sperm will have little chance of becoming fertilized. However, other studies have shown that high densities of oysters ($>400/m^2$) may result in deleterious effects on oysters themselves. Many aspects of oysters and their ecosystem must be considered when planning restoration projects. Those projects seeking to restore ecological function should bear in mind the complex relationships between oysters, the habitat they create as biogenic reef builders, and the water column in which they reside.

CULTURE TECHNIQUES APPLIED TO WILD BIVALVE BEDS IN GALICIA, NW SPAIN. **P. J. Pazo,** Delegación Territorial Conselleria De Pesca C/ Palma 4. 36202 Vigo Spain.

The region of Galicia is located in NW Spain. It has a coastline of 1,195 km. Galicia is the first producer of molluscs in Spain, taking advantage of natural oceanographic conditions: a seasonal upwelling and existence of positive estuary bays (Rias). Molluscs have been exploited in Galicia since prehistoric times. The present shellfishing situation is developed in two ways: the gathering of molluscs on foot, raking the substratum for macroinfaunal bivalves in the intertidal belt. The other type of shellfishing exploits the subtidal molluscs beds and involves the use of small boats. In order to maintain and enhance bivalve production in intertidal wild beds, a series of culture techniques are applied, acting on the bivalve population (lowering high densities, enlarging area beds, sowing and repopulating new areas), fighting against predators and competitors (starfishes, drilling gastropods), removing green algae of the bed surface to avoid deleterious effects in young bivalves, changing substrate granulometry by adding coarse sand to areas with mud and silt condition, and by other means. To act on the recruitment problems of two very valued species: butterfish clam (*Ruditapes decussata*) and European flat oyster (*Ostrea edulis*), a plan was established by the Fisheries Department of Galician Regional Government: "Plan Galicia". This Plan began in 1997 and was aimed to gradually transform traditional intertidal shellfish gathering into a professional activity by enhancing both the intertidal wild molluscan beds and the social organization of the mainly female population of shellfish gatherers. This Plan is presently developing and relevant achievements are being achieved, mainly in the social area.

THE INFLUENCE OF ENVIRONMENTAL FACTORS IN JAPANESE OYSTERS HEALTH CONDITION CULTIVATED IN THE SOUTH OF PORTUGAL. **A. L. Pereira,[1] F. A. Ruano,[1] L. Chicharo,[2] and D. Matias,[3]** [1]IPIMAR, Research Institute for Fisheries and Sea, Av. De Brasilia 1449-006 Lisbon, Portugal; [2]UCTRA, Algarve University, Campus De Gambelas, 8000-062 Faro, Portugal; and [3]CRIPsul, South Research Center of Ipimar, Av. 5 De Outubro, 8500 Olhao, Portugal.

The effect of environmental parameters as well as the organic contamination, heavy metals and tributytlin (TBT) upon the development of diseases in cultivated Japanese oysters *Crassostrea gigas* (Thunberg, 1793) was studied over 6 months. The results were also related with the condition index, growth and mortality rates. The study was performed in two different sites on a coastal lagoon in the South of Portugal. One site ("Elisamar") is located in a clean area whereas the other (Olhao), due to its proximity to urban areas and to an important fishing harbour, is exposed to higher contamination levels. In Olhao, nosological examinations showed the higher levels of lesions and the greatest incidence of parasites. Two ciliates, *Ancistrum* sp. and *Trichodina* sp., were the most abundant at the two areas. The intensity of the infections and the lesional picture observed at the two sites, didn't seem to affect significantly the condition, growth and mortality of the studied animals. However, the differences in the infection intensity registered in both sites could be an indicator of a lack of defensive response from the individuals submitted to more intense stressful conditions.

THE "EEEOHM" (EASTFIELDS' ENVIRONMENT ENHANCING OYSTER HOLDING MODULE). **P. Perina** and **D. Perina,** Eastfields Farms, Box 275, Mathews, VA 23109.

The "EEEOHM" (Eastfields' Environment Enhancing Oyster Holding Module) was originally developed to be used commercially, but the system is extremely versatile and is adaptable to a small garden size operation. The emphasis in developing the EEEOHM was on "Keep It Simple, Keep It Cheap." The EEEOHM module consists of three ADPI square-sided oyster cages (also called bags). Each cage has attached four 2-liter soda bottles for flotation. The cages are strung together with a 13-foot piece of crab pot rope or clothes line running laterally through their centers. The modules can also be separated into single floats as the needs of the grower dictate. The reason for using just three cages per module is simply "ease of handling". Whether employing the system from a dock or a skiff, it's a lot easier to detach and lift just three cages at a time than to struggle with maybe 40 or more cages all attached to a single rope. No heavy lifting. The EEEOHM can be floated under or along side of a dock, tied between posts, or deployed in rows secured to two parallel ropes anchored to the bottom at each end. The latter usually requires the use of a skiff. During the last 14 years, we at Eastfields have tried many ways of growing oysters. We've found the EEEOHM to be one of the most efficient and cost effective systems of off bottom culture. The ADPI cages last for many years, and the soda bottles are free. We at Eastfields Farms are proud to have developed the EEEOHM and would appreciate the opportunity to answer any questions concerning this environment enhancing system.

