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A REVIEW OF PUBLISHED WORK ON *CRASSOSTREA ARIAKENSIS*

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INTRODUCTION

Field research on the Asian (Suminoe) oyster, *C. ariakensis*, began in 1998 at the Virginia Institute of Marine Science (VIMS) in response to a resolution from the Virginia Legislature to initiate investigations on alternative species. All field trials have used sterile triploids. Initial research indicated promising performances in *C. ariakensis* in a variety of salinities for growth and disease resistance (Calvo et al. 2001). Research on this species continues at VIMS today, but in the meantime, the Virginia Seafood Council has run two commercial trials of *C. ariakensis* on their own with similar promising results. They have proposed a third for 2003 with about a million triploid *C. ariakensis*. The direction taken by industry clearly indicates a desire to proceed with larger and larger scale-ups of aquaculture using triploids. This notion was addressed in a symposium staged in 2001 (Hallerman et al. 2002) where the general consensus found that "it is difficult to consider the risks of aquaculture of triploid (infertile) *C. ariakensis* as separate from the risks of diploid (fertile) *C. ariakensis*. That is, there was consensus that triploid aquaculture would inevitably lead to some introduction of reproductive individuals in the Bay, with unknown outcomes for population growth." Part of the difficulty in assessing the risk of such a scenario comes from the inherent difficulty of predicting the consequences of an introduction generally. Another difficulty of assessing risk, especially for *C. ariakensis*, is the lack of information on this species.

The aim of this review was to provide an unabridged overview of the published works on this species. We may have missed some references that were obscure or indirectly referred to *C. ariakensis*. Many of the works on *C. ariakensis* were in other languages, principally Chinese. For Chinese articles, they were translated and are presented in somewhat more detail than those in English. Some were obtained while traveling to specific laboratories in China and would otherwise be difficult to obtain. We were as complete as possible give the timely need for this review.

We present the information uncritically. That is, we present the contents of the articles without analysis. Partly this is the result of space constraints. More importantly, it is unclear that data reported always apply to *C. ariakensis*. Morphologic confusion is common with *Crassostrea* species. For example, a considerable number of reports of *C. ariakensis* occur in west India and Pakistan, geographically isolated from the main populations in Japan, China, and Korea. It seems unlikely that this is the same species, but to judge so *a priori* would be to leave out this information. We expect scientists to consider the data critically and test it if appropriate.

The information we collected is organized into general categories so that one work may be cited repeatedly if it crosses categories. The content in each category in no way implies the importance of this information, merely what has been done. Conversely, categories missing information reflect the absence of data.

NOMENCLATURE

Harry (1981) described the history of the genus name *Crassostrea* Sacco, 1897 as follows: Over half a century ago Lamy (1929–1930) surveyed the living oysters and put all species in the genus *Ostrea* Linnaeus, 1758, including *Crassostrea ariakensis*. But since 1930, other authors, chiefly those interested in the commercial production of oysters (e.g., Thompson 1954), have separated *Crassostrea* from *Ostrea* on the basis that the promyal passage on the right side of the excurrent mantle chamber is closed in *Ostrea* and open in *Crassostrea*. Other differences on morphology and anatomy between these two genera can be found in Ahmed (1971 and 1975), Glude (1971), and Stenzel (1971). In this review, please note that *Ostrea* is cited from many old references.

Nomenclature is confusing for *C. ariakensis* (Carriker & Gaffney 1996) because the traditional oyster classification methods rely mainly on conchological characters, i.e., external and internal morphology of the shell, which express high phenotypic plasticity among environments (Hirase 1930). In addition, oyster eggs are fertilized in mass spawns that increase the possibility of hybridization and promote high variation (Guan & Li 1986). Therefore, species with the same name might be genetically distinct whereas the ones with different scientific names might be genetically the same. Species variously called *C. rivularis, discoidea, palmipes,* or *paulucciae* in previous literature (Carriker & Gaffney 1996) might be the same as the species we call *C. ariakensis* today. In general, it is accepted that *rivularis* is synonymous with *ariakensis*, although it is still possible that *rivularis/ ariakensis* was misclassified in certain publications. This review includes all the available publications with the above mentioned species names.

The authorship of *ariakensis* has been credited to Fujita (1913). However, we are confused by the description of Wakiya (1930) on the origin of the name *ariakensis*. He wrote his reference as "*O. ariakensis* (Wakiya M. S.) Fujita, ... 1913." Harry (1981) assumed that "Fujita proposed the name in 1913, based on a manuscript of Wakiya." Coan et al. (1995) seemed to agree by giving the reference in a way of "Fujita, 1913... *ex* Wakiya MS." Who proposed the name *ariakensis* first, Fujita or Wakiya? We were not able to locate Fujita (1913), so we cannot answer that question for sure. According to our publication collection, the species name *ariakensis* was not referred to as frequently as *rivularis* before mid 1990s, but it has been widely referred to in recent publications.

The history of species name *rivularis* can be traced back to 1861, when Gould described a new species called *Ostrea rivularis*, which in Latin means "oysters in small brooks." His original description was written in Latin. Translated to English, the shells he observed were "discoid, oblong, slender; inferior valve thick, purple, with remotely radiate ribs and fortified small tubes; superior valve simple, with ramosing less purple veins; cavity minimally deep, ovate; white ash-colored broad margin, weak hinge." He emphasized "the rays of the little tubes below, and the veins

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above, are unusually clear, distinctive characters." The dimension of the observed shells was "Diam. 60; Lat. 10 millim." It "inhabits the China Seas, as indicated by shells adhering to it."

There is serious ambiguity in the source of Gould's specimen. The title of his article indicates that his description was based on the collection of "the North Pacific Exploring Expedition," whereas according to Hirase (1930), it was based on a single specimen from China in Dunker's collection. Hirase did not explain whom Dunker is except for a reference listed as Dunker (1882). Several other authors mentioned China as the source of Gould's specimen (Ahmed 1971, Galtsoff 1964), but no additional references were offered for further confirmation. Hirase (1930) also questioned the completeness of Gould's description and its value for identification because it seems based on a single specimen, which seems to be comparatively young according to its size (60 mm). Gould's description of *rivularis* and those of others (see Morphology section) are incompatible. Thus, it is quite possible that *rivularis* of Gould (1861) is different from the species we call *rivularis* or *ariakensis* today.

O. (C.) rivularis Gould has been widely applied to oysters with similar conchological characters in many Pacific coastal countries, such as Japan, China, Pakistan, and India. Its taxonomic status in each country is still muddled. A review is summarized below.

In Japan, Ariake-gaki, Suminoe-gaki, and Kaki ("gaki" in Japanese means oyster) were some common names for *O. rivularis* (Amemiya 1928). This species was once classified as *O. gigas* by Fujimori (1929) but this was refuted by Taki (1933) and Imai and Hatanaka (1949). Wakiya (1930) surmised that *O. rivularis* of his in 1915 (Wakiya 1915) and that of Amemiya (1928) was the same as *O. ariakensis*, whereas the *O. rivularis* described by Lischke (1871) seemed to be the young of *O. ariakensis*.

In Pakistan, Awati and Rai (1931) indicated two names for the same species, *O. discoidea* and *O. rivularis*. Reeve (1871) described *O. discoidea* based on specimens from Fuji Island and New Zealand, but Ahmed (1971) stated that the figure and the shell characters published by Reeve were different from that of *O. discoidea*. According to Ahmed, Reeve's *O. discoidea* is rounded and flat to the extent that it looks like the windowpane oyster, *Placuna placenta* Linne, 1758, which is abundant in lagoons of Philippines and South East Asia (Abbott & Dance 1986). Based on his own experience, Ahmed believed that *O. discoidea* is not distinguishable from *C. rivularis*.

In China, the common name for *O. (C.) rivularis* is Jinjiang-muli ("jinjiang" in Chinese means "close to river" and "muli" means oyster). One of the long-standing debates on oyster classification involves two morphologically very similar variants that occur in the Pearl (Zhujiang) River estuary. One is called "white meat" oyster and the other is "red meat." Very experienced oyster farmers can separate these two variants by external appearance and the color of the soft body. Fei (1928) believed that both are *O. gigas*. However, Zhang and Lou (1956a) identified "white meat" as *O. rivularis* and "red meat" as a variant. The "white meat" oyster is considered better than the "red meat" because of meat quality and productivity in aquaculture, thus has higher commercial value. The "red meat" oyster is apparently more resistant to harsh conditions according to observations of it in culture (Guan & Li 1986). Further investigations by other researchers revealed other differences. A comparative study on the physiologic and biochemical indexes (Guan & Li 1986), such as oxygen consumption rate, fatty acid composition, and amino acid composition, demonstrated sufficient differences in physiology to suspect that

genetic differences are likely. Anatomically, Li (1989) found a difference in the connection of the body with the gills. In "white meat" both the left and right epibranchial chamber connect with the promyal chamber, whereas in "red meat" only the right epibranchial chamber connects with the promyal chamber. He believed the two belong to two different species. A study on genetic variation using starch gel electrophoresis (Li et al. 1988) demonstrated that they should belong to different species because their genetic identity was low ($I = 0.548$). The estimated divergence time of the two is 3×10^6 years. The comparison of genetic similarities and genetic distances suggests that "white meat" is *C. rivularis* and "red meat" is probably *C. iredalei*. Guan and Zheng (1990) studied the esterase isoenzyme of the two groups by polypropylene amide gel electrophoresis and agreed that they are different species. Above all, it was generally agreed that "white meat" is *C. rivularis*, but whether "red meat" is *C. iredalei* is still unconfirmed.

MORPHOLOGY

Conchological Characters

References on conchological characters of naturally occurring *C. ariakensis* come from three countries: China, Japan, and India. References from the United States (Pacific Northwest) are also included because the seed were introduced from Japan. Reports containing conchological data are listed individually following a general review to compare and contrast characters of what are called *O. (C.) rivularis*, now *C. ariakensis*. The major conchological characters presented in these reports are size; thickness and shape of the valves; outer structure of the valves; comparison between the left and right valve; color of outer and inner surface; size and color of ligament; color, size and position of the muscle scar; and hinge structure (Table 1).

Review

In China, it is commonly observed that valves of *Ostrea (Crassostrea) rivularis* are large and thick with varying shapes, basically round but sometimes elongated into oval, oblong, and even triangular shapes. The right valve is thinner, flatter, and smaller than the left. Both valves are covered with concentric lamellae (fluted shell margins on the external shell), with fewer layers of, but stronger, lamellae on the left valve. Density and shape of lamellae varies by age class, which are thicker and more layered in older oysters (Zhang & Lou 1956a, Zhang et al. 1960). Color of lamellae or the outer surface of valves ranged from gray, yellowish brown, brown, to purple or dark purple. Dark purple coloration is apparent in *C. ariakensis* grown in high-salinity areas of Chesapeake Bay (Zhou & Allen, unpubl.). The inner surface of valves is white or grayish white, purple on the edge. The ligament area is short and wide, and the ligament is usually purple black. The muscle scar is very large, mostly oval or kidney shaped, located in the mid-dorsal area, purple or light yellow in color.

The coloration of valves and muscle scars of *C. ariakensis* described in reports from Japan is different from those from China. In Japan, the outer surface of the valves was described as cream-buff or white, streaked with radial chocolate bands, violet bands, or almost uniformly violet (Hirase 1930, Torigoe, 1981, Wakiya 1929). The inner surface of the valves was strongly lustrous or partly opalescent (Hirase, 1930, Torigoe 1981). The muscle scar was usually white or sometimes stained with olive–ocher spots or

TABLE 1.

Characteristics of oysters by citation.

Gould (1861), China, *O. rivularis*
Valve shape size	Discoid, oblong, slender
Left, right valve:	Interior valve thick, purple, with remotely radiating ribs and fortified small tubes; superior valve simple, with ramosing less purple veins, cavity minimally deep, ovate
Shell color outer	Purple, white ash-colored broad margin
Shell color inner	—
Ligament	—
Muscle scar	—
Hinge:	Weak

Zhang and Lou (1959), China, *O. (C.) rivularis*, includes figure(s)
Valve shape size	Large and thick with various shapes, round, oval, triangle, and oblong; concentric scarce lamellae on outer surface.
Left, right valve	—
Shell color outer	Yellowish brown.
Shell color inner	—
Ligament	—
Muscle scar:	—
Hinge	—

Zhang and Lou (1956a), China, *O. (C.) rivularis*, includes figure(s)
Zhang et al. (1960), South China, *O. rivularis*, includes figure(s)
Similar descriptions from the above two references are combined as follows
Valve shape size	Valves large and thick with various shapes, round, oval, triangle, and oblong
Left, right valve	Right valve flatter and smaller than the left one, with yellowish brown or dark purple concentric lamellae on its surface. In 1 to 2-y-old individuals, lamellae thin, flat, and brittle, sometimes dissociated; on valves older than 2 ys old, flat but sometimes with tiny wavy shape at the edge; on valves several years old, thickly layered, strong as stone. Left valve is larger and thicker than right valve, stronger but fewer layers of lamellae. A few samples had inconspicuous radiating ribs or plication.
Shell color outer	Gray, purple, or brown
Shell color inner	White, grayish purple on the edge.
Ligament:	Ligament purple black. Ligament groove short and wide, like an ox horn. The length from the ligament to anterior is one sixth to one fourth of shell height
Muscle scar	Muscle scar very large, light yellow, irregular shape, mostly oval or kidney shaped, located in the middle of the dorsal area
Hinge:	—

Cai et al. (1979), China, *O. rivularis*, includes figure(s)
Valve shape size	Shells large and thick with various shapes, such as round, oval, triangle and oblong.
Left, right valve:	Right shell flatter and smaller than the left shell, with yellowish brown or dark purple lamellae on its surface. The lamellae are thin and flat, with not much layers and no radiating ribs, but usually with protuberance. The left shell is larger and thicker with irregular shape and similar lamellae as the right shell.
Shell color outer:	Yellowish brown or dark purple.
Shell color inner:	White or grayish white.
Ligament	Ligament purple black. Ligament groove short and wide.
Muscle scar	Muscle scar large, oval or kidney shaped, located in the middle of the dorsal area
Hinge	No denticulate on the hinge.

Li and Qi (1994), China, *C. rivularis*, includes figure(s)
Valve shape size:	Large variation in shell shape, usually oval or oblong.
Left, right valve:	Concentric lamellae tend to coalesce, no radiant ribs.
Shell color outer:	Light purple.
Shell color inner	White
Ligament	Wide ligament groove.
Muscle scar	Light purple.
Hinge:	—

Amemiya (1928), Japan, *O. rivularis*, includes figure(s)
Valve shape size:	It is either circular or oval in form, pronounced elongation as found in *O. gigas* is absent
Left, right valve:	—
Shell color outer:	—
Shell color inner:	—
Ligament	—
Muscle scar:	—
Hinge:	—

Cahn (1950), Japan, *O. rivularis*, includes figure(s)
Valve shape size:	Round, flat, smooth surfaced, plates thin, almost smooth, shell thick
Left, right valve:	—
Shell color outer:	Pale pink, radiating burnt lake strikes on shells
Shell color inner:	—
Ligament:	—
Muscle scar	—
Hinge:	—

Hirase (1930), Japan, *O. (C.) rivularis*, includes figure(s)
Valve shape size:	Orbicular, oval, elongated oval, though appearing somewhat subtriangular because of its rather long umbo. There are many intermediate forms, but on the whole the specimens are oval. The shell is fairly strong and thick, though not to the extent of *C. gigas*

TABLE 1.

continued

Left, right valve:	The right valve is somewhat smaller. The concavity of the left valve is larger. The anterior depression of the left valve is very obscure. The lamellae of the right valve are somewhat thin and almost smooth, and distinct placations are not apparent, but sometimes the lamellae are covered with somewhat irregularly tubular projections. It is noteworthy that smooth lamellae are more common in the young than in the adult. The color is cream-buff with many radial chocolate bands, but in adults these bands are fused into larger ones; their arrangement differs in each individual. In the left valve, the lamellae are generally indistinct, and may be close together or separate
Shell color outer:	The common color is pale rhodonite pink with radiating "burnt lake" striae.
Shell color inner:	The inner shell surface is generally white with strong luster, sometimes with a yellowish central part.
Ligament:	The ligament is "burnt lake" or black
Muscle scar:	The muscular impressions, elongated oblong with concave anterior side, are equal in size for the two valves and rather large in porportion to the inner shell area. The color of the impression is white, or rarely marked with olive-ocher spots; its surface is almost flat
Hinge:	—

Imai (1978), Japan, *C. rivularis*, includes figure(s)

Valve shape size:	Round or elliptical
Left, right valve:	The lower shell is shallow and the umbo cavity below the hinge plate is very small
Shell color outer:	The part near the hinge plate in the upper shell is violet–brown in color
Shell color inner:	—
Ligament:	—
Muscle scar:	—
Hinge:	—

Kira (1962), Japan, *C. rivularis*

Valve shape size:	Has a large and rather flat shell, of which the surface bears very coarse and widely spaced concentric lamellae
Left, right valve:	—
Shell color outer:	—
Shell color inner:	—
Ligament:	—
Muscle scar:	—
Hinge:	—

Torigoe (1981), Japan, *C. ariakensis*, includes figure(s)

Valve shape size:	Large sized (height 200 mm × length 112 mm, Hirase 1930). Outline orbicular to long spatulate form, mostly tongue form, subequivalves. Attachment area is small to medium, commonly behind the umbonal area
Left, right valve:	Both valves flat, but left valve weakly concave. Both valves have very faint dichotomous radial ribs, left valve more conspicuous than right valve. Growth squamae flat and stretched parallel to the grow lines. No commissural plication, or very weak even if present Commissural shelf small to medium. Umbonal cavity shallow. No chomata. The dorso-ventral section has chalky deposits between solid shell layers and no hollow chambers. Both valves are thinner than those of *C. gigas*, so chalky layers are very thin. The parts of chalky deposits are often intruded by worms.
Shell color outer:	"White in ground" (sic) color with pale purple streaks radiating from umbo.
Shell color inner:	Chalky white or partly opalescent
Ligament:	—
Muscle scar:	Reniform, dorse-anterior border concaved and close to ventro-posterior shell margin from the center of the valve. Lustrous white or sometimes with purple patches, particularly on right valve.
Hinge:	—

Wakiya (1929), Japan, Ostrea ariakensis

Valve shape size:	Shell usually circular or oval in shape. However, its shape varies considerably according to the hardness of the bottom on which it lives. When found imbedded in soft mud it has an extremely elongated shell so that it is very difficult to distinguish it from that of *O. laperousi* found on a mud bottom of lower salinity, only differing from *O. laperousi* in having the hinge of lower valve not very long and subequal to that of the upper one. *O. rivularis* Gould has, according to the original description, its lower valve provided with radiating, tube-shaped ribs set distantly. Therefore the species in which the ribs are absent from the lower valve or only very weakly developed, if present at all, cannot be the species of Gould
Left, right valve:	Lamellae imbricated rather compactly, lower valve concave, not provided with ribs; upper valve flat, length of hinge nearly equal to that of lower valve. Occasionally, weakly developed ribs are observed on the lower valve of the young of the species, but never on full-grown ones.
Shell color outer:	Whitish and streaked with violet, or almost uniformly violet.
Shell color inner:	Lead white; muscular impression faint, usually not specially colored but sometimes stained purple.
Ligament:	—
Muscle scar:	—
Hinge:	The hinge of the lower valve not so long as, as long as or a little longer than the breadth, no umbonal cavity below margin of hinge.

Coan et al. (1995), USA, *C. ariakensis*, includes figure(s)

Valve shape size:	Subtrigonal, flared ventrally, heavier and more rounded than *C. gigas*
Left, right valve:	Left valve moderately concave, with white to pale pink lamellae; right valve moderately flattend, with many thin commarginal lamellae, sometimes with dark brown to purple radial color bands. Both valves with densely layered, thin lamellae.
Shell color outer:	—
Shell color inner:	—
Ligament:	—
Muscle scar:	White to purple to olive
Hinge:	—

Galtsoff (1964), USA, *C. rivularis*, includes figure(s)

Valve shape size:	Orbicular strong and large.
Left, right valve:	Left, lower valve slightly concave, upper valve shorter and flat. The left valve has generally indistinct lamellae of pale pink color with radiating striae. The lamellae of the right valve are thin and most smooth, sometimes covered with tubular projections.

TABLE 1.
continued

Shell color outer:	The color of the right valve is cream buff with many radial chocolate bands, their arrangements greatly variable.
Shell color inner:	—
Ligament:	—
Muscle scar:	Situated near the center or a little dorsally, is white, occasionally with olive-ochre spots.
Hinge:	—
Langdon and Robinson (1996), USA, *C. ariakensis*, includes figure(s)	
Valve shape size:	This species differs from the Pacific oyster morphologically in that the shell is typically more rounded and the edges of shell layers are flat and not rippled like those of Pacific oysters (Torigo, 1981)
Left, right valve	—
Shell color outer:	—
Shell color inner:	—
Ligament:	—
Muscle scar:	—
Hinge:	—
Awati and Rai (1931), India, *O. discoidea* or *O. rivularis*	
Valve shape size:	Shell flat and of large size, rounded, foliaceous with conspicuous lines of growth
Left, right valve:	Lower valve lightly concave, upper valve of the same size and shape as the lower, slightly convex.
Shell color outer:	—
Shell color inner:	Clear and nacreous.
Ligament	Ligament area small
Muscle scar:	Oblong with a cloudy white or smoky white color.
Hinge:	No denticulations.
Rao (1987), India, *C. rivularis*, includes figure(s)	
Valve shape size:	Shallow shell cavity
Left, right valve:	—
Shell color outer	Imai (1978) has stated that the hinge part of the shell of *C. rivularis* is violet brown in color. The coloration may be caused by ecological conditions such as luxuriant growth of seaweeds in the vicinity or other factors and should not be considered of taxonomic importance.
Shell color inner:	—
Ligament:	—
Muscle scar:	Oblong white
Hinge:	—
Patel and Jetani (1991), India, *C. rivularis*	
Valve shape size:	Shell oval, narrow at anterior end and broader with posterior end.
Left, right valve:	Left valve has deep radial ridges from the hinge and tightly inter locked with upper right valve.
Shell color outer	Pink to brownish with tints.
Shell color inner:	—
Ligament:	Having narrow hinge-ligament
Muscle scar	White.
Hinge:	Having narrow hinge-ligament.

purple patches (Hirase 1930, Torigoe 1981, Wakiya 1929). Rao (1987) thought the difference in coloration might be caused by ecological conditions and therefore not considered a character of taxonomic importance. Reports from the United States are consistent with reports from Japan for coloration, which indicates that at least some part of coloration might be caused by genetic factors. *O. (C.) rivularis* from India are similarly described. Coloration of the inner surface of the valves and the muscle scar are close to Japanese reports.

Reports from Japan were often comparative between *C. ariakensis* and other species, such as *O. (C.) gigas* (Amemiya 1928, Hirase 1930, Torigoe, 1981) and *O. laperousi* (Wakiya 1929). *O. (C.) gigas* were believed to have stronger, thicker, and more elongated shells than *O. (C.) rivularis*, whereas *O. rivularis* is very difficult to distinguish from *O. laperousi* found on muddy bottom in lower salinity. *O. rivularis* differs from *O. laperousi* by having the hinge of the lower valve not very long and subequal to that of the upper one. Japanese reports agree that *O. (C.) ariakensis* has flat valves, with the left one weakly concave (Cahn 1950, Kira 1962, Torigoe 1981). Wakiya (1929) thought the various shapes of *O. ariakensis* were influenced by the hardness of the bottom because the ones with extremely elongated shells were found imbedded in soft mud. This is also a character of other *Crassostrea* spp. (Galstoff 1964).

The most confusing character through this review has been what Gould (1861), who first named *O. rivularis*, described as

remotely radiating ribs and fortified small tubes on the outer surface of left valve and veins on right valve. He emphasized that these are usually clear, distinctive characters of this species. His observation was based on a sample from China. However, no reports from China agreed with his description of such characters. Cai et al. (1979) and Li and Qi (1994) observed no radiating ribs in this species. Based on a large-scale investigation of oyster species all along the Chinese coast, Zhang and Lou (1956a) described inconspicuous radiating ribs or plication in a few samples of *O. (C.) rivularis*. Only one report from India described deep radial ridges from the hinge on the left valve (Patel & Jetani 1991), although the origin of the background specimen was unknown. From Japan, similar characteristics were described as indistinctive or occurring at very low frequency. Hirase (1930) and Galstoff (1964) mention that the lamellae are sometimes covered with tubular projections. Hirase (1930) and Cahn (1950) mentioned "radiating burnt lake strikes," which might or might not be the same feature we are discussing here. Torigoe's (1981) report said "both valves have very faint dichotomous radial ribs, left valve more conspicuous than right valve." Wakiya (1929) is more helpful in clarifying this confusion. He stated this species was "not provided with ribs...occasionally, weakly developed ribs are observed on the lower valve of young of the species (*Ostrea ariakensis*), but never on full-grown ones." Either Gould's original descriptions

were inappropriate for adult *C. ariakensis*, or he described a different species (Wakiya 1929). The latter possibility is quite high if Gould did get his specimen from China because there are around 20 oyster species there (Zhang & Lou 1956b, Cai & Li 1990, Li & Qi 1994, Guo et al. 1999), and classification based completely on morphologic characters is questionable.

ANATOMIC CHARACTERS

Review

Anatomic characters were not studied as broadly and completely as conchological ones. Reports mainly come from Japan and China. Researchers had different emphases in their anatomic studies. The only character described by more than one researcher is the mantle. Hirase (1930), Zhang et al. (1960), and Galtsoff (1964) were in agreement that the inner row of the mantle tentacles is aligned while the outer row is irregular. Details of anatomic characters are given in Table 2.

GEOGRAPHIC DISTRIBUTION

From an overview of the literature, *C. ariakensis* seems to have a wide geographical range. According to Kuroda and Habe (1952), *O. rivularis* encompassed latitudes 12–34°N, which covers the area from southern Japan to southern India. Ranson (1967) listed sources of *C. ariakensis* specimens in museums around the world, coming from Southern Japan to coasts bordering the South China Sea, including Hong Kong, Vietnam, and Sabah (formerly North Borneo), Malaysia. Several authors (Wakiya 1929, Cahn 1950, Kira 1962, Coan et al. 1995) mentioned its distribution in Korea. Anon (1996) mentioned that *C. rivularis* was also found from the Philippines and Taiwan to Thailand. Above all, this species seems to occur all along the west coast of the Pacific Ocean, from southern Japan to Pakistan (Angell 1986). Sparks (1965) even reported that *C. rivularis* was indigenous to Kenya. However, for most areas outside of Japan and China, no references are available to confirm these observations genetically as *C. ariakensis*.

Quite a few literature reports are available listing specific locations in a country where this species occurs naturally. Below we

TABLE 2.

Anatomical characteristics of oysters by citation.

Hirase (1930), Japan, *O. (C.) rivularis*

Mantle—In a specimen whose length and altitude are 96 mm and 45 mm, respectively, the mantle is united by the anterior 21 mm, or 0.22 of the body length. There is no siphon. The mantle margin is dark nigrosine violet or pinkish vinaceous, and the tentacles are arranged in two rows, the outer consisting of tentacles of irregular size and the inner of slender single tentacles. Fine tendons radiate from the posterior sides of the adductor muscle as usual.

Adductor muscle—The adductor muscle measures 20 mm in altitude and 22 mm in breadth and is suborbicular, with somewhat concave anterior face and convex posterior face. The distance between the anterior end of the adductor muscle and the anterior end of the body is 52 mm. A small portion of the posterior part of the adductor muscle is white as usual.

Heart—The pericardium, contiguous to the anterior face of the adductor muscle, is oval and measures 19 mm in altitude and 6 m in breadth. The heart runs obliquely from the antero-dorsal corner of the pericardium to the postero-ventral corner. The ventricle and the auricles are both flesh color. The ventricle measures 8 mm in altitude and 6 mm in breadth, while one of the auricles measures 8 mm in altitude and 3 mm in breadth.

Ctenidium—The posterior end of the ctendium curls up along the posterior face of the adductor muscle.

Alimentary system—The palps are as usually found in *Crassostrea*. The rectum begins at the dorsal region of the pericardium and ends just above the posterior end of the adductor muscle. About 3 mm of the terminal portion is free, differing from other oysters of this subgenus and shorter than in *Neopycnodonte cochlear*, whose free portion is 5 mm. The anal end has a ring. The distance between the mouth and the anus is 55 mm, its ratio to body length being 0.57.

Imai (1978), Japan, *C. rivularis*

C. ariakensis differs from *C. gigas* in that a part of the rectum and anus are away for the soft parts.

Torigoe (1981), Japan, *Crassostrea ariakensis*

Soft parts are similar to *C. gigas* but the coloration of soft parts is the palest of Japanese *Crassostrea* species.

Zhang et al. (1960), South China, *O. rivularis*

Mantle—The inner row of the mantle tentacles is aligned while the outer row is irregular.

Heart—Heart chamber is flesh pink.

Li (1989), China, *C. rivularis*

Promyal chamber—The left and right epibranchial chambers connect with the promyal chamber all together. In the cross section of this type, the ascending lamellae of the left and right outer demibranch attach to the mantel, whereas the other part of gills are free in the mantel cavity. The whole epibranchial chamber is connected with the promyal chamber. On the lateral view from the right side of the oyster, the joint of the two gills attaches to the visceral mass at and below the adductor muscle, while above the adductor muscle, the gills are dissociated so that the two rows of water tubes on the left as well as the two rows on the right of oyster body can be seen. The "white meat" Jinjiang oyster from Shenzhen Bay belongs to this group.

Nelson (1938) stated that oysters with a promyal chamber are adapted to low salinity and highly turbid waters, while oysters without it do better in high salinity, less turbid waters. Thomson (1954) had similar reports. The occurrence of the promyal construction in commonly cultured oyster species in China and their distribution are consistent with Nelson's statement. Oysters with the chamber inhabit mostly estuary and intertidal zones, where salinity and transparency are both low and the environmental factors fluctuate. The ones without the chamber inhabit mostly shallow seas with higher salinity and relatively stable environments. It is likely that the promyal chamber is an adaptation stemming from oysters moving into increasingly estuarine habitats.

Galsoff (1964), USA, *C. rivularis*

Mantle—Margin of the mantle is dark violet; the tentacles are arranged in two rows; those of the outer row are of irregular size; the inner tentacles in a single row are slender.

summarize this information by country, from north to south along the Pacific west coast.

Japan

Kira (1962) reported distribution of *C. rivularis* roughly from central Honshu to Kyushu (Fig. 1). Honshu is the largest island of Japan located in the center of the archipeligo. Kyushu is southern most. Cahn (1950) reported the restricted range of its distribution as western Kyushu, mainly in Ariake-kai ("kai" in Japanese means sea) and Yatsuchiro-wan ("wan" means bay). It is most abundant in the inner parts of Ariake-kai, the southern coast of Fukuoka and Saga prefecture. Hedgecock et al. (1999) found a similar distribu-
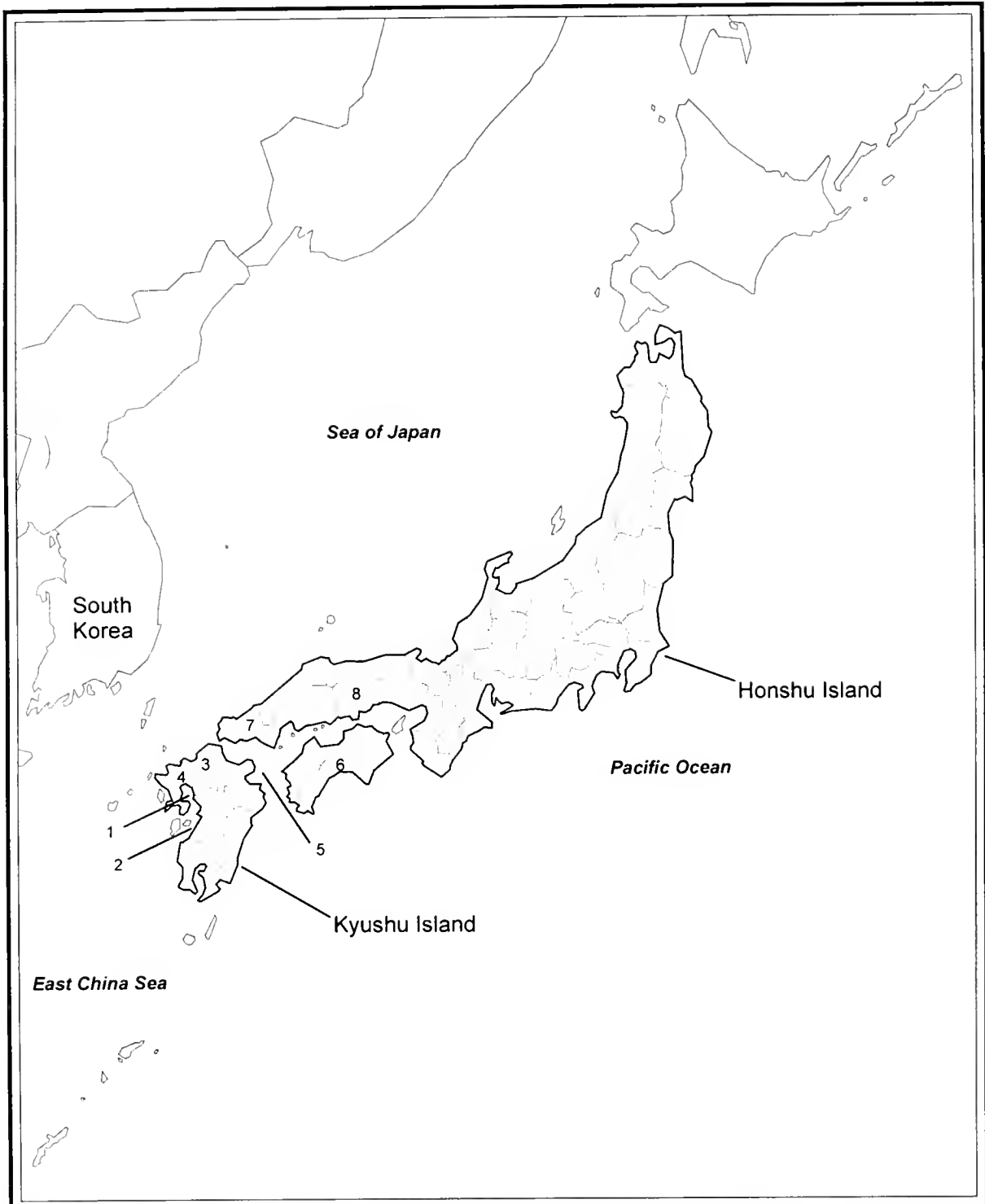

Figure 1. Locations reported with *C. ariakensis* populations in Japan. 1. Ariake-kai; 2. Yatsuchiro-wan; 3. Fukuoka prefecture; 4. Saga prefecture; 5. Shiranuhi Bay; 6. Kochi prefecture; 7. Yamaguchi prefecture; and 8. Okayama prefecture.

tion in the Ariake Bay. Ariake-kai or commonly called Ariake Bay, seems to be the most recognized natural habitat and the namesake of *C. ariakensis*, as it was mentioned most frequently (Wakiya 1929, Hirase 1930, Cahn 1950, Galtsoff 1964, Imai 1978, Hedgecock et al. 1999). In addition, Wakiya (1929) mentioned Shiranuhi Bay on the northeastern coast of Kyushu, and Cahn (1950) listed the Pacific coast of Kochi, the coast of Yamaguchi and Okayama prefecture.

China

China has an extensive coastline of about 18,000 km extending from the cold temperate north to the tropical south. Based on an extensive investigation on oyster species along the Chinese coast in 1956, *O. (C.) rivularis* was identified in each coastal province (Zhang & Lou 1959; Fig. 2). As Zhang et al. (1960) later stated, the distribution of this species covers the whole coastal region of China, with a latitudinal range of 15–40°N and a longitudinal range of 107–124°E. Table 3 lists the names of locations where *O. (C.) rivularis* has been reported. The locations underlined were considered by Zhang and Lou (1956b) as major production areas, which might not be true today. Among those, Xiaoqing River estuary in Yangjiaogou, Shandong province was specifically mentioned because a very large population of *O. rivularis* was found there. In certain localities, the population was so large that people call them "oyster hills" because individual oysters grew attaching to each other (Zhang & Lou 1956b, Zhang et al. 1960). It would be interesting to try to determine whether natural populations are still available in some locations, having possibly been shielded from exploitation because of their rarity (Table 3).

India

Although Ahmed (1971) mentioned that *C. rivularis* was distributed on both east and west coasts of the Indo-Pakistan subcontinent, other reports maintained that this species was found only on the west coast of India (Fig. 3). It was first reported along the coast of Bombay (Awati & Rai 1931). Durve (1986) gave a much wider range between Ratnagiri and Okha along the coast of Gujarat and Maharashtra area. Gujarat (Saurashtra) has a long coastline of 1500 km (Patel & Jetani 1991). Specific locations in this range were described by Mahadevan (1987) as Aramra, Poshetra, Port Okha, Porbandar, Sikka, Gagwa Creek, Singach Creek, Beet Kada, Khanara Creek, Laku Point, Gomati Creek (Dwarka), Harsad, Navibander (Madha Creek), Balapur, and Azad Island. In addition, Rao (1987) mentioned creeks of Kutch and Aramda Creek in Gujarat and Mahim, Ratnagiri and Jaytapur in Maharshtra. Durve (1986) also mentioned some trawling areas around Bahrain in the Arabian Gulf.

Pakistan

This species was found abundant on the coast of West Pakistan (Ahmed 1971; Fig. 3). The following locations have been mentioned in the literature: the coast of Sind (Ahmed 1971), Korangi Creek (18 miles south of Karachi) and Sonari (40 miles west of Karachi; Asif 1978b), Sandspit backwaters (Qasim et al. 1985, Barkati & Khan 1987, Aftab 1988), and Port Qasim (Gharo-Phitti saltwater creek system near Karachi; Ahmed et al. 1987, Barkati & Khan 1987).

ECOLOGY

Habitat

Below we summarize reports on the nature of the habitat described for *C. ariakensis* and the vertical and horizontal ranges of its distribution.

In Japan, *O. rivularis* was only reported from muddy beds (Amemiya 1928, Wakiya 1929, Hirase 1930). It generally adheres to other objects by the umbonal part of the left valve, but many specimens appear to have lived separately (Hirase 1930). Its vertical range is just above the low tide mark and closely restricted to the vicinity of the low tide line (Amemiya 1928, Wakiya 1929). Its horizontal range was determined by water temperature and salinity (Imai 1978). The salinity range of its natural habitat under ordinary conditions is 9–30 ppt (Amemiya 1928, Cahn 1950), the lower range of which is lower than many *Crassostrea* species. As Amemiya (1928) explained, these conditions are apt to change for one reason or another. For instance, during ebb tide the exposure of the beds to the air and sun inevitably make the surrounding water more saline due to evaporation. But because this species lives close to the low tide mark, exposure to high salinities is short. *C. ariakensis* can apparently tolerate low salinities as well. *O. rivularis* was found in places where the salinity falls occasionally much below 10 ppt, sometimes even in entirely fresh water (Amemiya 1928).