A BIOCHEMICALLY-BASED MODEL OF THE GROWTH AND DEVELOPMENT OF *CRASSOSTREA GIGAS* LARVAE. E. A. Bochenek,[1] E. N. Powell,[1] J. M. Klinck,[2] and E. E. Hofmann,[2] [1]Haskin Shellfish Research Laboratory, Rutgers University, Port Norris, NJ 08349 and [2]CCPO, Old Dominion University, Norfolk, VA 23529.

A biochemically-based model was developed to simulate the growth, development, and metamorphosis of larvae of the Pacific oyster, *Crassostrea gigas*. This model, which is the first of its type, defines larvae in terms of their gross biochemical composition: protein, neutral lipid, polar lipid, carbohydrate, and ash content. The model includes parameterizations for larval filtration, ingestion, and respiration, which determine growth rate, and processes controlling larval mortality and metamorphosis. Changes in the initial ratios of protein, carbohydrate, neutral lipid, and polar lipid occur as the larva grows and in response to the biochemical composition of available food. The model results show increased larval survival when low protein food sources are available. High-protein food sources result in insufficient carbohydrate and neutral lipid to cover metabolic costs and to permit successful metamorphosis. The result is larvae that are unable to successfully complete metamorphosis. Thus, food quality as well as food quantity appear to be primary controls on the ability of *Crassostrea gigas* larvae to reach the body condition needed for metamorphosis. Other simulations show that initial egg size (lipid content) controls the ability of the larva to sustain itself until it reaches a size where it can effectively filter and assimilate food. Large eggs produce larvae that are more able to withstand food-poor environments, suggesting that egg size variability may account for the range of larval sizes at which metamorphosis is attempted.

A FISHERIES MODEL FOR MANAGING THE OYSTER FISHERY DURING TIMES OF DISEASE. J. M. Klinck,[1] E. N. Powell,[2] J. N. Kraeuter,[2] and S. E. Ford,[2] [1]CCPO, Old Dominion University, Norfolk, Virginia 23529 and [2]Haskin Shellfish Research Laboratory, Rutgers University, Port Norris, NJ 08349.

In Delaware Bay, market-size oysters have been produced by moving oysters from the seedbeds to planted grounds in higher salinity, where oysters increased in size and meat condition. In 1989, the oyster disease Dermo became active on the planted grounds and lower seedbeds. The increase in disease reduced the desirability (loss of shell resource from seed areas) and profitability (high mortality) for moving oysters into higher salinity areas. As such, oyster production has focused on two production schemes: (1) direct harvest of oysters produced on the lower seedbeds, essentially a wild fishery and (2) the transplantation of oysters from the mid-estuary seed beds to the lower estuary seedbeds. Transplanted oysters are then harvested after the meat condition improves. One of the consequences of these approaches is the need to estimate the allowable production from the seedbeds each year, which is equivalent to setting a yearly quota. It seems clear that present oyster populations are below pre-disease levels, and that continued high disease levels will prevent recovery to pre-disease levels. The presentation describes a model developed for the management of fished oyster populations that lie over a salinity gradient and for which disease mortality is a controlling influence. We will present a review of the Delaware Bay stock assessment for 1998 and 1999. We will then describe a model developed to address management issues when B_{msy} and K are not appropriate options and apply it to Delaware Bay oyster populations.

SHELLFISH DATA MANAGEMENT AND REPORTING SYSTEM (SDMRS). J. Power[1] and D. B. Walker,[2] [1]Environment Canada, 224 West Esplanade, North Vancouver, British Columbia, V7M 3H7, Canada, and [2]E-Carta Services, 419 N 18th Ave. E, Duluth, MN 55812-1352.

The objective of this work was to design a user-friendly interface to digital watershed maps and hydrographic charts, thematic layers such as clam harvesting areas, farms, shellfish leases, and closures, and databases relating to pollution sources, water quality sampling, shellfish growing areas, locations of marine mammals and seabirds, fish farms and weather, for Canada's West Coast. Such an interface will allow scientists in the shellfish program of Environment Canada, Pacific and Yukon Region to readily generate data reports including maps detailing shellfish closures and shoreline assessments. The Shellfish Data Management and Reporting System (SDMRS) is an ArcView GIS application linked to an Access database, which requires no special knowledge of SQL queries of databases or GIS. From ArcView the user chooses the sector to be mapped and the additional area around the sector to display map information from drop down menus. The system then generates the appropriate base map by clipping out the underlying hydrographic charts and/or watershed maps. Then the user selects the themes and data sets to add to the map, again by choosing from drop down menus. The system adds the themes, uses ODBC to query the database and retrieve the appropriate data sets, converts them to the proper projection, and adds them to the map. When completed the map is then exported to the report document.

RESTORING OYSTER REEFS FOR FISH: ESTIMATING ENHANCED SECONDARY PRODUCTION OF RESTORED OYSTER REEFS.

S. P. Powers, C. H. Peterson, and **J. H. Grabowski,** Institute of Marine Sciences, University of North Carolina at Chapel Hill, 3431 Arendell Street. Morehead City. NC 28557.