In China, this species occurs widely among the river estuaries along the coast. It is found from the low tide line to 7–10 m below mean low water (Zhang & Lou 1956b, Zhang et al. 1960, Cai 1966, Cai et al. 1979, Xu et al. 1992). Sometimes it could be found around the high water mark (Zhang et al. 1960). According to Lu (1994), the temperature range of *C. rivularis* is 2–35°C. Normal salinity range was reported as around 10–30 ppt (Zhang & Xie 1960, Lu 1994) or 9–28 ppt (Zhang & Lou 1956b). Optimum salinity was reported as 10–25 ppt (Zhang et al. 1960) or 10–28 ppt (Nie 1991). It was observed that *C. rivularis* could tolerate salinity as low as 1–2 ppt for a short term (Zhang et al. 1960, Zhang & Xie 1960), as Nie (1991) reported its salinity range 1–32 ppt. Pure fresh water could cause mortality (Zhang et al. 1960). An interesting exception to the normal distribution of *C. ariakensis* was reported by Chen (1991) for Northern Jiangsu. The silty coast of Jiangsu province was not originally suitable for *O. rivularis*. Actually, few oysters were found in this province. Things changed when *Spartina anglica* was introduced. It was planted discontinuously along the coast of Jiangsu province, and by 1991, it occupied 377 km of coastline and 180 km² coastal area of the province. This plantation changed the local ecology. Chen reported that this plant kept clay around its growing area and gradually formed small ridges and backwaters in that area, which he believed was a critical condition for these oysters. *O. rivularis* was found at the seaward boundary of the *S. anglica* planting area, which was between high and middle tide mark with one-third to one-half time exposure. The density of its distribution was as high as 107 per m² and the average shell height of adult *O. rivularis* was 19.5 cm.

In India, *C. rivularis* was found on both hard grounds and in muddy creeks (Mahadevan 1987, Patel & Jetani 1991). Patel and Jetani (1991) reported its preference of muddy rocks, rocks covered by 3–4 inches of mud, although we have to think that settlement preceded the mud deposits. This oyster has been found in groups of four to five large and small individuals attached to isolated rocks and coral stones that came up in trawl-nets (Durve
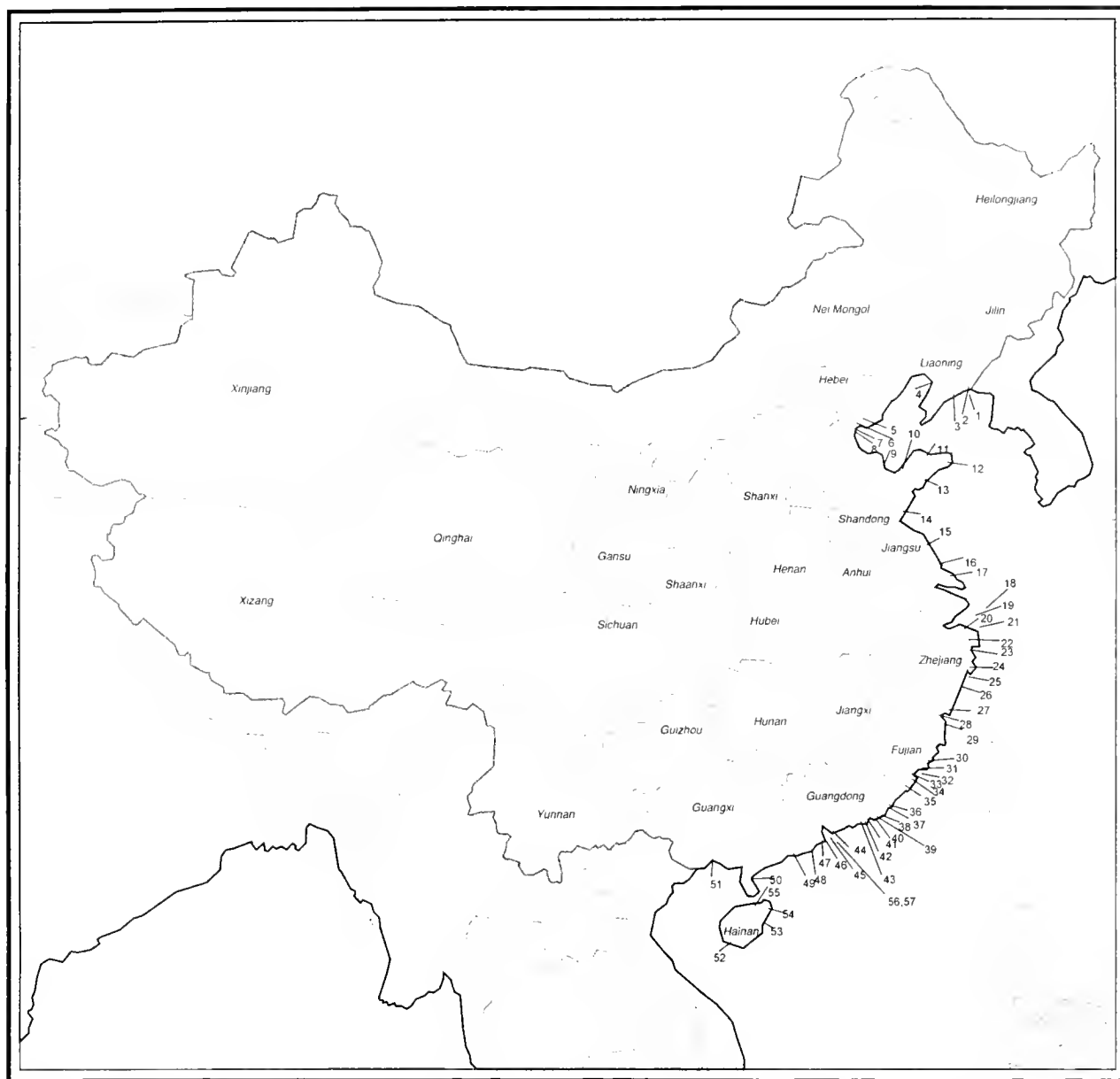
Figure 2. Locations reported with *C. ariakensis* populations in China. No distinction is made between aquaculture sites and natural populations. Underlined sites are considered major production areas. 1. Xindao (dao: island); 2. Andong (Dadonggou); 3. Zhuanghe; 4. Gaiping; 5. Fengnan; 6. Ninghe; 7. Beitang; 8. Tanggukou; 9. Yangjiaogou; 10. Yexian; 11. Yantai; 12. Rongchen; 13. Dingzigang; 14. Shijiusuo; 15. Sheyang; 16. Jianggang Bay; 17. Rudong; 18. Huijiao; 19. Daishan; 20. Zhenhai; 21. Dinghai; 22. Meilin; 23. Sanmen; 24. Wenling; 25. Leqing Bay; 26. Wenzhou Bay; 27. Xiapu; 28. Ningde; 29. Luoyuan Bay; 30. Huian; 31. Tongan; 32. Xiamen; 33. Longhai; 34. Haicheng; 35. Yunxiao; 36. Shantou; 37. Haimen; 38. Lanhiao, Huilai County; 39. Jiazi; 40. Jieshi; 41. Gaolno; 42. Shanwei ; 43. Qingcao; 44. Baoan; 45. Xiangzhou; 46. Tangjiahuan; 47. Nanshui; 48. Hengshan; 49. Zhanjiang Bay; 50. Qinzhouwan(Longmen); 51. Baoping Bay; 52. Boao; 53. Qinglangang; 54. Qiongshan; 55. Lofu Shan; and 56. Deep Bay.

1986) or solitary (unattached) in the littoral zone (Awati & Rai 1931). The vertical range of *C. rivularis* was described as the littoral zone (Awati & Rai 1931), sublittoral low waterline area or submerged offshore area (Durve 1986), intertidal (Mahadevan 1987, Rao 1987) or tidal region (Patel & Jetani 1991) and also at 9–15 m depth (Durve 1986).

In Pakistan, the preferred habitats of *C. rivularis* are the backwaters and creeks along the coast (Moazzam & Rizvi 1983). It seems that this species thrives in muddy environments (Ahmed

1971, Asif 1978b, Ahmed et al. 1987) and adheres to hard substrate such as stones (Ahmed et al. 1987). It occurs near the low water mark (Ahmed 1971, 1975, Ahmed et al. 1987, Barkati & Khan 1987) and the preferred tidal height for spat settlement is 0.5 ft mark (Ahmed et al. 1987).

Predators, Harmful Organisms, and Diseases

According to Zhang and Lou (1956b), in China, "red tide" is generally most harmful to oysters. It caused 50% mortality of

TABLE 3.

Locations where C. (O.) rivularis was reported in China.

Province	Locations Where C. (O.) rivularis was reported
Liaoning	Gaiping, Andong (Dadonggou), Xindao, Zhuanghe (Zhang & Lou 1959)
Hebei	Fengnan, Tanggukou, Beitang (Zhang & Lou 1959)
Tianjin City	Ninghe (Zhao et al. 1991)
Shandong	Rongchen (Zhang & Lou 1956b)
	Yangjiaogou, Dingzigang (Zhang & Lou 1956b, 1959)
	Shijiusuo (Zhang & Lou 1959)
	Yantai, Yexian (Zhao et al. 1991)
Jiangsu	Sheyang, Rudong (Zhang & Lou 1959)
	Northern coast (north of Jianggang Bay; Chen 1991)
Zhejiang	Sanmen (Zhang & Lou 1956b)
	Zhenhai, Daishan, Huijiano, Dinghai, Meilin, Wenling (Zhang & Lou 1959)
	Wenzhou Bay (Huang et al. 1981)
	Leqing Bay (Zhou et al. 1982)
Fujian	Xiamen (Zhang & Lou 1956b, 1959)
	Tongan, Haicheng (Zhang & Lou 1959)
	Luoyuan Bay (Xu et al. 1992)
	Yunxiao, Longhai, Huian, Ningde, Xiapu (Cai 1966)
Guangdong	Shanwei, Lanbiao (Zhang & Lou 1956b)
	Baoan, Tangjiahuan, Hengshan (Zhang & Lou 1956b, 1959)
	Shantou, Jiazi, Jieshi, Haimen, Nanshui (Zhang & Lou 1959)
	Qingcao, Gaoluo, Xiangzhou (Zhang et al. 1960)
	Zhanjiang Bay (Cai et al. 1992)
	Peal River estuary (Guan & Li 1986)
Guangxi	Longmen (Zhang & Lou 1959)
Hainan	Baoping Bay (Zhang & Lou 1956b, 1959)
	Qiongshan, Qinglangang, Boao, (Zhang & Lou 1959)
Hong Kong	Lofu Shan (Ke & Wang 2001)
	Deep Bay (Mok 1974)

cultured oysters in Baoan, Guangdong Province in 1953. Red tide could be caused by *Noctiluca* sp. diatom or the more harmful *Ditylum* sp. The carnivorous oyster drills *Thais gradata* (known as "huluo," which means tiger snail in China) and *Naticidae* sp. (known as "yuluo," which means jade snail) are also very harmful to oysters. Tiger snail can drill through the shell of a spat in 3 min and in 8 h for a 3-y-old oyster (Wu et al. 1997). Beside these, carnivorous crabs, such as *Scylla, Portunidae, Lithodidae,* sea urchin *Echinoidea,* and sea star *Aseroidea,* are also harmful to spat.

Below we list the available reports on these subject areas by publication year.

Harmful organisms to C. rivularis cultured in Zhanjiang Bay, Guangdong Province, China (Cai et al. 1992)

The effects of the predator *T. gradata* and *Balanus* spp. were reported in an important estuary for aquaculture. *T. gradata* was found harmful to 1-y-old oysters. Its density on oyster cultch could be as high as seven individuals/m^2. Mortality caused by *T. gradata* could be as high as 31%, 14% on average. *T. gradata* preferred living in groups, usually hiding in the shaded area of concrete posts. Its reproductive season was from the beginning of April to the middle of June peaking from the beginning of April to the beginning of May. Each female carried 50–100 oospores, with about 100 eggs in each oospore. Hatchability was very high, al-

most 100%. Incubation period was about 15–30 days. Barnacle *Balanus* spp. competed for setting space and food. In the worst situation, the oyster seed could be smothered with a total covering of *Balanus* spp. *Balanus* spp. set increased from the upper estuarine area toward the lower saltier regions. Highest density occurred in the low intertidal area. *Balanus* spp. larvae preferred the sunny side of a setting place.

Mass mortality putatively caused by Prorocentrum sp. bloom in Zhanjiang, South China (Zhang et al. 1995)

From late April to late May 1994, an episode of high mortality occurred at an *O. rivularis* farm close to the port of Zhanjiang, Fujian Province, South China. Mortality reached 98% over about 25 hectares. Water sampling and histopathological monitoring was conducted. During the outbreak, the water temperature increased from 18 to 30°C, pH fluctuated between 6.5 and 7.0, and salinity ranged 25.6–29.1 ppt. The water was blue-brown in color and all water samples revealed variable concentrations of phytoplankton, of which 96% were composed of *Prorocentrum* sp. with concentrations of 201–667 cells/mL over the period of observation. The temporal association of the mass mortality and a *Prorocentrum* bloom suggested that the bloom was probably the cause of the mortality. This assumption is supported by the histopathological findings that suggest toxicosis. In particular, the observed lesions were acute and corresponded with the outbreak.

Affected oysters were gray in color and had a softer than normal texture. The most outstanding microscopic lesion was intense accumulation of hemocytes in and around hemolymph channels, especially in the Leydig tissue. Close examination of the larger vessels revealed that hemocytes were actively infiltrating the vessel walls, as well as involved in transmigration into the Leydig tissue and the formation of intravascular thrombi. A diffuse, and less intense, hemocytosis was present in the interstitium between the digestive tubules, while a mild hemocytosis was detected in the gills. Oedematous changes were prominent around the digestive tubules and in the Leydig tissues where they were accompanied by tissue necrosis/lysis. The digestive tubules were empty and their epithelia were dysplastic, varying from low columnar to cuboidal and in some instances there was necrosis of the tubular epithelium. Brown cells were particularly prominent in the intertubular tissues. The pathology was consistent with a systemic toxicosis resulting from absorption of toxins from the digestive gland.

Bonamia-like parasite found in C. rivularis reared in France (Cochennec et al. 1998)

C. rivularis was imported from the Haskin Shellfish Research Laboratory in New Jersey in 1994. Seven months after introduction, some mortality occurred in quarantine. Histologic examination revealed the presence of an intracellular protozoan parasite in the connective tissues of nine dead specimens. Ultrastructure analysis suggested that the protozoan might belong to the genus *Bonamia. Bonamia* was likely transmitted to the experimental oysters from neighboring waters, which are endemic for bonamiosis, possibly when inlet water treatment lapsed.

An intracellular procaryotic micornganism associated with lesions in C. ariakensis in Pearl River estuary, South China (Wu & Pan 2000)

A series of mortalities of cultured oysters have occurred in Pearl River estuary since 1992, usually from February to May. The mortality peaks at 80–90% during April and May. The diseased
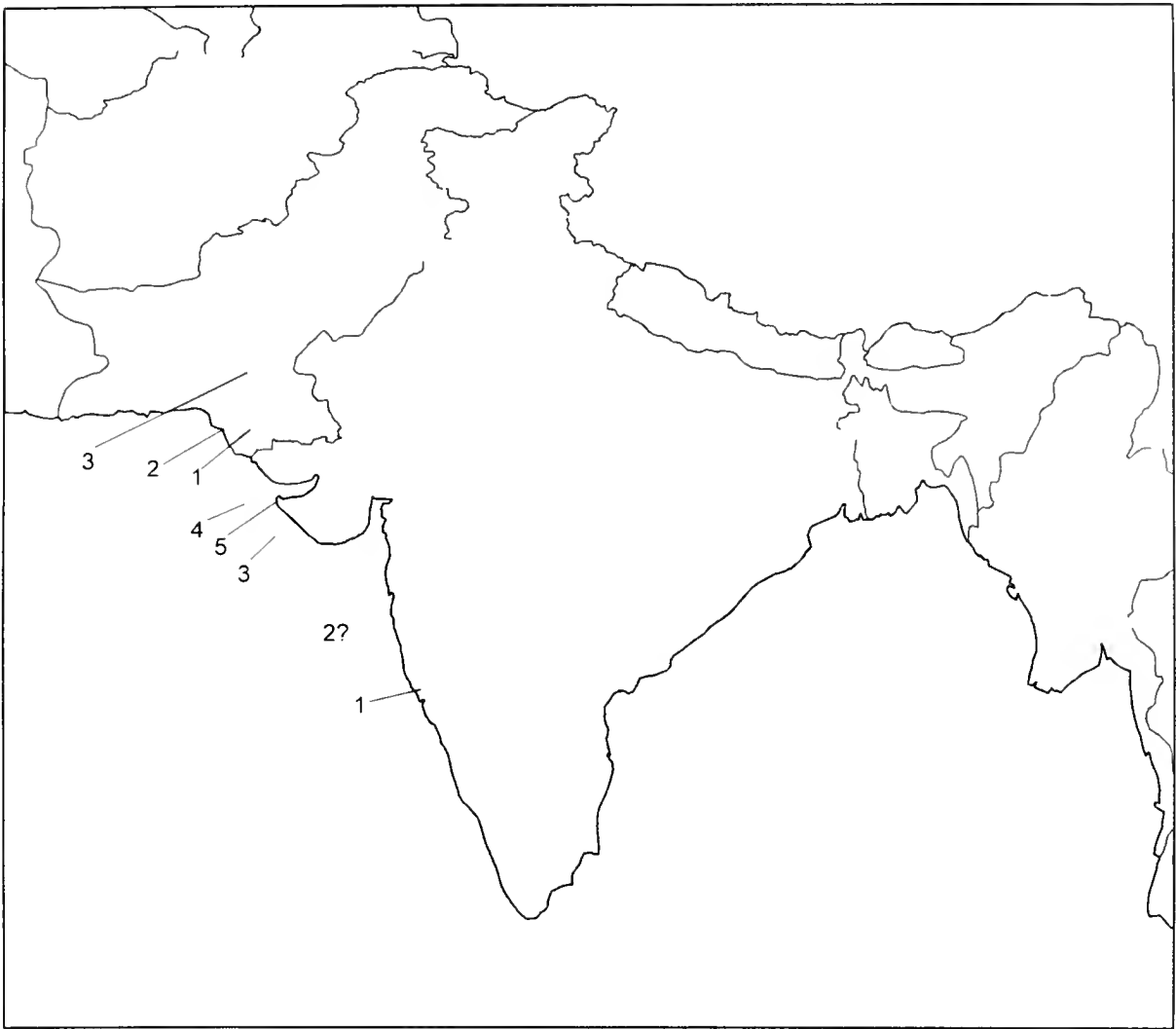
Figure 3. Locations reported with *C. ariakensis* populations in India and Pakistan. India: 1. Ratnagiri (16N, 73E); 2. Balapur (not located); 3. Porbander (Porbundar), Navibander (21N, 69E); 4. Dwarka (Gomati Creek) (22N, 68E); 5. Okha, Aramda Creek, Poshetra, Port Okha, Sikka (22N, 69E). Pakistan: 1. Korangi Creek (24N, 67E); 2. Karachi (24N, 64E); and 3. Port Qasim (27N, 68E).

oysters are generally aged 2–7 y. A rickettsia-like intracellular microorganism is present in the tissue of diseased oysters.

PHYSIOLOGY

Natural Reproduction

Hermaphroditism and Sex Reversal

Crassostrea are oviparous and protrandric hermaphrodites (c.f., Coe 1934). The occurrence of true hermaphrodites (both sexes simultaneously) is rare. Hasan (1960) stated that hermaphrodites do not exist in *O. discoidea* (= *C. rivularis*). In a study of hermaphroditism and sex reversal in *C. rivularis* from the coast of Karachi, Pakistan, true hermaphrodites were absent (Asif, 1979). Hermaphrodites observed were actually transitional stages of the sexes and used to study sex reversal. According to Asif, gonad generally appeared in *C. rivularis* at the age of 2–3 mo at a length of 0.4–0.6 cm and 62% were male. Protandric hermaphrodites were found in summer and autumn, which indicates the time of sex reversal. The percentage of males declines gradually with increasing size as is true for other *Crassostrea* spp. Cai et al. (1992) also

claimed that sex ratio of *C. rivularis* had an obvious regular change during the reproductive season (usually summer and autumn) and the ratio of females to males increased as the oysters got older. Hasan (1960) also mentioned that individuals with undistinguishable sex are fairly common throughout the spawning season. In Asif's study, the percentage of females increased over males beyond the size class 5.0–5.9 cm.

Spawning

Importance of temperature in gonad maturity and spawning of oysters is well known. Temperature influences the development of gonad (Orton 1936, Spark 1925, Nelson 1928). Temperature also directly influences the abundance of food, which is necessary for the development of gonad (Loosanoff & Engle 1942, Loosanoff & Tommer 1948). Periodic examinations of the gonad of *O. discoidea* showed that normal growth of the reproductive products was coincident with gradual rise of water temperature and food abundance in the summer months (Hasan 1960).

The combined effect of temperature and salinity on the start of

spawning was discussed by Hornell (1910, cited from Hasan, 1960) and confirmed by Hasan (1960) through an experiment on *O. discoidea* in Pakistan. The rise in water temperature helps the development of gonad, while decrease in salinity stimulates the gonad for spawning. Cai et al. (1992) also mentioned that oyster reproduction is closely related to environmental conditions. High temperature and low salinity could cause mass spawning of *C. rivularis* in Zhanjiang Bay, Guangdong province. Hu et al. (1994) presented a more detailed and slightly different discussion in his study of *C. rivularis* spat collection in Jiulong River estuary, Fujian province. He agreed that spawning is related to the change of water temperature and salinity. Water temperature could change with wind direction or strength. Salinity could be changed by precipitation, water current, and tides. However, he seemed to believe that simply a change of water temperature and salinity could be the trigger for spawning, whether an increase or decrease. According to his observation, whenever the tide changed from neap to spring, spring to neap, or during spring tide, oysters would spawn, as long as their gonad was well developed. If the wind direction happened to change from northeast to southwest, or cold air happened to pass by, spawning would increase. He explained that a temperature change of only about 1–2°C would stimulate *C. rivularis* to spawn.

Hasan (1960) studied two natural *O. discoidea* beds at Waugudar Creek, Pakistan. Spawning starts by the first week of July when temperature was about 28–29°C and salinity about 24 ppt. Number of spawning individuals remains almost constant during August and September, much reduced in November and almost nil in December

Several authors talked about reproduction of *C. rivularis* from China. According to Zhang and Lou (1956a), the optimum salinity for reproduction of *C. rivularis* is 10–25 ppt in China. Hu et al. (1994) reported that in Jiulong River estuary, Fujian province, gonad maturity reaches its peak from the middle of April until mid-May. Oysters spawn twice each year: spring spawn is from May to June and fall spawn, from the end of October to the beginning of December. During spring spawn, water temperature fluctuated between 20 and 30°C, salinity 5–25 ppt. Guan and Li (1986) mentioned that in Zhujiang River estuary, Guangdong province, the reproductive season is from June to September. Spawning is mainly during June and July. There might be a second spawning if appropriate environmental conditions are available. Guan and Li did not report the environmental conditions associated with spawning. Cai et al. (1992) reported that the reproductive season is generally from the beginning of April to the middle or end of June in Zhanjiang Bay, Guangdong. Environmental conditions in the study area (Shimen) are listed as follows: Annual water temperature ranged from 14 to 31.8°C. Daily water temperature changed 2 to 4°C. Water temperature was highest in June and lowest in January. Salinity ranged from 7.52 to 22.18 ppt in summer (but could drop to 0.00 ppt when flooded), 18 to 30 ppt in winter. pH ranged from 7.1 to 7.9 in summer and 7.9 to 8.1 in winter. Zhang et al. (1960) mentioned that reproduction occurred year round in South China Sea area. The reproductive peak is from late May to early September. Zhang et al. did not report environmental conditions during this time period.

According to Tanaka (1954), the spawning season of *O. rivularis* ranges from late May (20–22°C) to early September (28–26.5°C) in Ariake Bay, Japan. There are three major spawning periods during this season: early June (22–23°C), late June to early July (24–26°C), and the beginning to middle of August (30–

28.5°C). The eggs of *O. rivularis* measure 49–53 μm in diameter. The relation between salinity and developmental condition is shown in Table 4. The temperature varied from 24 to 27°C (Amemiya 1928). The above results are nearly identical to those of Huzimori (1920, cited from Amemiya, 1928).

Spatfall

The preferred tidal height of settlement for *C. rivularis* spat was reported to be at the 0.5 ft mark in Pakistan (Ahmed et al. 1987). A broader range was reported from China by Nie (1991): from the low tide line to a depth of 10 m, with the maximum setting at ± 0.4 m low water mark. Hu et al. (1994) reported the optimal water depth for spat collection is from the low tide mark to a depth of 1 m in Jiulong River estuary, China. Larvae settle 12–18 days after spawning. In southern China, spatfall occurs from June to August, the period of highest temperature and lowest salinity (Nie 1991, Cai & Li 1990).

Three reports on spatfall seasons from Pakistan are summarized below. One study was conducted at Paradise Point situated on the west coast of Karachi (Moazzam & Rizvi 1983). This is basically a rocky shore having frequent stretches of boulders and sand. The subtidal area along this shore is generally more deeply inclined than the rest of the coast. This is also a power plant site. *C. rivularis* occurs in the cooling system of the power plant, which has been made artificially "protected" and simulates conditions of a backwater environment. The environment conditions were reported as follows. Temperature dropped to its minimum of 20–22°C in December–January and reached its maximum of 28–30°C in June–July. Salinity remained fairly constant (35–36 ppt) except during the short spell of rains in July–August when salinity dropped to 28 ppt. The contents of suspended matter fluctuated between 0.003 mg/L in November and 0.116 mg/L in June. Transparency was less than 1 m in June–July. Maximum settlement of *C. rivularis* occurred in June and September–October. A considerable number were also observed in July–August.

The second report came from two natural oyster beds (Hasan 1960). One is situated between Korangi and Kadero creeks, south of the village Vagudar and about 16 miles southeast of Karachi. The other one is about 6 miles south of Dhabeji. The temperature and salinity profile were reported from Vagudar creeks. Temperature profile looks very similar to the one from the above report, except that it dropped even lower to 16–17°C in January. Salinity was reported only from April to September, with a maximum of 36–37 ppt in April–May and then dropped continuously to 21–22 ppt in September. The pattern of larval settlement of *O. discoidea* in this report is different from the one mentioned above. Settlement at Vagudar Creek occurred from July to December with mid-

TABLE 4.

Relationship between salinity and developmental condition, according to Amemiya 1928.

Salinity ppt	Sp. gr. at 0°C	Condition
ca. 7	ca. 1.0056	Minimum salinity
8–14	1.0064–1.0112	Much too low salinity
15–18	1.0120–1.0144	Too low salinity
19–25	1.0153–1.0200	Optimum salinity
26–30	1.0209–1.0241	Too high salinity
31–33	1.0249–1.0256	Much too high salinity
ca. 34	ca. 1.0273	Maximum salinity

September being the peak period. Moazzam and Rizvi related setting failure to the presence of high contents of suspended matter in seawater during the southwest monsoon period (June–September). This high content of suspended matter is believed to interfere with larval settlement of many invertebrates in this area (Ahmed et al. 1978).

The third report came form the Gharo-phitti saltwater creek system (Ahmed et al. 1987). Spat fall occurred from April to October with peak settlement from April to July. The maximum settlement occurred during the period June 24 to July 23. No environmental conditions were given in this report.

Growth

Growth Rate

C. ariakensis is well known for fast growth. In Pakistan, C. rivularis spat reached the size of 0.5 mm in about one week and 2.0 cm in about 1 mo (Ahmed et al. 1987). Hasan (1960) found that a size of 3.0 cm was reached 2 mo after settlement. In about one and half years, they become ready for market. Temperature and salinity data of Hasan's study is shown in the spatfall section. In China, C. rivularis can growth to 10–16 cm in 2 to 3 y (Zhang & Lou 1956b). In Japan, it attains full size (20 cm) in 2 or 3 y (Amemiya 1928). The results of Fujimori's study (1929) on the growth rate of O. rivularis was presented in two parts: spat / young oysters and the sexual adult. Fujimori found that the growth rate of the spat varies considerably according to their time of attachment. The size of adult O. rivularis in Kyushu was 5.5 cm shell height at 1 y, 9.7 cm at 2 y, 12.4 cm at 3 y, 15.2 cm at 4 y, 17.9 cm at 5 yr, and 19.7 cm at 6 y. In Japan, growth was most rapid in August and September (Cahn 1950). Environmental conditions were unavailable for the above reports, if not mentioned.

Shell Dimension

C. ariakensis reaches a large size. As Cahn (1950) mentioned, the maximum size attained by this species according to the literature is 257 mm with an estimated age of 20 y. The maximum length he recorded in Japan was 240 mm. A maximum shell height of about 200 mm was reported several times from Japan and the United States (Amemiya 1928, Hirase 1930, Coan et al. 1995). According to the growth rate of adult O. rivularis determined by Fujimori (1929), the estimated age of such size is more than 6 y old. Generally, adult specimens reach 6–7 inches (or 150–170 mm) in height, as reported from four countries (Hirase 1936, Galtsoff 1964, Ahmed 1971, Rao 1987).

Allometric Growth

A study of the allometric (relative growth) relationship between shells and tissues of C. rivularis was presented by Barkati and Khan (1987) from Pakistan. Shell length was defined as the maximum distance between the tip of the anterior margin and the posterior margin. Shell width was defined as the maximum distance between the lateral margins. The following points were reported. Shell width increased faster than shell length ($r = 0.85$). Shell length increased faster than dry tissue weight ($r = 0.52$). An exponential relationship exists between shell length and shell weight with faster growth in length compared with shell weight ($r = 0.84$). Dry tissue weight increased faster than shell weight ($r = 0.74$). Condition index (the proportion of dry tissue weight to total dry weight of shell and dry tissue) increased with increasing shell length ($r = 0.41$). No linear variable was useful to accurately predict other variables due to low coefficient of correlation (r),

probably due to irregular growth in various shell dimensions (length and width).

For example, Asif (1978b) reported variation in shell growth in two populations of C. rivularis caused by setting density in Pakistan. One population in Korangi Creek was exploited and densities were low. Another population in Sonari was crowded. In the Korangi Creek, the oysters are attached to rocks or stones horizontally, whereas those of Sonari grow upward with the umbo downwards. Generally, the wild stock of C. rivularis of the Korangi Creek are round and shallow whereas the Sonari population is elongated and deeply cupped. In the majority of the Korangi Creek population, height plus width varies closely with length of the shell while in the Sonari population, shell height plus width varies twice as much as the length.

Feeding

Food Selectivity

According to Cai et al. (1992), C. rivularis (collected in Zhanjiang Bay, Guangdong Province, China) is a selective feeder. It preferred small articles to long-chain groups or large articles. The majority of its food is composed of phytoplankton such as Coscinodiscus sp., Nitzschia sp. and Cyclotella sp.

Feeding Habits

Zhang et al. (1959) did an extensive study on the feeding habits of O. rivularis in relation to time, tides, season (change of temperature and salinity) and suspended particles. The experiment was conducted in the Pearl River estuary and some nearby bays. Most of the sampled oysters were 3 to 4 y old at the time of examination. These oysters were collected from the wild as spat and cultivated in oyster farms. The percent of O. rivularis that are feeding at any given time (incidence of feeding) was not related to periods of light and darkness, nor to the periods of tides, or the density of suspended particles. Salinity and temperature did have certain influences, as summarized below.

According to examinations at five different times of the year, the highest average incidence of feeding for O. rivularis was a little more than 80%. It was also found that feeding time of O. rivularis adds up to 16–19 h everyday with irregular intervals. Feeding habits of O. rivularis were not related to change of sea level or direction or speed of water flow caused by tidal change.

In Pearl River estuary, feeding incidence of O. rivularis was highest from October to April (50–100%), when temperature ranges between 10 and 25°C and salinity between 15 and 30 ppt. During summer, the natural reproductive season of O. rivularis, when temperature is much higher (22–30°C) and salinity is much lower (3–26 ppt), feeding incidence is lower (0–70%). Feeding incidence seems to be more closely related to salinity according to monthly records. Although O. rivularis is known to tolerate low salinity, feeding rate was significantly retarded if salinity was lower than 5 ppt. Above 10 ppt, feeding was active.

Increase in suspended particles in the seawater (higher turbidity) failed to influence feeding incidence of O. rivularis. In this case, the authors maintained that these suspended particles served as a food source for the oysters.

Oxygen Consumption

Guan and Li (1988) did an extensive study on oxygen consumption of C. rivularis. A Warburg manometer was used to measure the oxygen consumption of dissected gill tissue of C. rivularis taken from the Shenzhen Bay Oyster Farm. Oxygen consumption

varied with the change of seawater temperature. A negative correlation was found between oxygen consumption and the oyster age. The older and heavier the oyster, the less oxygen was consumed by its gill tissue. Oxygen consumption differed significantly in different reproductive periods.

BIOCHEMISTRY

Biochemical composition

Qasim et al. (1985) determined the following biochemical parameters for *C. rivularis* from Pakistan. Water contributes 78% of soft body wet weight. Of soft body dry weight, 35.7% was crude protein, 22.5% glycogen, 23% lipid, and 11.2% total inorganic substances. These are the averages from sampling over a period of time (sample interval was not stated in the article). Higher value for lipids (31%) was reported from India (Patel 1979, cited from Qasim et al. 1985). This difference is probably the result of geographical variation, seasonal variation, or both.

Qasim et al. (1985) mentioned that the ratio between glycogen and protein changes with reproductive state of an oyster (no specific information available). Another report on biochemical indexes of *C. rivularis* from the Pearl River estuary, China (Guan & Li 1986) showed seasonal change of lipid content and its close relationship with reproductive physiology of the oysters. As the authors discussed, reproductive season in the Pearl River estuary is from June to September, of which June and July are primary spawning periods. There could be a second spawning in September if environmental conditions were appropriate. In their study, lipid content was highest in May (2.88% of wet weight), then dropped dramatically from June until it reached the lowest point 1.06% in October, the end of the reproductive season.

For protein, amino acid profile determines the nutritive quality of tissues. Such a profile of *C. rivularis* tissue protein has been reported from the Pearl River estuary, China (Guan & Li 1986) and Pakistan (Aftab 1988). There are only slight differences between the two reports. From China, specimens were tested in May, and the amino acid profiles are presented in Table 5 (Guan & Li 1986). Glutamine and asparagines are most abundant. From Pakistan, 14 amino acids were analyzed. Methionine and arginine were not detected. Glycine and aspartic acids were most abundant. Seasonal variation in bound amino acid content is shown in Table 6 (from Aftab 1988)

The shells of *O. rivularis* have been used as traditional Chinese medicine. Zhao et al. (1991) examined the content of calcium carbonate, trace elements and amino acids in shells of *O. rivularis* collected from Tianjin, Shandong, Zhejiang, and Fujian provinces. Calcium carbonate in raw shells was 92.0–95.5% and in calcined shells, 96.4–96.9%. Calcined shells have had organic materials removed. The raw shells contain large amounts of Ca, small amounts of Mg, Na, Sr, Fe, Al, Si, and traces of Ti, Mn, Ba, Cu, etc. Shell decoctions (an extract obtained by boiling the shells) contain small amounts of Ca, Na, Mg, K, and trace element of Sr, P, Pb, Zn, Ni, V, Ba, Li, Mn, Ti, Cu, Cr, Mo, As, Hg, etc. The oyster shells contain 17 amino acids. Total amino acid content amounted to 0.16 to 0.24% in raw shells.

Li et al. (1994) studied the medicinal value of "oyster complete nutritional tablet," a dietary supplement made from extracts of both shells and soft body of *O. gigas* and *O. rivularis* from South China Sea. The tablet contains a high content of eighteen amino acids, especially the eight essential to the human body. Putative benefits are attributed to the liver, kidney, spleen and intestine to a certain extent.

TABLE 5.

The amino acid compositions and their contents in *C. rivularis* sampled in May, 1984 (Guan & Li 1986).

Amino Acid	Contents in Dried Samples (%)
Alanine	2.04
Arginine	1.95
Asparagine	3.30
Cystine	0.28
Glutamine	4.06
Glycine	2.15
Histidine	0.76
Isoleucine	1.18
Leucine	1.87
Lysine	2.23
Methionine	0.57
Phenylalanine	1.05
Serine	1.39
Threonine	1.48
Tyrosine	1.34
Valine	1.32

Heavy Metals and Toxins

Lu (1994) did a preliminary study on the feasibility of using *O. rivularis* as a monitoring agent for heavy metals, like Cu, Zn, Cd, Pb, along the Guangdong coast, China. He found that profiles of Cu, Zn and Cd content in the oyster correlated with the distribution of industrial discharge along Guangdong province. Also see Ke and Wang 2001. Further investigations on the suitability of *O. rivularis* as a biomonitor of specific metals or other chemicals are presented below.

Zn

According to Lu et al. (1998a), Zn accumulated continuously in the tissues of the oyster through 12 days of exposure. Accumulation was linear with time. Loss of Zn from *C. rivularis* was not observed over 35 days of depuration. Zn accumulated less readily with increasing salinity. The author concluded that in general *C. rivularis* is a reliable indicator of Zn in marine systems.

TABLE 6.

Seasonal variation in the protein and amino acid composition of tissue protein hydrolysate of *C. rivularis* (Aftab 1988).

Component	February	May	August	November	Average
Protein % d.w.	40.56	41.25	52.50	55.00	47.33
Alanine	6.04	6.91	9.00	9.67	7.90
Aspartic acid	6.78	9.83	12.56	6.58	8.94
Glutamic acid	6.30	7.98	11.26	5.08	7.65
Glycine	13.27	11.88	6.55	9.10	12.10
Histidine	2.07	1.87	2.72	1.91	2.14
Isoleucine	2.38	1.80	3.12	2.08	2.32
Leucine	3.98	2.89	5.34	3.63	3.96
Lysine	1.59	1.74	1.44	1.28	1.51
Phenylalanine	1.75	1.30	1.94	1.55	1.63
Proline	0.46	2.05	0.79	2.40	1.43
Serine	4.07	6.01	7.82	3.51	5.35
Threonine	3.85	5.74	6.92	3.24	4.93
Tyrosine	0.85	0.60	0.91	0.79	0.79
Valine	3.64	3.75	5.55	2.98	3.98

Cd

Lu et al. (1998b) studied Cd absorption in *C. rivularis*. The content of Cd in body tissues of *C. rivularis* accumulates in linear proportion to Cd concentration in the water and to exposure time. Accumulated Cd attenuates slowly with a biologic half-life of 77 days. With increased salinity, rate of accumulation decreases while rate of Cd loss slows down. *C. rivularis* seems to be a reliable bio-monitor of Cd pollution.

Cu

Cu absorption in *C. rivularis* was examined by Lu et al. (1998c). It continuously accumulated in the tissues of the oyster through the exposure to a concentration of 100 µg/L over 12 days. Accumulation was linear with time and decline of Cu concentration was slow, with a half-life about 131days. Rate of Cu accumulation was significantly slower with increased salinity, but rate of decline in Cu concentration was not significantly related to salinity.

Total Petroleum Hydrocarbons (TPHs)

Lin et al. (1991) looked at concentration of TPHs in the Pearl River estuary, China. TPHs in *C. rivularis* tissues decreased with time during the period leading to sexual maturity. The rate of decrease was about 0.24 µg/g, dry weight. The biologic half-life was 43 days. Aromatic hydrocarbon compounds with smaller molecular weight were released sooner from oyster tissues than those with greater molecular weight. The concentrations of TPHs in oyster tissues were not significantly related to those in waters and sediments, and not clearly dependent on the contents of lipids in oyster tissues during the study period (September 1986 until February 1987).