The recognition that oyster reefs provide an important resource, but also provide habitat for a variety of other species, has encouraged restoration of oyster reefs as a method to enhance production of finfish and shellfish within estuaries. Here, we describe an approach for determining the augmented secondary production of bottom areas that were sand/mud flats and restored to oyster reefs. First, through a review of published and unpublished studies, we compared densities of animals on oyster reefs to nearby areas without biogenic structure. We divided those species that showed enhanced densities on reefs into three groups: (1) species that recruited exclusively to reefs, (2) species that had higher recruitment to reefs, but still recruited and used non-structured habitats, and (3) species that showed higher aggregations around reefs, but were not limited by reef habitat based on diet and life-history analyses. For this first group, all production is attributed to the reef. Because some proportion of the enhanced density of species belonging to this second group would probably have recruited to other habitat, the production attributed to the reef is adjusted by a coefficient of reef-habitat exclusivity (CRE) that we developed using diet analysis and life history information. For the final group of animals, the reef only receives credit for that fraction of growth that is enhanced by the presence of the reef, this determination is made through the application of a CRE. Applying this approach to proposed oyster reef restoration in Tampa Bay, FL, we estimated the augmented secondary production of 10 m^2 of oyster reef to be 2.57 kg y^{-1}.

RESTORING THE LITTLENECK CLAM RESOURCE FOR NATIVE AMERICAN SUBSISTENCE USE IN THE PRINCE WILLIAM SOUND, ALASKA.

R. RaLonde, University of Alaska, School of Fisheries and Ocean Sciences, Anchorage, AK 99508-4140.

Natural and man caused disasters decimated the littleneck clam (*Protothaca staminea*) populations on the intertidal beaches of Prince William Sound. Alaska. Subsequently, Native American Villages have been unable to harvest clams to meet their subsistence needs. Since 1995, the Quteckak Native Corporation has been actively pursuing restoration of the clam populations by developing seed production technology, conducting site selection studies, and managing growout trials. The initial success of their restoration efforts now enables communities to harvest clams from the restored beaches and broaden the program. This presentation will describe the research and restoration results of the project and the human impact of the restored resource on the Native Villages of Prince William Sound, Alaska.

DEVELOPMENT OF *CRASSOSTREA VIRGINICA* MICROSATELLITE MARKERS FOR A GENETIC LINKAGE MAP AND GENETIC MONITORING OF RESTORATION PROJECTS.

K. M. Reece, W. L. Ribeiro, K. L. Hudson, and **S. K. Allen Jr.,** Virginia Institute of Marine Science, The College of William and Mary, Gloucester Point, VA 23062.

Dermo and MSX have had significant impacts on natural populations of the eastern oyster *Crassostrea virginica* and have been a detriment to oyster aquaculture development. A potential solution to this problem is genetically improved disease-resistant strains of *C. virginica* that can grow to market size despite disease challenge. Traditional selective breeding programs have resulted in strains of oysters that are being assessed for disease resistance. One means of accelerating selective breeding programs is to identify genetic markers associated with traits such as disease resistance or growth rate. A goal of this project is to develop genetic markers for constructing a linkage map and to identify markers associated with disease resistance to use in marker-assisted selection programs. In addition, markers developed in the genomics project are being surveyed for use in genetic monitoring of reef restoration projects. To assess relative genetic contributions of wild and planted stocks to restored reefs; markers are being identified that are able to genetically distinguish selected strains and natural populations in Chesapeake Bay. *C. virginica* microsatellite markers are being developed in an ODRP funded genome mapping project. Several di-, tri-, and tetranucleotide repeat sequences have been identified. Primers for use in the polymerase chain reaction have been designed to anneal to regions flanking 39 microsatellites and amplification reactions for 21 loci have been optimized. F1 individuals from four reference families have been screened at twelve microsatellite loci for generating a genetic linkage map. Microsatellite allelic profiles of selected strains and natural populations are being examined.

OYSTER REEF RESTORATION RESEARCH IN MOBILE BAY, ALABAMA.

D. B. Rouse,[1] **R. K. Wallace,**[2] and **F. S. Rikard,**[2] [1]Department of Fisheries and Allied Aquacultures, Auburn University, Auburn, AL 36849 and [2]Mobile, AL 36615.

Oyster reef restoration in Mobile Bay has consisted primarily of shell planting on active reefs in the lower sections of the bay.

Efforts are now underway to restore reefs in the mid-bay area. Studies are being conducted to determine why these reefs are no longer productive and what should be done to restore them to a productive state. Bottom surveys were performed to quantify cultch availability. Sediment traps were deployed to determine rates of sediment accretion and spat collectors were used to evaluate natural oyster set. Spat were deployed on the bottom and on platforms 20 cm and 40 cm above bottom. Data loggers were deployed on bottom and 40 cm above bottom to measure temperature, salinity and oxygen concentration. Surveys revealed hard bottoms but little exposed cultch on non-productive reefs. Sedimentation was high and consisted mainly of silt with more than 10% organic matter. Single peak oyster sets occurred in the fall. Oysters at the three experimental levels grew to approximately 60 mm in the first year. During the second year, total mortality was observed at all three levels when oxygen levels dropped to 0 mg/L for 5 consecutive days. Similar periodic low oxygen events may be occurring at the study site and on other relic reefs that will hinder their successful restoration. Water quality studies suggest that cultch mounding will be necessary to elevate oysters above anoxic bottom conditions.

TRANSPLANTING BROODSTOCK OYSTERS, *CRASSOSTREA VIRGINICA*, ONTO RECONSTRUCTED OYSTER REEFS TO INCREASE SPAT RECRUITMENT IN THE PIANKATANK RIVER. **D. C. Sherwood,** Sandston, VA 23150.