GENETICS

Karyotype

So far, research on the cupped oyster species of the genus *Crassostrea* shows a common diploid chromosome number of $2n = 20$, and their karyotypes include only metacentric and submetacentric chromosomes. The proportion of these chromosome types can be different interspecifically (Leitão et al. 1999).

Chromosome number of $2n = 20$ was confirmed in *C. ariakensis* (Ieyama 1975) and in *C. rivularis* from West Pakistan (Ahmed 1973) and China (Yu et al. 1993). Yu et al. reported the karyotype of *C. rivularis* sampled in Southern China had 10 metacentric pairs. A more recent karyological study (Leitão et al. 1999) on an American population of *C. ariakensis* originally introduced from Japan shows that it consists of eight metacentric and two submetacentric (nos. 4 and 8) chromosome pairs. A variable number of one to three Ag-NORs (nucleolus organizer regions) was observed terminally on the metacentric pairs 9 and 10. About 68% of the silver stained metaphases showed Ag-NORs only on pair 10.

Polyploidy

Rong et al. (1994) reported their attempts to produce tetraploid *C. rivularis*. Newly fertilized eggs of *C. rivularis* from south China were treated with physical and chemical methods in the first three minutes before the cleavage of zygotes or at the onset of first cleavage. Induction rates of tetraploids were 28% for heat shock, 30% for cold shock, 28% for chlorpromazinum treatment and 35.8% for "traditional Chinese medicine" treatment as indicated by chromosome spreads from larvae. Production of viable spat was not reported.

Hybridization

Gaffney and Allen (1993) reviewed previous hybridization reports among *Crassostrea* species and pointed out that most of reports of successful hybridization suffer from one or more of the following: 1) ambiguities in classification; 2) possible contamination during spawning; 3) absence of experimental controls for assessing the quality of gametes as well as larval viabilities; and 4) the absence of genetic confirmation of hybrid status. They conclude that there was virtually no unequivocal evidence for the formation of viable interspecific hybrids among *Crassostrea* species.

Early studies on cross-fertilization between *C. gigas* and *C. rivularis* gained little success (Miyazaki 1939, Imai & Sakai 1961), but was reported successful by Zhou et al. (1982) and Downing (1988a,b, 1991). Asif (1978a) reported successful production of trochophore larvae 4–5 h for the cross of *C. rivularis* with *C. glomerata* and *Saccostrea cuccullata*. For the reasons mentioned above, these should be viewed with caution.

Hybridization of *C. gigas* and *C. rivularis* was re-examined by using specimens originally introduced from Japan to the United States (Allen & Gaffney 1993). Such crosses are of interest because of the disease resistant properties of these species (Calvo et al. 1999, 2001). In addition, the hardiness and apparent disease resistance of *C. gigas* and the high temperature, low salinity tolerance of *C. rivularis* could lead to promising variants for aquaculture, especially if the diploid is sterile. Three replicates of a 2 × 2 factorial mating of *C. gigas* and *C. rivularis* were produced to examine the viability of this cross. Fertilization rate, yield of 48-h-old larvae, and survival of fertilized eggs was lower in the hybrids than in pure crosses. All crosses showed similar larval growth rates, except *C. rivularis* (female) × *C. gigas*, which grew more slowly. Isozyme electrophoresis and flow cytometry confirmed hybridization. Triploid hybrids were produced using tetraploid *C. gigas* and diploid *C. ariakensis* (Que & Allen 2002).

Hybridization between *C. ariakensis* and *C. virginica* failed (Allen et al. 1993). Cytogenetic and electrophoretic analysis revealed the formation of hybrid zygotes and larvae between *C. virginica* and *C. rivularis*, but larval survival was limited to a maximum of 10 days. Larvae stopped growing at about day 4, reaching a maximum length of about 80 um. Studies on larval feeding using fluorescent beads indicated that growth limitation apparently was not caused by an inability to feed. Induced triploidy did not rescue hybrid failure.

Population Genetics

A number of studies have used molecular markers of various sorts to distinguish among *Crassostrea* species, including *C. ariakensis*. Among the earliest was work by Buroker et al. (1979) who estimated levels of genetic variation for six *Crassostrea* and three *Saccostrea* species based on electrophoretic variation in proteins in about 30 loci, *C. rivularis* among them. Liu and Dai (1998) used RAPD techniques to differentiate *C. talienwhanensis* and *C. plicatula* from *C. rivularis*. Li et al. (1988) used electrophoretic markers to separate four *Crassostrea* species, and concluded that the "white oyster" was *C. rivularis* and the "red oyster," *C. iredalai*.

C. rivularis was also among those used by Littlewood (1994) to establish the first phylogenetic estimates for this species based on nuclear DNA. Since then, a number of other studies employing

molecular markers have been applied to *C. ariakensis*, mostly to discriminate among species (O'Foighil et al. 1995, Gaffney & O'Biern 1996, Hedgecock et al. 1999, Francis et al. 2000). Hedgecock et al.'s study confirmed the occurrence of *C. ariakensis* in the northern regions of the Ariake Sea and re-emphasized the need for genetic confirmation for species identification.

AQUACULTURE

References to aquaculture of *C. ariakensis* come mainly from Japan and China, and are discussed accordingly.

Aquaculture in Japan

Of the five edible oysters species in Japan, only *O. gigas* and *O. rivularis* were cultured commercially (Cahn 1950). *O. rivularis* was second to *O. gigas* in commercial importance (Amemiya 1928)

According to Amemiya (1928), cultivation of *O. rivularis* began in Ariake Bay in the late 1890s and seed were later transplanted to Kozima Bay in Okayama Prefecture around 1928. An even earlier report of cultivation in Ariake Bay in the 1860s was given by Wakiya (1929). Both Wakiya and Langdon and Robinson (1996) mentioned that the culture of Suminoe oyster were conducted in the Suminoe river, Saga Prefecture from the beginning of the Meiji period in the mid-19th century. Discrepancy between Cahn and Wakiya on the start of *C. rivularis* aquaculture might rest on their definition of cultivation. Cahn (1950) described two types of culture systems at the mouth of the Suminoe-gawa ("gawa" in Japanese means river or stream), Ariake Bay, a primitive one and a more developed one. Cahn did not say when the primitive culture started, but he implied that the more sophisticated culture started after 1885. The primitive culture consisted simply of gathering natural oysters and storing the larger individuals for a short time on the bottom of the Sumino-gawa, later to be shipped to Nagasaki at the proper season for sale.

Aquaculture of *O. rivularis* began fortuitously. For some reason during the winter of 1884 these oysters were not shipped for sale to Nagasaki. The next year they were considerably larger by size and weight. From this observation, a new type of culture evolved in the local area. Young oysters about 2.5 cm in length were gathered from every possible growing place from July until March and were placed on oyster beds at the mouth of the river. To prevent loss, they were heaped close together in masses. They were washed and cleaned twice or three times each month during low tide. In April individual oysters were stuck in the mud vertically, hinge down and ventral margins uppermost. As the mud was very firm, the oysters fared and grew well. As they grew, they were thinned and replanted to give them more growing space. Growth was most rapid in August and September.

Aquaculture in China

C. rivularis is the most economically important marine shellfish species cultured in South China (Zhang et al. 1995), primarily in Fujian, Guangdong and Guangxi Province. The history of its culture in Guangdong is over 300 y old (Cai et al. 1979). The Pearl River (Zhujiang) estuary, Guangdong was considered the most famous cultivation site of this species (Zhang & Xie 1960). Some other places mentioned in the literature are Yangjiaogou, Shandong Province (Zhang et al. 1960), Leqing Bay, Zhejiang Province (Zhou et al. 1982) and in Deep Bay, Hong Kong (Mok 1974). In 1996, China produced 2.3 million tonnes of oysters from aquaculture, among which *C. rivularis* accounts for 20–30% (Guo et al.

1999). In Guangdong province, *C. rivularis* production was about 40% of total sea culture production (Qiu & Li 1983).

The primitive method of oyster culture was to improve growth and reproduction with procedures like fishing restrictions and protection from diseases and predators (Zhang & Xie 1960). The advanced method involves collecting natural spat and artificial grow-out. Modern oyster culture includes larval culture and breeding. Larval culture and breeding of *C. rivularis* larvae has been successfully accomplished on a research scale in South China (Liang et al. 1983, Cai et al. 1989) but has not been used in large-scale commercial culture. Hatchery production of seed is seen as a step to increase the reliability of seed production.

Spat collection and artificial grow-out is still the most popular. This is composed of four steps: spat collection, grow-out, fattening, and harvest. For spat collection, cultch material to collect spat was traditionally oyster shell and gravel (Nie 1991). Since the 1960s, cement plates (17–24 cm × 14–19 cm) or cement bars (40–80 cm long × 4–6 cm^2) reinforced with embedded bamboo stakes were used. Stakes are used increasingly since they are easier to handle, provide more surface area, and are not so readily covered by silt. Season and location of spat fall is summarized in Physiology. Oyster larvae in the water are monitored to ensure the best time of planting the clutch. Spat collectors are placed in rows in rectangular blocks, usually 30 to 37.5 × 10^3 stakes or 100 to 135 × 10^3 plates per hectare. Further details follow below for specific culture techniques.

The age of harvest is generally 3.5 to 4 y (Qiu & Li 1983), but varies from 2 to 5 y depending on culture location where the environment, the specific culture technique, and even the expected market size could be different. For example, Guo et al. (1999) reported 2 to 3 y in Guangxi where oysters maintain rapid growth throughout the first 3 y and are usually harvested at a size of 10–15 cm. The culture technique used there is concrete bars or shell strings hanging on rafts and long lines. In Pearl River estuary, Guangdong, oysters were usually harvested at 3 y of age by bamboo stake culture (Zhang & Xie 1960). Cai and Li (1990) reported the period to be 3 to 5 y in Southern China.

Cai and Li (1990) summarized oyster culture techniques in China. The ancient bottom culture techniques, including bamboo stake, stone and concrete culture, are still the major methods, but farmers are becoming increasingly aware of the advantage of off-bottom culture, like the rack and raft culture. The various techniques are described below (reproduced from Cai and Li's work, 1990).

Rock (Stone) Culture

Rock culture is usually applied in areas that have hard substrate. Marble flagstones approximately 90 cm × 25 cm wide and 10-cm thick are preferred for this method. Stones may be arranged one-by-one vertically, resembling tombstones or two stones may be arranged in an "A" shape. Three stones may be arranged to form a tripod. Average spacing between stone groups is 70 cm. Another choice of rock is irregularly shaped natural boulders of 4 to 5 kg. The traditional arrangement of the boulders, called "stars in the sky," involves uniform distribution over the substrate. Two modifications were used along the coast of Guangdong and Hainan Provinces. One is called "plum blossom" with five or six boulders grouped together. Another is called "small house" with three flagstones arranged to form a shed or an upside-down "U." Both kinds of rocks are thoroughly washed and then covered in limewash 10 days before use.

In Guangdong and Fujian Provinces, the rocks are set out in early May to June or in November. Maximum spatfall is expected in May. Spat collected in June is usually subject to heavy mortality due to high temperatures and strong sunlight during attachment. Spat collected late in the season usually grew poorly because of to low water temperatures. Oysters are grown to market size at the site of spat collection.

Approximately 60,000 stones are required for one hectare, and *C. rivularis* may be harvested in 3 to 5 y. Production is moderate, ranging from 750 to 3000 kg per hectare. The oysters grown on rocks are more subject to predation by starfish and other organisms than are oysters grown on stakes, so considerable time must be invested in predator control.

Concrete Culture

Prefabricated posts or tiles are a derivative of the traditional rock culture technique for the culture of *C. rivularis* and has been used since 1950 in Guangdong Province. Spatfall occurs most of the year, but optimum periods are April and May. To prevent the tiles or posts from sinking into the mud, they are removed and rearranged around May, September, and December. Concrete culture requires a 4-y cycle. Spat collection and growth occupies the first year from June to April. The second and the third years involve a cultivation period yearly from May to August. Market size is attained in 2.5 to 3 y and involves a progressive increase in the spacing of the concrete tiles or posts. The cultivation cycle is completed by a fattening period extending from September to January. For fattening, oysters are transferred from the spat collection/grow-out area to prime growing grounds, usually in the low intertidal zone. For this culture method, in Guangdong, harvest generally occurs in February to April of the fourth year, when growth rates begin to decline sharply. Expected production from the concrete method is 7.5 to 15 tons of meat per hectare.

Rack Culture

Since 1965, rack culture has been used to cultivate *C. rivularis* in Guangdong Province. The racks may be constructed of bamboo, wood, stone or concrete. Because wood and bamboo are rapidly destroyed by shipworms and stone is heavy and awkward to handle, concrete is preferred. The form of the rack varies greatly, but consists basically of members driven into the substrate to form a horizontal frame, which supports the oyster cultch 2.5 to 3 m above the substrate.

Several types of material are used for spat collection. The most popular one is punched oyster shells, separated by 3 cm bamboo or plastic spacers, and strung on 2 m lengths of galvanized wire or polypropylene line. Concrete tiles, approximately 10 cm² with a central hole, may be substituted for the oyster shell. Concrete poles between 70 and 130 cm in length may also be used. The cultch is suspended from the rack, with spacing proportional to the density of spat settlement and the character of the growing area. The number of racks accommodated varies widely between the growing sites. Production is estimated at 10 to 20 tons per hectare.

Raft Culture

According to Qiu and Li (1983), raft culture started in Japan in 1950. Since 1979, the Fisheries Research Institute of the South China Sea has conducted experimental raft culture of *C. rivularis* in Guangdong Province. The fattening period lasts from September to May, and three crops may be harvested, because 2 mos are sufficient under optimal seasonal conditions. The ratio of meat production to shell is some 60% higher in raft-fattened oysters than in oysters harvested directly from bottom culture.

C. rivularis can be marketed in less than 3 y using rafts, and that the condition factor will be increased by more that 22% and the meat quality will be superior to oysters cultivated by the traditional bottom methods (Qiu & Li 1983). Though initial costs are higher, the increased production and working advantages of floating raft culture are apparent, and it is expected that raft culture will account for a steadily increasing share of oyster production in China (Qiu & Li 1983). Nie (1991) also mentioned that raft culture gives faster growth and a higher yield. A raft of 84 m² will produce in 2 y what 667 m² of bottom culture will in 4 y. Rafts seem to withstand typhoons better than originally thought.

DISCUSSION

C. ariakensis shares many life history traits with other *Crassostrea* species. It is clearly an estuarine species with salinity tolerances similar to *C. virginica*. Its occurrence in river systems and apparent responsiveness to salinity changes for spawning cues suggests that its reproductive strategy is somewhat different than *C. virginica*. There are indications that larval behavior differs from that of *C. virginica* (M. Luckenbach, VIMS, pers. comm.), perhaps an adaptation to fluvial existence. Many other questions about its ecology are unanswered or incomplete and a number of research priorities have been identified (Rickards & Ticco 2002). One of the principal problems with extrapolating life history from the available literature is the uncertainty over species designation. Some reports are clearly referring to *C. ariakensis*, e.g., those from southeast China where aquaculture activity is concentrated and there is a long history of working with this species. Other reports are not so clearly *C. ariakensis*, especially ones deriving from western India and Pakistan. Also because of likely morphologic confusion, the geographic range for *C. ariakensis* is incompletely described. For example, it seems likely that its range should include the coast of Vietnam, yet there seem to be no direct accounts of this. There are accounts of its occurrence as far as Borneo, the Philippines, and Thailand, but these are unconfirmed. From a practical standpoint, *C. ariakensis* from China are probably an appropriate starting stock for an introduction, should that proceed, because of similarities in latitude. From that respect, this area seems a most appropriate focus for obtaining more information on the species. Korea and Japan are possible sources as well. We did not encounter reports of *C. ariakensis* from Korea except as casual remarks. Stocks in Japan seem to be limited in abundance.

It is unclear whether *C. ariakensis* is a "reef-forming" oyster, depending on how you define "reef." Clearly, *Crassostrea* species, and oysters in general, benefit from aggregation and adults or their shells provide substrate for recruitment in subsequent generations. Some accounts of *C. ariakensis* describe "oyster hills" that would clearly qualify as reefs (Zhang & Lou 1956b, Zhang et al. 1960). Apparently, it is common knowledge among fishermen in China that *C. ariakensis* forms reefs. Other accounts have *C. ariakensis* occurring as small aggregates and singles. In our travels to China, we encountered several sites that had "natural" populations of *C. ariakensis* (Allen et al. 2002). There seem to be natural populations in proximity to Xiamen although we did not observe this first hand. They were available in the local market and reportedly from natural populations that were harvested. There are natural sets of *C. ariakensis* near Hong Kong on the shores of Deep Bay, but this

could be from culture activity in the area. Seed is imported from the Pearl River estuary, so there are likely sources of "natural" populations in the Pearl River delta system. We observed, first hand, collection (harvesting) of *C. ariakensis* adults from sections of the Shiman River near Guan Du in close proximity to Zhanjiang Ocean University. According to the diver on hand, they occur in various assemblages, mostly stuck onto available substrate such as large rocks. They also occur in the Dafeng River in Guangxi province near Beihai. There are probably many other natural populations along the coast of China. By way of caveat, it is difficult to attest to the "naturalness" of resident *C. ariakensis* populations. That is, those that we observed or heard about first hand were populations that occurred relatively deep (3–10 m) in river systems. Whether at some time in the past populations of *C. ariakensis* were distributed in higher reaches of the water column (i.e., before they were exploited over the millennia) is difficult to establish. It is also difficult to distinguish whether spat fall is from natural populations or from aquaculture operations.

There are clearly big questions concerning basic physiology in the kind of detail that exists for other congeners. *C. ariakensis* seems to exhibit growth rates that are extraordinary in head to head trials with *C. virginica*. Yet, these trials have been carried out in disease endemic areas where *C. virginica* could be sick or dying. Growth rates of *C. virginica* in, for example, the Gulf of Mexico, approach those seen in trials of *C. ariakensis* in the Chesapeake

Bay or reported growth rates from the literature. Similar knowledge gaps exist for larval biology, reproductive physiology, predation, competition, etc.

In our opinion, *C. ariakensis* is an underused resource around the world. It clearly has aquaculture applications in estuarine areas that are marginal or unsuitable to *C. gigas*, the most popular culture species. It seems hearty, fast growing, and highly marketable. Of course, utilization of this species would require introduction, as in the Chesapeake Bay. From that perspective, it would be useful to have more basic research on *C. ariakensis* with which to guide decisions about movement of this potentially valuable oyster species.

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CONSUMER RATINGS OF NON-NATIVE (*CRASSOSTREA GIGAS* AND *CRASSOSTREA ARIAKENSIS*) VS. NATIVE (*CRASSOSTREA VIRGINICA*) OYSTERS

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ABSTRACT Given suggestions that a non-native oyster be used to replace the depleted native oyster, consumer preference evaluations were conducted to determine how two non-native oysters, *Crassostrea gigas* and *C. ariakensis*, when grown in North Carolina estuaries, were rated by consumers. Tests compared the taste, appearance, and/or aroma of both raw and cooked non-native oysters to similarly prepared native oysters, *C. virginica*. In the first series of tests, consumers exhibited a slight preference for raw *C. virginica* over raw *C. gigas*. When cooked, both species were rated equal. In the second series of tests, a larger group of participants ranked the taste, appearance, and aroma of *C. virginica*, *C. gigas*, and *C. ariakensis*. Participants that tasted raw oysters collectively preferred *C. virginica* over both non-native species. This preference remained strong regardless of the frequency with which participants consumed oysters. Preferences for appearance and aroma varied; however, ratings never indicated a preference for either non-native species over *C. virginica*. Participants as a whole preferred the taste of cooked *C. virginica* better than *C. gigas*, whereas a taste preference did not exist between cooked *C. virginica* and *C. ariakensis*. Given that participants collectively preferred the taste of both raw and cooked *C. virginica* to *C. gigas*, the suitability of *C. gigas* for substitution in either the raw or steamed oyster market is questionable. For oysters of similar length (80 to 110 mm), dry tissue weight of *C. ariakensis* was twice that of *C. virginica*. This higher per-oyster yield suggests that *C. ariakensis* might be more suitable for a steamed or packaged oyster market where oysters are sold by meat weight rather than by number. However, these markets often command much lower prices, perhaps rendering unfeasible the aquaculture of this introduced oyster. Before large-scale introduction of non-native oyster species occurs, consumer preferences should be incorporated into economic evaluations that include additional economic (oyster prices, market demand and supply functions) and biological information (growth and survivorship). Profitability expectations generated by the model then need to be weighed against the potential ecological risks and ecosystem benefits of aquaculture or introduction to the wild for each non-native oyster species.