Virginia oyster reef restoration in the form of three-dimensional structures began in the Piankatank River, Virginia in 1993. From 1993 to 1998, fifteen artificial reefs were built in tributaries of the Chesapeake Bay. In December 1996, reproductively active broodstock oyster from Tangier and Pocomoke Sounds were transplanted to the Shell Bar reef in the Great Wicomico River, Virginia. Surveys in the fall of 1997 indicated a high spat recruitment both on the reefs and nearby oyster bars. With this success, broodstock oysters from Tangier and Pocomoke Sounds were transplanted in December 1997 to two of the four reconstructed reefs in the Piankatank River. Since total quantity as well as density of broodstock was believed to be limiting factors for recruitment in this river, adding stock was expected to raise spat recruitment. Spat recruitment data was collected via dive surveys on the reefs, and via dredge and patent tong surveys on the natural oyster bars. The recruitment of spat to both reefs and bars was significantly higher in 1998 ($P < 0.001$) than in the previous four years and a positive interaction ($P < 0.005$) was seen between the reefs and the year 1998. Based on these data, stock enhancements in the Piankatank River successfully improved recruitment and suggest oyster restoration may be facilitated in other areas of the Chesapeake Bay by strategic enhancement of spawning stocks.

HARMFUL ALGAL BLOOMS AND SHELLFISH RESTORATION: CAN THEY CO-EXIST? **S. E. Shumway,** Department of Marine Sciences, University of Connecticut, 1080 Shennecossett Road, Groton, CT 06340

Habitat quality is an obvious and important consideration for all shellfish restoration efforts including aquaculture and reseeding. Harmful algal blooms (HABs), worldwide threats to habitat quality, are naturally occurring phenomena and their number and frequency are increasing. These blooms impact ecosystem integrity, species interactions, aquatic animal health, population growth, human health, economy, industry, and ecology and often pose a threat to wild and cultured shellfish populations. While algal species that impact human health receive the most attention, there are numerous HABs that cause the destruction and demise of shellfish beds and aquaculture operations. These HABs can also dictate the successful citing of restoration efforts. This presentation will review our knowledge of harmful algal-shellfish interactions worldwide including threats associated with the presence of recently identified problem species, e.g., *Pfiesteria* spp. and *Heterocapsa*, and discuss ways in which shellfish restoration efforts may be undertaken successfully in the face of these imposing threats. Mitigation of these HABs has become an important focus for coastal research. Data will be presented on recent studies on the use of clay as a means of alleviating the impacts of HABs. Careful management-science interaction, not eradication, remains the only safe and functional means of mitigation currently available.

A COMPARISON OF TWO OYSTER (*CRASSOSTREA VIRGINICA*) STRAINS FOR PRODUCTIVITY AND SUITABILITY FOR USE IN OYSTER REEF RESTORATION EFFORTS. **L. A. Sorabella,**[1] **M. W. Luckenbach,**[2] and **F. X. O'Beirn,**[2] Virginia Institute of Marine Science, College of William and Mary, P.O. Box 1346, Gloucester Point, VA 23062, Virginia Institute of Marine Science, College of William and Mary, P.O. Box 350, Wachapreague, VA 23480.

Over the past 5 years, eastern oyster (*Crassostrea virginica*) restoration efforts in Virginia have focused on constructing reef structures to act as sanctuaries. Increasingly, shell plants are stocked with hatchery-produced brood stock oysters that spawn and increase recruitment to the reefs. This involves rearing hatchery-produced seed oysters in floating containers and out-planting them onto reefs when they reach an appropriate size (20–30 mm). To assist with the labor involved in this process, we recruited citizen and student oyster-gardeners who raised the bulk of the oysters for transplant onto the sanctuaries. Two strains were hatchery-reared for transplantation onto these reefs: CROSBreed oysters and wild-caught oysters from the lower Chesapeake Bay. The CROSBreed strain has been selectively bred since 1962 for resistance to the parasite Haplosporiduium nelsoni that causes MSX, and has more recently been bred for resistance to Perkinsus marinus, that causes Dermo. The second strain were large wild-caught oysters collected from the Lynnhaven River (Chesapeake Bay, VA) where the oysters were surviving under presumed high pressure from both parasites. The objective of this research is to evaluate the performance of the CROSBreed stock and wild stock oysters deployed on sanctuary reefs in the Lafayette River (Chesapeake Bay, VA). Evaluation compares the two strains based on female fecundity, growth, survival, and incidence of Perkinsus marinus and Haplosporiduium nelsoni infection measurements. As large-scale restoration projects proceed, it will be fundamentally important to assess which stock is most appropriate for use in the oyster restoration effort.

OYSTER RESTORATION AND THE UNIVERSITY OF MARYLAND: INTERACTIONS BETWEEN RESEARCH, INDUSTRY, AND THE PUBLIC.

S. M. Tobash and **D. W. Meritt**, University of Maryland, Center for Environmental Science, Horn Point Laboratory, PO Box 775, Cambridge, MD 21613.

Maryland oyster populations have been declining for many years. The University of Maryland is committed in its involvement to assist in restoration programs, placing an emphasis on the ecological contribution that oysters provide to the overall health of the Chesapeake Bay. Research efforts are focused on diseases, genetics, culturing techniques and basic oyster biology and ecology. The efforts of the Maryland Oyster Recovery Partnership and other concerned groups, along with increasing public involvement has enabled the oyster hatchery at the University of Maryland Center for Environmental Science Horn Point Laboratory to increase production of disease-free hatchery seed. These seed oysters are then used in a variety of restoration, education, and outreach activities statewide. Educational programs are targeted to a wide audience, ranging from nonprofit organizations of school-aged children to commercial watermen. Outreach projects promote the involvement of concerned citizens like the Chesapeake Bay Foundation's Oyster Gardening Program. Cooperation between commercial fishermen, management agencies, and concerned groups and combination of resources are key to a successful restoration program. The University of Maryland represents an important link in this process. The ongoing commitment of UMCES Horn Point Laboratory together with cooperative programs will continue to strengthen the future of the Chesapeake Bay oyster restoration effort.

EFFECTS OF WATERSHED ALTERATIONS ON OYSTER POPULATIONS IN SOUTHWEST FLORIDA ESTUARIES: AN ECOLOGICAL APPROACH.

A. K. Volety, **M. Savarese**, and **S. G. Tolley**, College of Arts and Science, Florida Gulf Coast University, 10501 Fgcu Boulevard South, Fort Myers, FL 33965.

Southwest Florida is one of the country's fastest growing regions. Consequently, watersheds are heavily managed to accommodate development. Studies on the effects of altered watershed in this region involving valued ecosystem components, like 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. Measurements of oyster spatial distribution, condition index, spat recruitment, energy reserves, and disease prevalence of *Perkinsus marinus* are underway using a "spatial homologue approach" (among-estuary comparisons at hydrologically and geomorphically similar locations along the salinity gradient). Preliminary results indicate that in summer months, depending on the location, mean prevalence of P. *marinus* infection in oysters varied between 33–73%, whereas the mean condition index varied between 2.4–4.7. The distribution of reefs, regions of maximum living density, and maximum oyster productivity are shifted seaward in altered relative to pristine systems when water management practices are supplying excessive freshwater to estuaries. These populations, however, exhibit lower *P. marinus* prevalence because of the greater freshwater influence. This project represents the first study of watershed alteration on oysters in Southwest Florida and will help provide target environmental conditions for restoration efforts.

THE VIRGINIA OYSTER HERITAGE PROGRAM.

J. A. Wesson[1] and **L. B. McKay**,[2] [1]Virginia Marine Resources Commission, 2600 Washington Ave., 3rd Floor, Newport News, VA 23607-0756 and [2]Virginia Department of Environmental Quality, 629 E. Main St., Richmond, VA 23219.

The Virginia Oyster Heritage Program (VOHP) is a bold initiative to capitalize on recent advances and consensus on strategies for oyster restoration. The VOHP seeks to restore oyster populations and oyster reef habitat, and to, thereby, improve ecological function, water quality and the oyster industry in Virginia's portion of Chesapeake Bay and its seaside bays. Initiated in 1999 by the Department of Environmental Quality and Marine Resources Commission, the VOHP is a partnership of Federal, State, and private entities. Phase One focuses on the Lower Rappahannock River, with a goal of rebuilding 8 to 10, three-dimensional, oyster sanctuary reefs and restoration of more than 200 acres of oyster beds for direct harvest. The combination of funding from all sources totaled more than $1,500,000 in the first year, resulting in six reefs constructed, and 85 acres of harvest area restored. The Lower Rappahannock River, closed to harvest for six years, is an area of high salinity with consistent exposure to oyster diseases. Annual monitoring during this closure demonstrated that a significant proportion of the native oysters survived and grew, and that spatset became dependable. We believe that the lack of cultch has limited the increase in oyster populations and that enough natural broodstock has accumulated to colonize these restored areas. With proper management of sanctuary and harvest areas, we also believe that a sustainable fishery can be established while simultaneously increasing the standing stock of oysters. We anticipate comparable funding in 2001, and the expansion of this model into other areas.

ENHANCING AND SUSTAINING NORTH SHORE SHELLFISHING THROUGH AQUACULTURE. **J. J. Whitten**, Merrimack Valley Planning Commission, 160 Main Street, Haverhill, MA 01830.

Soft-shell clamming has long been a vital commercial fishery on the North Shore of Massachusetts. Earlier in the century, the Merrimack River Estuary alone produced up to 100,000 bushels of soft-shell clams per year (among the highest of shellfish harvesting communities on the East Coast). During the past decade, the fishery has seen significant declines and wide fluctuations in productivity. Landings have plummeted as much as 80% along the North Shore. The dramatic downturn in this once prominent industry is due to a combination of factors such as: natural "boom-and-bust" reproduction cycles, predation from non-native species such as the green crab, over harvesting, as well as regulatory closures of previously undocumented contaminated areas. The wide, unpredictable fluctuations in bed productivity make it impossible to ensure a sustainable and predictable harvest. The seeding of Massachusetts' North Shore shellfish beds with hatchery-reared soft-shell clams offers the potential to moderate this cyclical and declining nature of productivity. Efforts to improve shellfishing through restoration/enhancement efforts in other states have demonstrated several methods by which a more consistent and sustainable harvest can be achieved. This presentation examines regional efforts conducted by the Merrimack Valley Planning Commission (MVPC) in collaboration with the coastal communities of Gloucester, Ipswich and Rowley Massachusetts to enhance and sustain soft-shell clam (*Mya arenaria*) populations. Ultimately a more sustainable harvest provides more secure employment opportunities for shellfishermen as well as other marine-based job opportunities.