KEY WORDS: *Crassostrea ariakensis, Crassostrea gigas, Crassostrea virginica,* economic feasibility, native versus non-native oysters, raw versus cooked oysters, frequent versus inexperienced consumers, taste test

INTRODUCTION

Landings of the eastern oyster, *Crassostrea virginica* (Gmelin 1791), have declined by over 90% during the past century in the major estuaries of the eastern United States (MacKenzie 1983, Hargis & Haven 1988, Frankenberg 1995). Habitat degradation from destructive harvesting techniques (Rothschild et al. 1994, Lenihan 1999) and mortality induced by bottom-water hypoxia/anoxia, sedimentation, and parasitic diseases (Seliger et al. 1985, Ford & Tripp 1996, Lenihan & Peterson 1998, Lenihan et al. 1999) collectively have contributed to this decline. In North Carolina, efforts to sustain the oyster fishery over the past several decades through shell plantings have contributed to but not restored landings, which are less than 1% of historic maxima achieved in the late 1800s (Frankenberg 1995). Introduction of non-native species such as *C. gigas* (Thunberg 1793) or *C. ariakensis* (Fujita 1913) is a possible alternative or supplement to continued efforts to restore native populations, and could resuscitate the oyster industry in the eastern United States.

The Pacific oyster, *C. gigas*, accounts for over 80% of the world's aquaculture production of oysters (Ayers 1991), and thrives in shallow, sub-tidal estuaries at higher salinities (Calvo et al. 1999). Native to Japan and the Korean peninsula (Mann et al.

1991), it has been successfully introduced to France, Oregon, Washington, western Canada, Australia and New Zealand (Shatkin et al. 1997). *C. gigas* often establishes populations successfully when introduced and is successfully cultured in part because it is highly resistant to the protozoan diseases MSX, *Haplosporidium nelsoni*, and dermo, *Perkinsus marinus* (Calvo et al. 1999). MSX and dermo continue to impede recovery of native oyster populations along the eastern coast of the US (Ayers 1991, Mann et al. 1991). *C. gigas* also typically reaches harvest size more quickly than native oysters, leading many culturists to prefer growing *C. gigas* over native species (Pollard & Hutchings 1990, Ayers 1991, Parameswar 1991).

In contrast to *C. gigas*, the Suminoe oyster, *C. ariakensis*, currently does not contribute substantially to oyster fisheries of the world. Despite some taxonomic confusion with *C. rivularis*, the native distribution of *C. ariakensis* is thought to range from Pakistan to Japan, and extends into quite low salinities within the estuaries that it inhabits (Breese & Malouf 1977, Langdon & Robinson 1996). Like *C. gigas*, *C. ariakensis* grows more quickly than most other oyster species (Byrne 1996, Calvo et al. 2001), partly explaining why many fishermen in North Carolina and Virginia are advocating its introduction. This species can be grown to market size in 12–18 mo in colder waters along the west coast of the U.S. (Langdon & Robinson 1996). Calvo et al. (2001) also demonstrated that *C. ariakensis* is resistant to MSX and dermo. Long-term failure of management to restore native oyster populations coupled with higher growth rates and disease-resistance of *C. gigas* and *C. ariakensis* have created the impetus within industry to promote triploid aquaculture of and even intentional introduction

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of diploid non-native species along the Atlantic coast of North America.

Previous intentional and accidental introductions of commercial fishery species have resulted in many well-documented negative impacts (Naylor et al. 2001). For example, the predatory oyster drill, and both MSX and dermo, have been introduced unintentionally through oyster introductions (Carlton 1999, Burreson et al. 2000). Because of the risks associated with introducing a new fisheries species, including possible introduction of non-native diseases, competitors, and predators, importation of harmful microbes, and induction of competition with native species (Ruiz et al. 2000, Naylor et al. 2001), assessing and contrasting the potential risks and benefits associated with any proposed introduction should precede taking action. Here we present results of controlled trials assessing how oyster consumers rate the palatability of the two non-native species under consideration for introduction as compared with *C. virginica.*

MATERIALS AND METHODS

Two series of tests were conducted to determine consumer responses to non-native oysters grown in eastern North Carolina and to compare those responses to native oysters. In both series of tests, preferences among native, *Crassostrea virginica* (eastern oyster) and non-native species, *Crassostrea gigas* (Pacific oyster), and *Crassostrea ariakensis* (Suminoe oyster), were tested separately for raw and cooked oysters. Regulations set forth by the Shellfish Control Authorities in North Carolina mandated that we inform participants that they were consuming raw or steamed oysters, the location where oysters were grown (non-natives) or harvested (natives), and the species of oysters that were being offered. Participants in the tests were drawn from the local coastal community surrounding Morehead City, NC and represented a diverse

range of ages (20-81 y old), professions, and knowledge of local fisheries. Of the 31 individuals that participated in the first taste test, a few also were among the 96 participants in the second. Each participant completed and signed a waiver form regarding risk of raw seafood consumption, completed a demographic survey, and provided information on oyster consumption. Finally, participants were offered water and crackers to assist them to cleanse their pallets between tasting oysters.

In the first series of tests (conducted on 21 August 2000), we compared consumer responses to taste and appearance of *C. virginica* to *C. gigas.* Triploid *C. gigas* (approx. 30 mm in length) obtained from the Virginia Institute of Marine Sciences (VIMS) had been cultured since February 2000 in plastic mesh vexar cages held on racks above the sea bottom in Chadwick Bay, Onslow County, North Carolina. *C. gigas* achieved a length of approx. 80 mm by August 2000 and were removed from the field and stored in upwellers at the Institute of Marine Sciences in Morehead City, North Carolina. Wild *C. virginica* oysters were harvested in August 2000 from both the Newport River and Bogue Sound (Carteret County, North Carolina). Participants were asked to rate unlabeled raw or cooked oysters in paired contrasts. Separate trials were performed for raw and cooked oysters. Some participants were involved in both trials. To begin a trial, two oysters (either raw or cooked) on the half-shell were presented to each participant, who then rated each oyster's appearance and (separately) taste on a scale of 1 (least desirable) to 5 (Fig. 1). Each participant also specified whether either oyster tasted unappetizing, and, if any difference was perceived, which one tasted saltier, was more watery, and was more preferable overall (including an explanation for any preference). A second pair of oysters was presented to each participant, who then answered the same set of questions. One of the pairs of oysters presented a contrast of the two species, whereas

Circle the most appropriate response

1. Have you eaten raw oyster before?   Yes   No

2. Approximately how many times a year do you eat raw oysters?   0   1   2   3   4   5   >6

| 1st Test Oyster # _____ (A) vs. # _____ (B) |

1. Rate the appearance of each oyster on a scale from 1 to 5 with 5 being the best and 1 being the worst.

        A = 1   2   3   4   5   B =   1   2   3   4   5

2. Rate the taste of each oyster on a scale from 1 to 5 with 5 being the best and 1 being the worst.

        A = 1   2   3   4   5   B =   1   2   3   4   5

3. Did one or both of the oysters taste unappetizing?   Yes   No

    If so which one(s):   A   B   Both

4. Did one oyster taste saltier than the other?   Yes   No

    If so which one: A   B

5. Did one oyster taste more watery than the other?   Yes   No

    If so which one: A   B

6. If you preferred one oyster over the other briefly explain why.

_____

_____

Figure 1. Survey form used in first taste test.

OYSTER TASTE PANEL

Panelist Code # _____ Sample: Raw_____ Steamed _____ Date: _____

Procedure: Three samples of oysters (either raw or steamed) will be placed in front of you. We would like for you to taste each of the oysters and evaluate them for their quality attributes by answering the questions listed below. Please rate each sample according to their four digit code by placing a mark across the unmarked line that best reflects your opinion, e.g., like greatly (far right), neither like or dislike (middle) and dislike greatly (far left).

Note that you are not required to chew or swallow the oyster samples. You may spit the sample out at any time you need to into the cup provided. You are expected to drink (wash mouth) between samples with water. If you feel a need to be less fatigued in terms of flavors, aromas and textures blending together between samples, then you should eat crackers and drink some water.

Q1: When you eat oysters either at home or in a restaurant, what quality attributes are most important to you?

_____

Q2: How does the appearance of the samples appeal to you? What appearance characteristics do you like? Dislike?

Dislike Greatly_____ Like Greatly

Q3: How does the aroma of the samples appeal to you? What aroma characteristics do you like? Dislike?

Dislike Greatly_____ Like Greatly

Q4: How does the texture of the samples appeal to you? What texture characteristics do you like? Dislike?

Dislike Greatly_____ Like Greatly

Q5: How does the flavor of the samples appeal to you? What flavor characteristics do you like? Dislike?

Dislike Greatly_____ Like Greatly

Q6: What other attributes do you perceive in the samples?

_____

Please dispose of any left over samples in the appropriate trash container. Be sure to turn your sensory survey sheet to the project assistant when you leave the room. We appreciate your time in this study! Results will be available from the project coordinator. **THANK YOU!**

Figure 2. Survey form used for second taste.

the other presented two *C. virginica* with one from each site to determine if grow-out location affected the test results.

The second series of taste tests (conducted on 6 and 7 February 2002) evaluated consumer responses to appearance, aroma and taste of *C. virginica, C. gigas,* and *C. ariakensis.* Triploid *C. gigas* and *C. ariakensis* (approx. 30 mm in length) had been obtained from VIMS and planted at Chadwick's Bay (3 April 2001) and in the Newport River (23 March 2001). Oysters were cultured using the cage and rack method and achieved harvestable size by January 2002. *C. virginica* was also harvested in January 2002 from Chadwick's Bay and the Newport River in close proximity to culture operations. In this second set of taste tests, we requested more subtle distinctions by asking participants to rate each oyster tasted by placing a mark on a continuous line that ranged from least to most desirable (Fig. 2). Each participant was presented three oys-

ters (one of each species, either all raw or cooked) on the half-shell, and asked to rate the appearance, taste, texture and aroma of each oyster. To quantify a participant's ratings of each oyster, we measured the distance of the mark along the line, creating a scale from 0 cm (least desirable) to 10 cm (most desirable). We asked participants to indicate profession, age group and the frequency with which they eat oysters (either raw or cooked, depending on whether they were tasting raw or cooked oysters) to determine if these factors influence their ratings.

We also quantified the wet and dry weights of 50 replicate oysters (80–110 mm shell length) for each of the three species to determine whether percent dry tissue or total dry tissue differed among the three species. We determined that the shell length of oyster specimens did not vary among the three species with a one-factor analysis of variance (ANOVA; $F_{2,147}$, 1.06, $P = 0.35$).
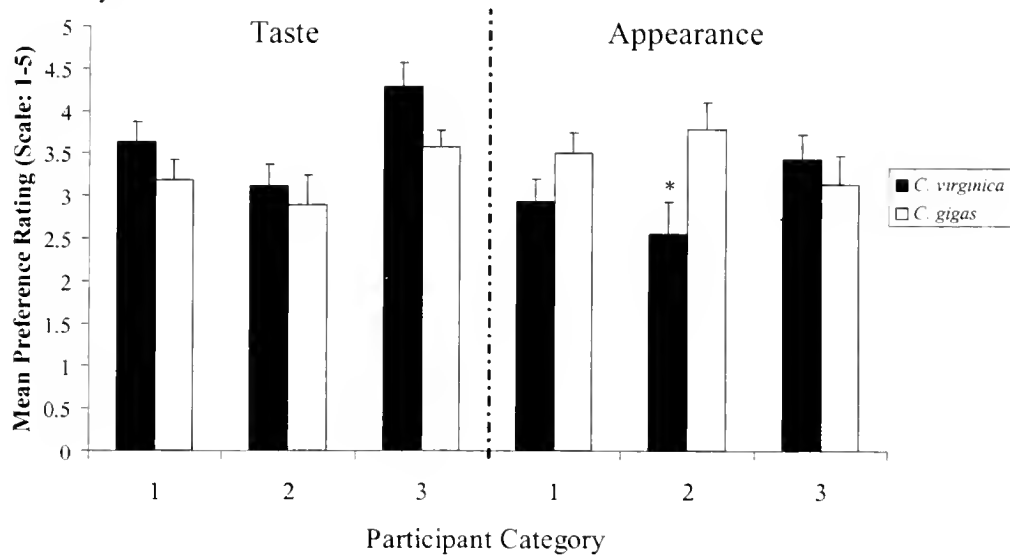
TABLE 1.

Results of Wilcoxon signed rank tests comparing consumer ratings for taste and appearance of *Crassostrea virginica* with *C. gigas* in the first series of taste tests.

Oyster Feature	All Participants[a]		Infrequent Oyster Consumers		Frequent Oyster Consumers	
	Taste	Appearance	Taste	Appearance	Taste	Appearance
Raw oysters						
No. of 0 differences[b]	2	1	1	0	1	1
No. of ranks < 0[b]	4	11	3	8	1	3
No. of ranks > 0[b]	10	4	5	1	5	3
Z value	−1.38	−1.25	−0.56	−2.19	−1.57	−0.63
P value	0.17	0.21	0.58	0.03	0.12	0.53
Cooked oysters						
No. of 0 differences[b]	2	2	1	0	1	2
No. of ranks < 0[b]	6	7	1	2	5	5
No. of ranks > 0[b]	7	6	3	3	4	3
Z value	−0.21	−1.12	−0.37	−0.27	−0.06	−1.26
P value	0.83	0.26	0.72	0.79	0.95	0.21

[a] Raw data were analyzed collectively and then reanalyzed by subgroup to determine whether those participants who rarely eat oysters have different preferences from those that frequently eat oysters.

[b] No. of 0 differences indicates the number of participants that rated species equally, no. of ranks <0 indicate participants who rated *C. gigas* as better than *C. virginica*, and the no. of ranks >0 indicates participants who rated *C. virginica* as better than *C. gigas*.
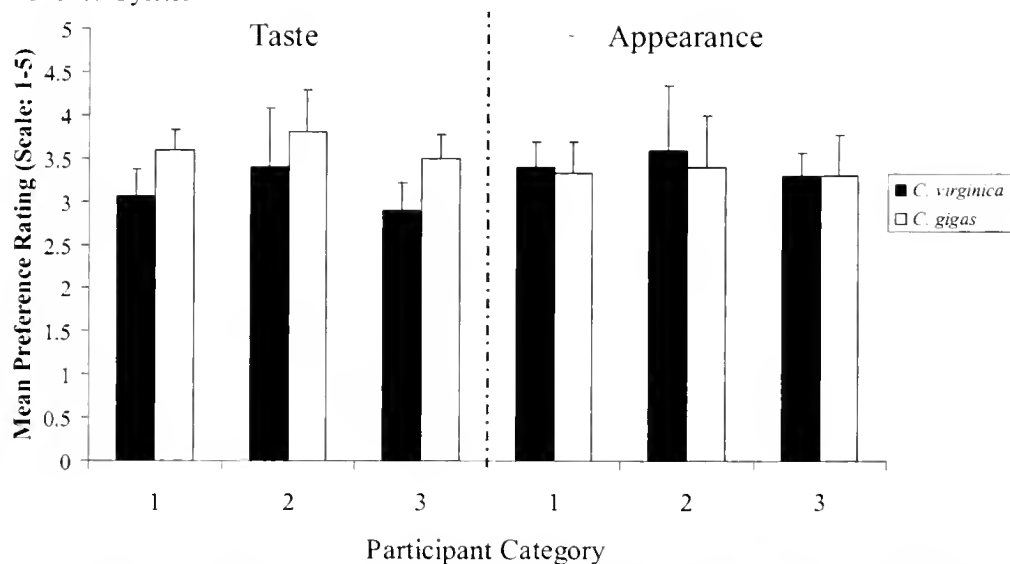

Figure 3. Results from taste test 1. Taste and appearance ratings of (a) raw and (b) cooked oysters (*Crassostrea virginica* vs. *C. gigas*) are presented for the following participant categories: 1) all participants, 2) infrequent consumers of raw oysters, and 3) frequent consumers of raw oysters. The test in which *C. virginica* was ranked significantly lower than *C. gigas* is marked with an asterisk. Error bars indicate +1 SE.

Soft tissue was removed from each oyster, placed in a pre-weighed aluminum pan, and weighed using a Mettler balance (0.001 g). Tissue was then dried at 60 C in a drying oven for 48 h, and weighed again to obtain a dry tissue weight (dry weight with pan minus pan weight). The proportion of each oyster's soft tissue that is biomass was calculated by dividing the dry weight (tissue weight minus water weight) by the initial wet weight.

Statistical Analyses

Results from the first taste test were analyzed using the Wilcoxon signed rank test. *C. virginica* from Bogue Sound were first compared with *C. virginica* from the Newport River. Because rankings of native oysters from Bogue Sound and the Newport River did not differ from each other (in taste: $P = 0.97$; in appearance: $P = 0.33$), we concluded that grow-out site did not affect the taste of native oysters in our study and we analyzed rankings for *C. gigas* versus *C. virginica* from both sites collectively. Separate *C. virginica* versus *C. gigas* tests were conducted for appearance and taste of raw and cooked oysters. Additional tests were conducted to determine if results varied between groups that (1) rarely and (2) frequently (three or more times per year) eat oysters to determine if the frequency with which participants eat oysters affected preferences for native versus non-native oysters. Results from the second taste test were also analyzed using the Wilcoxon signed rank test to determine whether participants preferred the taste, appearance, or aroma of raw and cooked *C. virginica* better than *C. gigas* or *C. ariakensis*. Each measure of *C. virginica* quality was first compared with *C. gigas* and then to *C. ariakensis* for raw and cooked oysters. Two additional series of Wilcoxon signed rank tests were conducted on the results of the second series of taste tests (raw and cooked) to determine if rank-

ings of people that eat oysters less frequently differ from those that often consume oysters. Finally, percent and mean dry tissue weights of all three species were compared using separate one-factor ANOVA tests. Cochran's test for homogeneity of variance was performed for both response variables (Underwood 1981). Student–Newman–Keuls (SNK) post hoc tests were conducted on significant ANOVA results ($P < 0.05$) to determine which of the three species differed from each other. The SNK test was selected because we conducted a balanced experiment with *a priori* predictions and a fixed factor (Day and Quinn 1989).

RESULTS

First Series of Tests (C. virginica versus C. gigas)

Collectively, survey participants ranked the taste of raw *C. virginica* slightly higher and its appearance slightly lower than *C. gigas*, but neither difference was significant (Table 1; Fig. 3). Of the 16 participants offered raw oysters, 10 preferred *C. virginica*, three preferred *C. gigas*, and three had no preference. Only two of the 16 considered *C. gigas* unappetizing and only one replied that *C. virginica* was unappetizing. Of the nine raw oyster tasters who rarely eat raw oysters, the appearance of *C. gigas* was ranked significantly higher than *C. virginica*, but the taste ratings were similar. Among the seven raw oyster tasters who frequently consume raw oysters, the taste of *C. virginica* was rated slightly higher than *C. gigas*: five of the seven preferred *C. virginica*, but low sample size more than likely rendered this difference non-significant (Table 1). Ratings of the appearance of the two species did not differ among this subgroup of tasters.

Collectively, tasters of cooked oysters did not distinguish between species in taste or appearance (Table 1; Fig. 3). Of the 15

TABLE 2.