COMMUNITY-BASED OYSTER RESTORATION IN AN URBAN ESTUARY: DEVELOPING AN OYSTER CULTURE AND REEF RESTORATION PROGRAM IN THE HUDSON-RARITAN ESTUARY. **A. Willner**, NY/NJ Baykeeper, Building 18, Sandy Hook, Highlands, NJ 07732.

Goals, progress, and challenges to development of a restoration program led by a nonprofit organization in an urban estuary will be discussed. The Hudson-Raritan Estuary supports a sparse oyster population that has collapsed since the beginning of the 1900s. Major natural oyster beds were exhausted by commercial shellfishing in the early 1800's, since the closure of the industry in the 1920s the population has continued to decline to the current state of small groupings of individuals located in disparate reaches of the system. Prompted by the success of oyster restoration in other estuaries, NY/NJ Baykeeper, with guidance from a scientific advisory board, has begun to investigate the feasibility of restoring reefs to provide habitat for larval settlement and population recovery. Spat surveys conducted in 1998 demonstrating minimal recruitment, lead to the 1999 experiment of placing a large amount of shell on the historic footprint of an oyster population in New York Harbor to provide a greater area to increase probability of recruitment. Preliminary results demonstrate the potential for reef construction to increase oyster recruitment. To stock the reef in an effort to further improve recruitment success, a community-based oyster-culturing program has recently been established involving schools, marinas, civic groups and families. Oyster restoration in estuaries with pollution concerns such as this provide the opportunity to investigate factors affecting population recruitment and maintenance as well as challenges to public education and outreach in urban areas.

THE NATIONAL SHELLFISHERIES ASSOCIATION

The National Shellfisheries Association (NSA) is an international organization of scientists, management officials and members of industry that is deeply concerned and dedicated to the formulation of ideas and promotion of knowledge pertinent to the biology, ecology, production, economics and management of shellfish resources. The Association has a membership of more than 1000 from all parts of the USA, Canada and 18 other nations; the Association strongly encourages graduate students' membership and participation.

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Sergio Ragonese, Patrizia Jereb, and Earl Dawe
A comparison of growth performance across the squid genus *Illex* (Cephalopoda, ommastrephidae) based on modelling weight-at-length and age data 851
Chandrika Liyana-Pathirana, Fereidoon Shahidi, and Alan Whittick
Comparison of nutrient composition of gonads and coelomic fluid of green sea urchin *Strongylocentrotus droebachiensis* 861
B. R. Moore, S. N. Kleeman, and R. J. G. Lester
The development of a positive non-infectious control for the detection of *Perkinsus* using the Ray test 871
S. J. Nichols and D. Garling
Evaluation of substitute diets for live algae in the captive maintenance of adult and subadult freshwater unionidae 875
Abstracts of technical papers presented at the 4th International Conference on Shellfish Restoration, Hilton Head Island, South Carolina, November 15–18, 2000 883

COVER PHOTO: Red crab (*Geryon quinque dens*) are found in deep water off of the coast of North America. These crabs were caught 140 miles south of New England aboard the *Diamond Girl.* Photo by Richard W. Dionne, Jr. with special thanks to the Sakonnet Times.

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Patrick Rice, Sammy M. Ray, Sherry D. Painter, and Gregg T. Nagle
An intrinsic membrane protein in oyster sperm stimulates spawning behaviors in *Crassostrea virginica:* implications for aquaculture 715

Qiaoxiang Dong, Benoit Eudeline, Standish K. Allen, Jr., and Terrence R. Tiersch
Factors affecting sperm motility of tetraploid Pacific oysters 719

Amy D. Nickens, Jerome F. La Peyre, Eric S. Wagner, and Terrence R. Tiersch
An improved procedure to count *Perkinsus marinus* in Eastern oyster hemolymph 725

Stephen J. Jordan, Kelly N. Greenhawk, Carol B. McCollough, Jessica Vanisko, and Mark L. Homer
Oyster biomass, abundance, and harvest in northern Chesapeake Bay: trends and forecasts 733

Andrew G. Jeffs, B. J. Dunphy, and R. M. G. Wells
Experimental effects of water temperature on the gametogenic development of broodstock in the oyster, *Ostrea chilensis* 743

Raymond E. Grizzle, Jamie R. Adams, and Linda J. Walters
Historical changes in intertidal oyster (*Crassostrea virginica*) reefs in a Florida lagoon potentially related to boating activities 749

Sonia Rodríguez-Astudillo, Marcial Villalejo-Fuerte, Federico García-Domínguez, and Rafael Guerrero-Caballero
Biochemical composition of *Spondylus leucacanthus* Broderip, 1833 (Bivalbvia: Spondylidae) and its relationship with the reproductive cycle at Isla Danzante, Gulf of California, Mexico 757

Ana Bratoš, Jakša Bolotin, Melita Peharda, and Jakica Njire
Seasonal distribution of the oyster *Ostrea edulis* (Linnaeus, 1758) larvae in the Bay of Mali Ston, Adriatic Sea 763

Alan J. Power, Ellie Covington, Todd Recicar, Randal L. Walker, and Nelson Eller
Observations on the egg capsules and hatchlings of the knobbed whelk, *Busycon carica* (Gmelin, 1791) in coastal Georgia 769