Results of Wilcoxon signed rank tests comparing consumer ratings for taste, appearance, and aroma of *Crassostrea virginica* with *C. gigas* and *C. ariakensis* during the second series of raw oyster taste tests.

Oyster Feature	C. virginica vs. C. gigas			C. virginica vs. C. ariakensis		
	Taste	Appearance	Aroma	Taste	Appearance	Aroma
All participants[a]						
No. of 0 differences[b]	2	3	15	5	2	16
No. of ranks < 0[b]	22	35	31	32	40	35
No. of ranks > 0[b]	64	53	44	51	49	39
Z value	−4.75	−2.4	−0.88	−2.96	−0.14	−0.50
P value	<0.0001	0.02	0.38	0.003	0.89	0.62
Infrequent consumers of raw oysters						
No. of 0 differences[b]	0	2	3	0	1	5
No. of ranks < 0[b]	5	13	15	9	14	15
No. of ranks > 0[b]	24	17	14	20	17	12
Z value	−3.43	−0.61	−0.28	−2.32	−0.27	−0.99
P value	0.0006	0.54	0.78	0.02	0.79	0.32
Frequent consumers of raw oysters						
No. of 0 differences[b]	2	1	12	5	1	11
No. of ranks < 0[b]	16	22	16	22	26	20
No. of ranks > 0[b]	40	35	29	31	31	26
Z value	−3.65	−2.48	−1.29	−2.07	−0.35	−1.22
P value	0.0003	0.01	0.20	0.04	0.72	0.22

[a] Raw data were analyzed collectively and then reanalyzed by subgroup to determine whether participants who rarely eat raw oysters have different preferences from those who frequently eat them.

[b] No. of 0 differences indicates the number of participants who rated species equally, no. of ranks <0 indicate participants who rated the non-native species as better than *C. virginica*, and the no. of ranks >0 indicates participants who rated *C. virginica* as better than the non-native species.
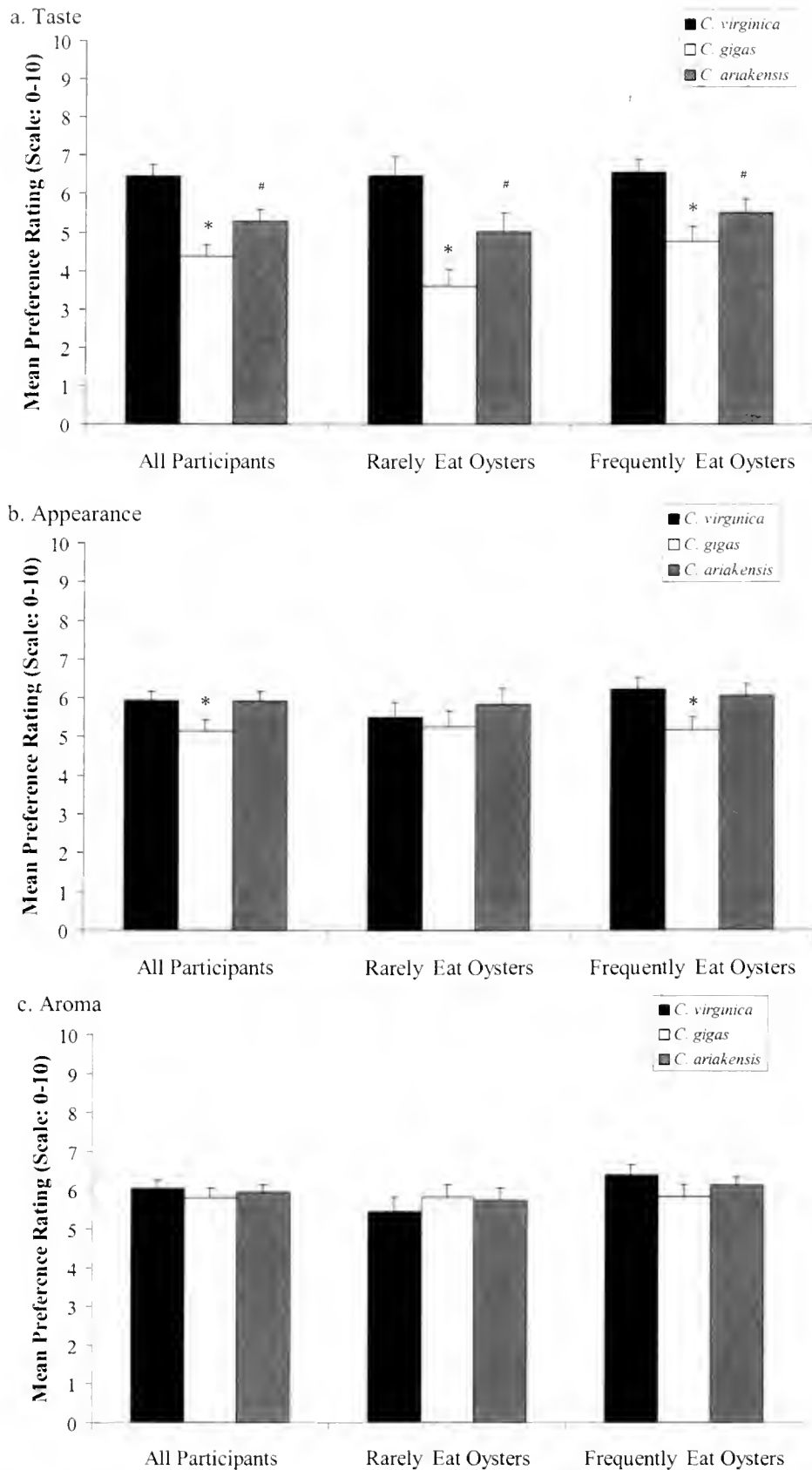
Figure 4. Results from taste test 2: raw oysters. (a) Taste, (b) appearance, and (c) aroma ratings of raw *Crassostrea virginica*, *C. gigas*, and *C. ariakensis* for the following participant groups: 1) all participants, 2) infrequent consumers of raw oysters, and 3) frequent consumers of raw oysters. Tests in which *C. virginica* was ranked higher than non-native oysters are marked with * for *C. gigas* and # for *C. ariakensis*. Error bars indicate +1 SE.

<div align="center">

TABLE 3.

Results of Wilcoxon signed rank tests comparing consumer ratings for taste, appearance, and aroma of *Crassostrea virginica* with *C. gigas* and *C. ariakensis* during the second series of cooked oyster taste tests.

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Oyster Feature	*C. virginica* vs. *C. gigas*			*C. virginica* vs. *C. ariakensis*		
	Taste	Appearance	Aroma	Taste	Appearance	Aroma
All participants[a]						
No. of 0 differences[b]	3	3	15	3	3	14
No. of ranks < 0[b]	33	49	37	38	40	36
No. of ranks > 0[b]	54	39	38	47	47	38
Z value	−2.55	−0.89	−0.23	−0.68	−1.22	−0.003
P value	0.01	0.38	0.82	0.49	0.22	0.99
Infrequent consumers of cooked oysters						
No. of 0 differences[b]	0	2	4	0	2	3
No. of ranks < 0[b]	13	19	15	13	16	17
No. of ranks > 0[b]	19	12	12	18	14	10
Z value	−1.98	−1.53	−0.32	−0.36	−0.51	−1.59
P value	0.05	0.13	0.75	0.72	0.61	0.11
Frequent consumers of cooked oysters						
No. of 0 differences[b]	3	1	11	3	1	11
No. of ranks < 0[b]	19	29	21	24	24	18
No. of ranks > 0[b]	35	27	26	29	32	28
Z value	−1.83	−0.15	−0.57	−0.90	−1.82	−1.26
P value	0.07	0.88	0.57	0.37	0.07	0.21

[a] Cooked oyster data were analyzed collectively and then reanalyzed by subgroup to determine whether participants who rarely eat cooked oysters have different preferences from those that frequently eat them.

[b] No. of 0 differences indicates the number of participants who rated species equally, no. of ranks <0 indicate participants who rated the non-native species as better than *C. virginica*, and the no. of ranks >0 indicates participants who rated *C. virginica* as better than the non-native species.

participants tasting cooked oysters, seven preferred *C. virginica*, six preferred *C. gigas*, and two had no preference. Only one of the 15 considered cooked *C. gigas* to be unappetizing, whereas two replied that *C. virginica* was unappetizing. Splitting participants out into inexperienced and frequent eaters of cooked oysters failed to detect any pattern of species preference in taste or appearance of the cooked oysters (Table 1; Fig. 3).

Second Series of Tests (C. virginica versus C. gigas or C. ariakensis)

In the second taste test, raw oyster tasters collectively ranked the taste of *C. virginica* significantly higher than both *C. gigas* and *C. ariakensis* (Table 2; Fig. 4). Appearance of *C. virginica* was rated significantly above *C. gigas* but not above *C. ariakensis* (Table 2; Fig. 4). Neither of the paired species contrasts distinguished native from non-native oysters by aroma. Infrequent oyster eaters ranked the taste of raw *C. virginica* significantly above both *C. gigas* and *C. ariakensis*, but rankings by appearance and aroma did not vary among the three species (Table 2; Fig. 4). Frequent oyster eaters ranked the taste of raw *C. virginica* significantly above both non-native species and the appearance of *C. virginica* over *C. gigas* but not different from *C. ariakensis*. Aroma rankings did not differ in either contrast of pairs of oysters (Table 2; Fig. 4).

Tasters of cooked oysters collectively rated the taste of cooked *C. virginica* significantly more than *C. gigas* (Table 3; Fig. 5) but did not distinguish between cooked *C. virginica* and *C. ariakensis*. Ratings of appearance and aroma did not differ between cooked native and non-native oysters in any contrast. The subgroup formed by infrequent consumers of cooked oysters also ranked the taste of cooked *C. virginica* significantly better than *C. gigas* but failed to distinguish between cooked *C. virginica* and *C. ariakensis* (Table 3; Fig. 5). These relatively inexperienced oyster eaters did

not rate the appearance or aroma of native oysters differently from non-native species. Finally, frequent oyster eaters ranked the taste of *C. virginica* marginally above *C. gigas* but not significantly higher than *C. ariakensis*. For these experienced oyster eaters, aroma and appearance rankings did not differ significantly between cooked native and non-native oysters, though the appearance of *C. virginica* was ranked marginally higher than *C. ariakensis* (Table 3; Fig. 5).

Dry Weight

Percent dry weight of soft tissues (dry weight/wet weight) did not significantly differ among the three species (Table 4). Prior to this analysis, percent dry weight data were transformed using a square root transformation to remove heterogeneity among variance groups. Total dry tissue weight (g) of *C. ariakensis* was significantly greater than that of *C. gigas* or *C. virginica*, and the dry tissue weight of *C. gigas* was greater than that of *C. virginica* (SNK post hoc comparisons; Fig. 6). Because average shell length did not differ among species, this analysis reflects biomass for oysters of a fixed range of harvestable lengths (80–110 mm).

DISCUSSION

As managers consider use of non-native species to enhance or restore fisheries, they should weigh carefully the risks and potential benefits. Decisions on species introductions are driven by a variety of social and political pressures, often with insufficient attention to potential ecological risks or economic benefits (Andrews 1980). In North Carolina, it is unclear, for example, how current market prices would adjust to an increase in oyster supply (Lipton & Kirkley 1994). Oyster and clam markets in the state have already endured low demand and reduced prices that threaten the economic viability of both culture operations and wild harvest
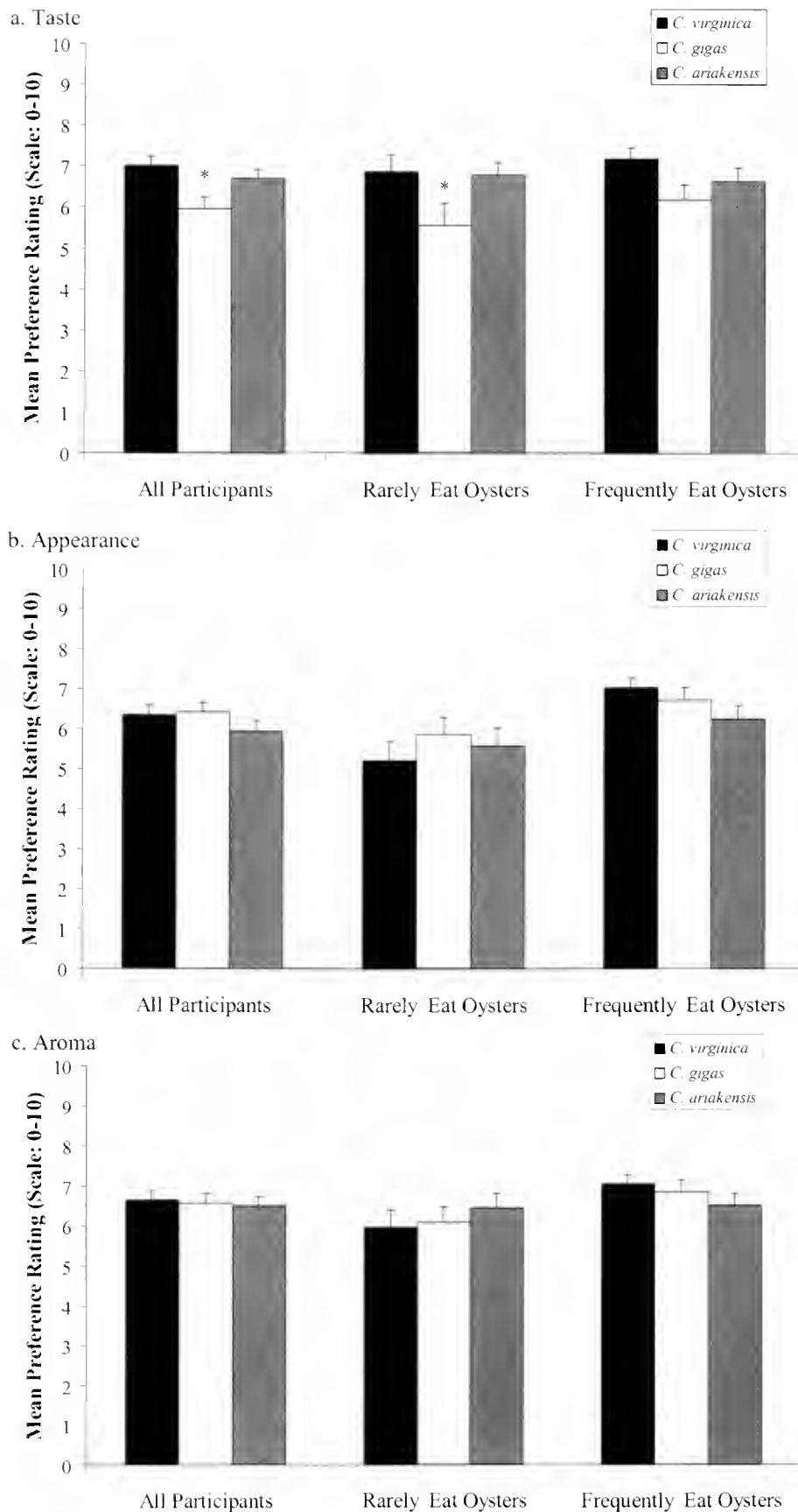
Figure 5. Results from taste test 2: cooked oysters. (a) Taste, (b) appearance, and (c) aroma ratings of cooked *Crassostrea virginica*, *C. gigas*, and *C. ariakensis* for the following participant groups: 1) all participants, 2) infrequent consumers of cooked oysters, and 3) frequent consumers of cooked oysters. Tests in which *C. virginica* was ranked higher than non-native oysters were marked with * for *C. gigas* and # for *C. ariakensis*. Error bars indicate +1 SE.

less preferable than the native eastern oyster in our study and that producing cultured oysters from triploid seed is expensive, successful culture of triploid oysters would require a substantial biological benefit in the form of shorter time to market and/or higher survival. Inclusion of this information into a comprehensive economic analysis of potential benefits and costs of introduction would enable managers to assess whether the environmental risks are worth taking. Finally, restoration of any oyster will have positive effects in restoring water quality and compensating for estuarine eutrophication (Jackson et al. 2001, Newell et al. 2002), such that this ecosystem benefit should be included in a complete economic evaluation of any potential oyster introduction. If the introduced oyster were to form reefs, then further ecosystem benefits of habitat enhancement (Lenihan et al. 2001) should also be incorporated.

ACKNOWLEDGMENTS

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TAXONOMIC STATUS OF FOUR *CRASSOSTREA* OYSTERS FROM CHINA AS INFERRED FROM MITOCHONDRIAL DNA SEQUENCES

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ABSTRACT   It has been presumed that there are four common *Crassostrea* oyster species along the coast of China: the Pacific oyster (*Crassostrea gigas*), Zhe oyster (*C. plicatula*), Suminoe oyster (*C. ariakensis*), and Dalianwan oyster (*C. talienwhanensis*). Classification and species identification of these *Crassostrea* oysters have been difficult because of morphologic plasticity. In this article, phylogenetic analysis was performed to clarify taxonomic status of these species using mitochondrial DNA sequence data. Nucleotide sequences of a 443-bp fragment of ribosomal RNA gene and a 579-bp segment of cytochrome *c* oxidase I gene were obtained through sequencing and used for analysis. Genetic distances among the four species, using *C. virginica* as outgroup, were computed based on the sequence data, and phylogenetic trees for the five species were generated. The divergence between *C. gigas* and *C. talienwhanensis* was very low, as was that between *C. plicatula* and *C. ariakensis*. Phylogenetic analysis showed that haplotypes of *C. gigas* and *C. talienwhanensis* clustered in one clade and those of *C. plicatula* and *C. ariakensis* in another one. Our data suggest that *C. gigas* and *C. talienwhanensis* may be the same species. However, the lack of divergence between *C. plicatula* and *C. ariakensis* samples may indicate that the *C. plicatula* specimen we sampled could actually be a morph of *C. ariakensis* living in high salinity habitats. More work is needed for confirmation.

KEY WORDS: *Crassostrea* oysters, taxonomy, phylogenetic analysis, 16S rDNA, COI gene, nucleotide sequences

INTRODUCTION

Among the over 20 species of oysters recorded in China, four *Crassostrea* species are most common and of commercial importance: the Pacific oyster (*Crassostrea gigas*), Zhe oyster (*C. plicatula*), Suminoe oyster (*C. ariakensis*), and Dalianwan oyster (*C. talienwhanensis*; Zhang et al. 1956, Qi 1989). The Pacific oyster, which occurs naturally along the coast of China, is a well-recognized species. However, most of the Pacific oysters cultured in China were originally introduced from Japan or Korea (Wang et al. 1993). The Zhe oyster is commonly found along the entire coast of China. It is relatively smaller in body size than the Pacific and Suminoe oysters and thin-shelled (Qi 1989, Guo et al. 1999). Suminoe oysters are also distributed along most of the coast of China with two major populations, one in the estuaries of Yellow river and the other in Guangxi and Guangdong in southern China. It can tolerate a wide range of salinity but prefers low-salinity estuaries and riverbeds (Torigoe 1981, Li & Qi 1994). The Dalianwan oyster occurs mainly in areas along the coast of Liaoning and Shandong provinces in the North (Zhang et al. 1956, Qi 1989).

Because of the morphologic plasticity, there have been disagreements about the taxonomic status of the four *Crassostrea* types and difficulties in their identification. Some believed that the Pacific and Dalianwan oysters are different species (Zhang et al. 1956, Qi 1989), whereas others argued that the Dalianwan oyster, described by Zhang et al. (1956), is the Pacific oyster, or a variety of Pacific oyster (Torigoe 1981, Li & Qi 1994). In addition, sometimes the discrimination of Pacific and Suminoe oysters was ambiguous with shell morphology, although it is distinguishable with some body anatomic features (Li & Qi 1994). The most common oysters found in the rocky intertidal zone and extensively cultured in the south are generally believed to be the Zhe oyster, although

Li and Qi (1994) assumed it was the Pacific oyster. Liu et al. (1998) compared RAPD data from several *Crassostrea* species and concluded that the Dalianwan oyster, Zhe, and Pacific oysters were sister species with each other.