Dario Savini, Juliana M. Harding, and Roger Mann
Rapa whelk *Rapana venosa* (Valenciennes, 1846) predation rates on hard clams *Mercenaria mercenaria* (Linnaeus, 1758) 777

N. Chaitanawisuti, S. Kritsanapuntu, and Y. Natsukari
Economic analysis of a pilot commercial hatchery-based operation for spotted babylon, *Babylonia areolata* Link 1807, juveniles in Thailand 781

P. T. Gibson, D. G. Worthington, C. Blount, and N. L. Andrew
Incidental damage of blacklip abalone (*Haliotis rubra*) by commercial divers in New South Wales, Australia 787

Meegan E. Vandepeer, Patrick W. Hone, Jon N. Havenhand, and Robert J. Van Barneveld
The effect of nonnutritive fillers on the digestibility of a manufactured abalone diet 793

Meegan E. Vandepeer, Patrick W. Hone, Jon N. Havenhand, and Robert J. Van Barneveld
The digestibility of whole and dehulled lupins (*Lupinus angustifolius*) fed to juvenile greenlip abalone, *Haliotis laevigata* 799

C. D. Hawkins and J. B. Jones
Larval escape through abalone culture effluent systems: an analysis of the risk 805

Qi Li, Choulji Park, and Akihiro Kijima
Isolation and characterization of microsatellite loci in the Pacific abalone, *Haliotis discus hannai* 811

Carolyn S. Friedman, Wendy Biggs, Jeffrey D. Shields, and Ronald P. Hedrick
Transmission of withering syndrome in black abalone, *Haliotis cracherodii* leach 817

Ma. Del Carmen Álvarez Tinajero, Jorge Cáceres-Martínez, and José Guadalupe Gonzáles Avilés
Histopathological evaluation of the yellow abalone *Haliotis corrugata* and the blue abalone *Haliotis fulgens* from Baja California, México 825

María Georgina Gluyas Millán, Casimiro Quiñonez Velazquez, and Jesús Talavera Maya
Effect of El Niño 1997–98 on the snail *Astraea undosa* (Wood) population along the Baja California western coast.... 831

Martha E. Sandoval Quintero and Adolfo Gracia
Reproduction of the spotted pink shrimp, *Farfantepenaeus brasiliensis* (Decapoda: Penaeidae) 835

R. Rosa and M. L. Nunes
Influence of the reproductive cycle on the biochemical composition of deep-sea decapod *Parapenaeus longirostris* (Lucas, 1846) in the Portuguese south coast 843

CONTENTS CONTINUED ON PREVIOUS PAGE

Sizhong Wang, Peter F. Duncan, Wayne Knibb, and Bernard M. Degnan
Byssal attachment of *Amusium balloti* (Bernardi, 1861) (Bivalvia: Pectinidae) spat 563

Maite Narvarte and Marina Kroeck
Intraspecific variation in the reproductive cycle of the tehuelche scallop *Aequipecten tehuelchus* (Pelecypoda, Pectinidae), in San Matías Gulf, Patagonia, Argentina 571

G. Román, M. J. Campos, J. Cano, C. P. Acosta, Pilar Iglesias, and Oscar García
Reproductive and reserve storage cycles in *Aequipecten opercularis* (L., 1758) in Galicia, NW Spain 577

Gonzalo Gajardo, Margarita Parraguez, and Nelson Colihueque
Karyotype analysis and chromosome banding of the Chilean-Peruvian scallop *Argopecten purpuratus* (Lamarck, 1819) 585

Mohsin U. Patwary, Akelia Wanchope, Timothy W. Short, and Edward J. Catapane
Molecular cloning and characterization of a fructose-1,6-biphosphate aldolase cDNA from the deep-sea scallop *Placopecten magellanicus* 591

Rosalío Maldonado-Amparo and Ana M. Ibarra
Comparative analysis of oocyte type frequencies in diploid and triploid catarina scallop (*Argopecten ventricosus*) as indicators of meiotic failure 597

Martin LaFrance, Helga Guderley, and Georges Cliche
Low temperature, but not air exposure slows the recuperation of juvenile scallops, *Placopecten magellanicus*, from exhausting escape responses 605

Jose L. Rueda and Aad C. Smaal
Selective ingestion of pelagic versus benthic algae by the cockle *Cerastoderma edule* (Linné, 1758) 619

Donald Brown, Bernardita Campos, and H.-Jörg Urban
Reproductive cycle of the bivalve clams *Semele solida* (Gray, 1828) (Semelidae) and *Gari solida* (Gray, 1828) (Psammobiidae) from Chile 627

K. L. Heck, Jr., L. D. Coen, and D. M. Wilson
Growth of northern [*Mercenaria mercenaria* (L.)] and southern [*M. campechiensis* (Gmelin)] quahogs: influence of seagrasses and latitude 635

C. Tirado, C. Salas, and J. I. López
Reproduction of *Callista chione* L., 1758 (Bivalvia: Veneridae) in the littoral of Málaga (southern Spain) 643

S. Novoa, D. Martínez, J. Ojea, P. Soudant, J.-F. Samain, J. Moal, and J.-L. Rodríguez
Ingestion, digestion, and assimilation of gelatin-acacia microcapsules incorporating deuterium-labeled arachidonic acid by larvae of the clam *Venerupis pullastra* 649

William S. Arnold, Dan C. Marelli, Melanie Parker, Philip Hoffman, Marc Frischer, and John Scarpa
Enhancing hard clam (*Mercenaria* spp.) population density in the Indian River Lagoon, Florida: a comparison of strategies to maintain the commercial fishery 659

Stefán Áki Ragnarsson and Gudrún G. Thórarinsdóttir
Abundance of ocean quahog, *Arctica islandica*, assessed by underwater photography and a hydraulic dredge 673

Stefano Peruzzi and Ximing Guo
Tetraploid induction by meiosis inhibition with cytochalasin B in the dwarf surfclam, *Mulinia lateralis* Say: effects of temperature 677

Alexandra Leitão, Raquel Chaves, Sara Santos, Pierre Boudry, Henrique Guedes-Pinto, and Catherine Thiriot-Quiévreux
Cytogenetic study of *Ostrea conchaphila* (Mollusca: Bivalvia) and comparative karyological analysis within ostreinae 685

Eric N. Powell, Kathryn A. Ashton-Alcox, Joseph A. Dobarro, Meagan Cummings, and Sarah E. Banta
The inherent efficiency of oyster dredges in survey mode 691

E. Kenchington, C. J. Bird, J. Osborne, and M. Reith
Novel repeat elements in the nuclear ribosomal RNA operon of the flat oysters *Ostrea edulis* C. Linnaeus, 1758 and *O. angasi* Sowerby, 1871 697

Maria Eugenia Valdez-Ramirez, Anne Donval, and Marcel Le Pennec
Ultrastructural and histochemical criteria for determining normality in mature oocytes of the Pacific oyster *Crassostrea gigas* 707

2128 057

CONTENTS CONTINUED ON PREVIOUS PAGE

JOURNAL OF SHELLFISH RESEARCH

Vol. 21, No. 2 December 2002

CONTENTS

IN MEMORIAM
Harold Haley Haskin (1915–2002) 441

Eugene Burreson
Honored Life Member: Jay Donald Andrews 445

Kenneth Chew
Honored Life Member: Neil F. Bourne 447

Loren D. Coen and M. Yvonne Bobo
Honored Life Member: Victor G. Burrell 449

Richard A. Lutz
Honored Life Member: Herbert Hidu 451

George R. Abbe
Honored Life Member: Sandra E. Shumway 453

F. H. MacKintosh and E. A. Smith
Evaluation of Mist Alert™ rapid test kits for the detection of paralytic and amnesic shellfish poisoning toxins in shellfish 455

Ana María Gayoso, Stacie Dover, Steve Morton, Mark Busman, Peter Moeller, Vanesa K. Fulco, and Lucie Maranda
Diarrhetic shellfish poisoning associated with *Prorocentrum lima* (Dinophyceae) in Patagonian Gulfs (Argentina) 461

Graham C. Fletcher, Brenda E. Hay, and Margaret F. Scott
Reducing neurotoxic shellfish poison (NSP) in Pacific oysters (*Crassostrea gigas*) to levels below 20 mouse units · 100 g^{-1} 465

F. M. Harper, E. A. Hatfield, and R. J. Thompson
Recirculation of dinoflagellate cysts by the mussel, *Mytilus edulis* L., at an aquaculture site contaminated by *Alexandrium fundyense* (Lebour) Balech 471

Rodrigo Gaspar Soria, Marcela Susana Pascual, and Víctor Hugo Fernández Cartes
Reproductive cycle of the cholga paleta, *Atrina seminuda* Lamarck, 1819 (Bivalvia: Pinnidae) from northern Patagonia, Argentina 479

Qin-Feng Gao, Ka-Kei Mak, and Siu-Gin Cheung
The physiological ecology of black-ribbed mussels, *Septifer virgatus* (Wiegmann) (Bivalvia: Mytilidae) on a sub-tropical wave-exposed shore in Hong Kong 489

Néstor Fernando Ciocco and Ana María Gayoso
Microalgal food of the ribbed mussel *Aulacomya atra* (Molina, 1782) in Golfo Nuevo (Patagonia, Argentina) 497

John Brake, Jeffrey Davidson, and Jonathan Davis
Triploid induction of *Mytilus edulis* using 6-dimethylaminopurine 503

Pauline Kamermans and Aad C. Smaal
Mussel culture and cockle fisheries in The Netherlands: finding a balance between economy and ecology 509

Dirk A. Campbell and Maeve S. Kelly
Settlement of *Pomatoceros triqueter* (L.) in two Scottish lochs, and factors determining its abundance on mussels grown in suspended culture 519

Per Dolmer
Mussel dredging: impact on epifauna in Limfjorden, Denmark 529

Katherine A. Ross, John P. Thorpe, Trevor A. Norton, and Andrew R. Brand
Fouling in scallop cultivation: help or hindrance? 539

Laura Schejter, Claudia S. Bremec, Rut Akselman, Daniel Hernández, and Eduardo D. Spivak
Annual feeding cycle of the Patagonian scallop *Zygochlamys patagonica* (King and Broderip, 1832) in Reclutas bed (39S–55°W), Argentine Sea 549

Karin B. Lohrmann, Andrew R. Brand, and Stephen W. Feist
Comparison of the parasites and pathogens present in a cultivated and in a wild population of scallops (*Argopecten purpuratus* Lamarck, 1819) in Tongoy Bay, Chile 557

CONTENTS CONTINUED ON INSIDE BACK COVER

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