Because of this confusion, further study, especially with DNA markers, is needed. DNA polymorphisms are useful tools for ecological, genetic, and evolutionary studies of both terrestrial and marine organisms, and DNA sequences can be used to detect differences among species, populations, or individuals. Proper identification of oyster stocks will assist management, including conservation and the sustainable use of these resources. Past efforts to investigate and identify differences among populations and species of oysters along the coast of China have provided useful but inconclusive information (Liu et al. 1998, Yang et al. 2000).

Because of its fast sequence evolution and maternal, nonrecombining nature of inheritance in animals, mitochondrial genes have proved a powerful tool in phylogenetic studies and species identification (Banks et al. 1993, Littlewood 1994, Jozefowicz et al. 1998, Lapegue et al. 2002). The 16S rRNA and COI gene fragments are popular choices for phylogenetic analysis (O'Foighil et al. 1995, O'Foighil et al. 1998, Canapa et al. 2000). In this study, mitochondrial 16S rRNA and COI gene fragments from these four putative species were amplified and sequenced for phylogenetic analysis.

MATERIALS AND METHODS

Sampling and Polymerase Chain Reaction (PCR) Amplifications

Crassostrea gigas samples (eight specimens) were obtained from a hatchery broodstock in Shandong province; *C. ariakensis* samples (seven individuals) were collected from estuaries of the Yellow River, in Yantai, Shandong province, which is a typical habitat of this species in north China. *C. talienwhanensis* was sampled from Dalian (five individuals), Liaoning province and Rongcheng (five individuals), Shandong province. *C. plicatula*

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samples were collected from Qingdao (five specimens), Shandong province and Wenzhou (five specimens), Zhejiang province. Sampling sites are showed in Figure 1. *C. virginica* was collected from Delaware Bay in the United States. Morphologic identification was made according to that described in Zhang et al. (1956), Qi (1989), Torigoe (1981), and Li and Qi (1994).

Total DNA was extracted from mantle tissue using an extraction kit (Pure Gene, Gentra, USA). Fragments of the 16S rDNA and COI gene were amplified using two pairs of universal primers: 16sar-L/16sbr-H: 5'-GCCTGTTTATCAAAAACAT-3'/5'-CCGGTCTGAACTCAGATCACGT-3' (Palumbi 1991); COIL1490/COIH2198: 5'-GGTCAACAAATCATAAAGATAT-TGG-3'/ 5'-TAAACTTCAGGGTGACCAAAAAATCA-3' (Folmer et al. 1994).

Amplification of the products was performed using a PTC-100 thermal cycler (MJ Research, USA). The 100-µL amplification reaction contained 2.0 mM MgCl$_2$; 200 µM of each dNTP; 0.2 µM each primer; 2.5 µL of template DNA; and 2.5 units of Taq polymerase (Sangon, Canada) with supplied buffer. For all amplifications, hot-start PCR was initiated by addition of polymerase and primers after an initial 2-min denaturization at 80°C. The PCR cycling profile was as follows: 35 cycles at 94°C/45 sec, 48°C (COI) or 50°C (16S)/1 min and at 72°C/1 min, with a final extension at 72°C for 7 min.

Sequencing

PCR products were purified using UNIQ-5 Column PCR Product Purification Kit (Sangon, Canada), ligated into pMD18-T Vector by following instruction of Takara DNA Ligation Kit ver.2 (Takara, Japan) and used to transform competent JM109 *Escherichia coli* cells using standard protocols. Recombinant colonies were identified by blue-white screening. Inserts of the correct size

Figure 1. A map of sampling area with sampling sites underlined.

were detected via restriction enzyme digestion by *Eco*RI and *Hind*III. Vector DNA containing the desired insert was further purified using Pharmacia EasyPrep Kit. Sequencing was performed for both strands of every sample on an ABI PRISM 377XL DNA Sequencer using ABI PRISM BigDye™ Terminator Cycle Sequencing Ready Reaction Kit with AmpliTaq DNA Polymerase, FS (Perkin–Elmer, USA).

Data Analysis

The 16S and COI sample sequences, along with those already obtained for *C. gigas* and *C. ariakensis* (O'Foighil et al. 1995, 1998; courtesy of Dr. D. O'Foighil) were aligned with CLUSTAL W (Thompson et al. 1994). For clarity and convenience in comparing with other published sequences, the sequences were trimmed to the same length as published sequences after alignment. Parsimony analysis was made with Phylip (Ver.3.56C, Felsenstein 1989) using the program DNAPARS with *C. virginica* as the out-group. Bootstrap analysis with 1000 replication was performed by the SEQBOOT and CONSENSE programs. Consensus phylogenetic trees were drawn with DRAWGRAM program in the Phylip package. Pair-wise sequence divergence between haplotypes and species were estimated by the DNADIST program of Phylip according to Kimura's two-parameter model (Kimura 1980).

RESULTS

A PCR fragment of 488 bp from the mitochondrial 16S ribosomal gene and a fragment of 649 bp from the mitochondrial COI gene were obtained and sequenced for 37 individuals of five species (including two *C. virginica* specimens). Figure 2 shows the alignment of 16S sequences of the seven haplotypes detected among all specimens in this study, along with those of *C. gigas* and *C. ariakensis* from O'Foighil's study. Eight specimens of *C. gigas* and 10 of *C. plicatula* exhibited only one genotype, whereas seven *C. ariakensis* and 10 *C. talienwhanensis* individuals had two haplotypes each. The two haplotypes of *C. talienwhanensis* came from different sampling locations. Including the outgroup, 80 nucleotide positions were variable in the 16S data set. Six insertion/deletion sites were detected between *C. virginica* and all other haplotypes.

Similarly, the alignment of the seventeen COI haplotypes detected in our study and those two of *C. gigas* and *C. ariakensis* from O'Foighil's study are shown in Figure 3. The 17 haplotypes in our study included one for *C. gigas* (gigas1, 8 individuals), seven for *C. plicatula* (plica1, 2, 3, 6 and 7, one individual for each; plica4, three individuals; plica5, two individuals), three for *C. ariakensis* (ariaken1, 4 individuals; ariaken2, two individuals and ariaken3, one individual), five for *C. talienwhanensis* (talienw1, 2 and 3, one individual each; talienw4, four individuals; talienw5, three individuals), and one for *C. virginica* (virg1, two individuals). Including the outgroup, 170 positions are variable. No insertions/deletions were detected for this protein-coding gene fragment.

Pair-wise genetic distances of 16S sequences among all nine haplotypes and those of COI sequences among all 19 haplotypes were computed, then the mean genetic distances were obtained (Table 1). In the 16S sequence, the genetic divergence between *C. gigas* and *C. talienwhanensis* was low, 0.81%, and so was that between *C. ariakensis* and *C. plicatula*, 0.13%. The sequence divergences between *C. gigas* or *C. talienwhanensis* and *C. ariakensis* or *C. plicatula* were higher, ranging from approx. 1.74 to

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                                                                               80
gigas1   GCAATACCTG CCCAGTGCGA AATATTACTG TAAACGGCCG CCCTAGCGTG AGGGTGCTAA GGTAGCGAAA TTCCTTGCCT
talienw1 .......... .......... .......... .......... .......... .......... .......... ..........
gigasO   .......... .......... .......... .......... ......GC.. .......... .......... ..........
talienw2 .......... .......... .......... .......... .......... .......... .......... ..........
plica1   .......... .......... .......... .......... .......... .......... .......... ..........
ariaken1 .......... .......... .......... .......... .......... .......... .......... ..........
ariaken2 .......... .......... .......... .......... .......... .......... .......... ..........
ariakenO .......... .......... .......... .......... ......GC.. .......... .......... ..........
virg1    ...G...... .......... C.ATAAGTC. .C........ ...T...... .......... .......... ..........

                                                                               160
gigas1   TTTGATTGTG GGCCTGCATG AATGGTTTAA CGAGGGTTTG ACTGTCTCTA AATTTTTTAT TGAAATTGTA CTGAAGGTGA
talienw1 .......... .......... .......... .......... .......... .......... .......... ..........
gigasO   .......... .......... .......... .......... .......... .......... .......... ..........
talienw2 .......... .......... .......... .......... ...A...... .......... .......... ..........
plica1   .........A .......... .......... .......... ...A...... .......... .......... ..........
ariaken1 .........A .......... .......... .......... ...A...... .......... .......... ..........
ariaken2 .........A .......... .......... .......... ...A...... .......... .......... ..........
ariakenO .........A .......... .......... .......... ...A...... .....AAA.. .......... ..........
virg1    .......... .......... .......... .......G.. ......C..T G........T G......... G..T......

                                                                               240
gigas1   AGATACCTTC ATTTAAAAGT TAGACAAAAA GACCCCGTGC AACTTTGAAA A--TTAACTT TATTCAGGAG TAAAAGATTT
talienw1 .......... .......... .......... .......... ......--.. .......... .......... ..........
gigasO   .......... .......... ......G.. A......... .......... --........ .......... ..........
talienw2 .......... .......... .......... .......... .--..G.... .......... .......... ..........
plica1   .......... ...A...... .......... .......... .--..G.... .......... .......... ..........
ariaken1 .......... ...A...... .......... .......... .--..G.... .......... .......... ..........
ariaken2 .......... ...A...... .......... .......... .--..G.... .......... .......... ..........
ariakenO .......... ...A...... C......G.. A......... .--....... .......... .G........ ..........
virg1    .A........ ...AAG.... ......G... .......... ....GC.A.G..G A...A.TA... ..........

                                                                               320
gigas1   TTAGGTGGGG CGCCTAGAAA GCAAG-TCTA ACCTTT-CTG AATAACT--A ACTCTTTCCG GATTTGACCC GATTATATTC
talienw1 .......... .......... .....-.... ......-... ......--.. .......... .......... ..........
gigasO   .......... .......... .....-.... ......-... ......--.. .......... .......... ..........
talienw2 .......... .......... .....-.... ......-... ......--.. .T........ .......... ..........
plica1   .......... .......... .T...-C... ......-... .G.....--. .......... .......... ..........
ariaken1 .......... .......... .T...-C... ......-... .G.....--. .......... .......... ..........
ariaken2 .......... .......... .T...-C... ......-... .G.....--. .......... .......... ..........
ariakenO .......... .......... .T...-.... ......-... ..C.TT.--. GT........ .C........ ..........
virg1    ..G....... ......A.G. .T..AA.T.. ....C.GT.. ...T..ATA. .T...AA.TA .........T ..C.TC.G..

                                                                               400
gigas1   GATCATAGGA GAAGTTACGC CGGGGATAAC AGGCTAATCC TTTAGTAGAG TTCGTATTGG CTAAAGGGAT TGGCACCTCG
talienw1 .......... .......... .......... .......... .......... .......... .......... ..........
gigasO   .......... .......... .......... .......... .......... .......... .......... ..........
talienw2 .......... .......... .......... .......... .......... .......... .......... ..........
plica1   .......... .......... .......... .......... .......... .......... .......... ..........
ariaken1 .......... .......... .......... .......... .......... .......... .......... ..........
ariaken2 .....C.... .......... .......... .......... .......... .......... .......... ..........
ariakenO .......... .......... .......... .......... .......... .......... C......... ..........
virg1    .......... .......... .......... ....C..... .C.....A.. .......-.AA .........C. ..........

                                                              443
gigas1   ATGTTGAATC AGGGATAATA GCTTCAAGGC GTAGAGGCTT TGA (8)
talienw1 .......... .......... .......... .......... ... (5)
gigasO   .......... .......... .......... .......... ... (7)
talienw2 .......... .......... .......... .......... ... (5)
plica1   .......... .....G... .......... .......... ... (10)
ariaken1 .......... .....G... .......... .......... ... (5)
ariaken2 .......... .....G... .......... .......... ... (2)
ariakenO .......... .....G... .......... .......... ... (5)
virg1    .......... .....T... C...T..... .......... .A. (2)
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Figure 2. Alignment of seven oyster haplotypes of a 443-bp fragment of the mitochondrial 16S rDNA obtained in this study (*C. virginica* as outgroup) with published sequences for *C. gigas* and *C. ariakensis* (O'Foighil et al. 1995, 1998). gigasO and ariakenO designate the sequences of *C. gigas* and *C. ariakensis* from O'Foighil's study, respectively. Haplotype names are abbreviated as: gigas for *C. gigas*, talienw for *C. talienwhanensis*, plica for *C. plicatula*, ariaken for *C. ariakensis*, and virg for *C. virginica*. Additional haplotypes per species are numbered consecutively. Dots indicate nucleotide identity to the first sequence presented, gigas1. Dashes indicate inferred nucleotide indels relative to *C. virginica*. The number of individuals observed for each haplotype is indicated in parentheses at the end of sequence.

2.45%. The same pattern appeared in the COI data set: the corresponding numbers were 1.08% between *C. gigas* and *C. talienwhanensis*, 0.59% between *C. ariakensis* and *C. plicatula*, and approx. 10.72 to 11.43% for the same comparisons mentioned above. It is worth noting that the COI sequence was more variable than the 16S sequence.

Consensus phylogenetic trees based on a parsimony analysis of the 16S and COI fragments sequenced are presented in Figures 4 and 5, respectively. Two groups (clades) in the 16S tree were clearly distinguishable: *C. ariakensis* and *C. plicatula* vs. *C. gigas* and *C. talienwhanensis*, whereas three groups (clades) were ap-

parent in the COI tree: (1) *C. ariakensis* and *C. plicatula*; (2) *C. gigas* and *C. talienwhanensis*; (3) *C. ariakensis* from O'Foighil's study.

DISCUSSION

Oysters are among the most extensively studied and morphologically variable marine invertebrates. However, our knowledge of oyster phylogeny and systematics is still limited. There had been over one hundred recorded species of oysters until 1970s, but two thirds of them could be synonymous with each other according to

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                                                                                      80
gigas1    GCTGTTCTTG CGGGAACTAG GTTTAGGTCT CTTATTCGTT GGAGACTTTA TAACCCTGGA GCTAAGTTTT TAGACCCCGT
talienw1  .......... .......... .......... .......... .......... .......... .......... ..........
gigasO    .......... .......... .......... .......... .......... .......... .......... ..........
talienw2  .......... .......... .......... .......... .......... .......... .......... ..........
talienw3  .......... .......... .......... .......... .......... .......... .......... ..........
talienw4  .......... .......... .......... .......... ...G...... .....C.... .......... ..........
talienw5  .......... .......... .......... .......... ...G...... .....C.... .......... ..........
plica1    .......... ....G..... .......... .......... .A..G..A.. C.....C..G ..C....... ....T..A..
ariaken1  .......... ...G...... .......... .......... .A..G..A.. C.....C..G ..C....... ....T..A..
plica2    .......... ...G...... .......... .......... .A..G..A.. C.....C..G ..C....... ....T..A..
plica3    .......... ...G...... .......... .......... .A..G..A.. C.....C..G ..C....... ....T..A..
plica4    .......... ...G...... .......... .......... .A..G..A.. C.....C..G ..C....... ....T..A..
ariaken2  .......... ...G...... .......... ...C...... .A..G..A.. C..T..C..G ..C....... ....T..A..
plica5    .......... ...G...... .......... .......... .A..G..A.. C..T..C..G ..C....... ....T..A..
ariaken3  .......... ...G...... .......... .......... .A..G..A.. C..T..C..G ..C....... ....T..A..
plica6    .......... ...G...... .......... .......... .A..G..A.. C..T..C..G ..C....... ....T..A..
plica7    .......... ...G...... .......... .......... .A..G..A.. C..T..C..G ..C....... ....T..A..
ariakenO  ..C....... TA........ ...C..A... .......... .A..TT.G.. ..C...A..G .......... ....C..T..
virg1     ..A...T.A. .T..G..C.. T......... .......... .A..T..A.. ..CT.....G .....A.... ....G..T..

                                                                                     160
gigas1    GACTTATAAT GCAGTTGTAA CTAGGCATGC GTTGGTTATG ATTTTTTTCT TTGTTATACC TGTAATAATT GGGGGGTTTG
talienw1  .......... .......... .......... .......... .......... .......... .......... ..........
gigasO    .......... .......... .......... .......... .......... .......... .......... ..........
talienw2  .......... .......... .......... ....C..... .......... .......... .......... ..........
talienw3  .......... .......... .......... .......... .......... .......... .......... ..........
talienw4  .......... .......... .......... .......... .......... .......... C..G...... ..........
talienw5  .......... .......... .......... .......... .......... .......... C..G...... ..........
plica1    .......... .......... .......... A......... .......... .......... ...G..A..G ..........
ariaken1  .......... .......... .......... A......... .......... .......... ...G..A..G ..........
plica2    .......... .......... .......... A......... .......... .......... ...G..A..G ..........
plica3    .......... .......... .......... A......... .......... .......... ...G..A..G ..........
plica4    .......... .......... .......... A......... .......... .......... ...G..A..G ..........
ariaken2  .......... .......... .......... A......... .......... .......... ...G..A..G ..........
plica5    .......... .......... .......... A......... .......... .......... ...G..A..G ..........
ariaken3  .......... .......... .......... A......... .......... .......... ...G..A..G ..........
plica6    .......... .......... ...A...... A......... .......... .......... ...G..A..G ..........
plica7    .......... .......... ...A...... A......... .......... .......... ...G..A..G ..........
ariakenO  A..C.....C .......... .C..A..... A..A....A ..C....T. .......... ...G......C ..........
virg1     TGTG.....C ..T..G.... .G..T..C.. ...A..A... .......C.. .......... ...G. A......... ..T.......

                                                                                     240
gigas1    GTAACTGGCT TATCCCTTTG ATGCTTCTAG TAGCAGACAT GCAATTTCCT CGATTAAATG CATTTAGATT TTGAGTTTTG
talienw1  .......... .......... .......... .......... .......... .......... .......... ..........
gigasO    .......... .......... .......... .......... .......... .......... .......... ..........
talienw2  .......... .......... .......... .......... .......... .......... .......... ..........
talienw3  .......... .......... .......... .......... .......... .......... .......... ..........
talienw4  .......... .......... .......A.. .......... .......... .......... .......... ..........
talienw5  .......... .......... .......A.. .......... .......... .......... .......... ..........
plica1    .......... ...T.....A .......A.. .......T.. A.....C... .GC....C.. .......... ......C...
ariaken1  .......... ...T.....A .......A.. .......T.. A.....C... .GC....C.. .......... ......C...
plica2    .......... ...T.....A .......A.. .......T.. A.....C... .GC....C.. .......... ......C...
plica3    .......... ...T.....A .......A.. .......T.. A.....C... .GC....C.. .......... ......C...
plica4    .......... ...T.....A .......A.. .......T.. A.....C... .GC....C.. .......... ......C...
ariaken2  .......... ...T.....A .......A.. .......T.. A.....C... .GC....C.. .......... ......C...
plica5    .......... ...T.....A .......A.. .......T.. A.....C... .GC....C.. .......... ......C...
ariaken3  .......... ...T.....A .......A.. .......T.. A.....C... .GC....C.. .......... ......C...
plica6    .......... ...T.....A .......A.. .......T.. A.....C... .GC....C.. .......... ......C...
plica7    .......... ...T.....A .......A.. .......T.. A.....C... .GC....C.. .......... ......C...
ariakenO  .A..T..... ......A..A .......A.. .......T.. ...G...... .G........ .......... C..G......
virg1     ....T..... ...T..GC.T ..A...GA.. .G........ ...G..C... .......... .T........ C.....A...

                                                                                     320
gigas1    CCAGGGTCTC TTTATCTTAT GCTTATGTCT AACATTGTAG AAAACGGAGT TGGGGCAGGG TGAACAATTT ACCCTCCTTT
talienw1  .......... .......... .......... .......... .......... .......... .......... ..........
gigasO    .......... .......... .......... .......... .......... .......... .......... ..........
talienw2  .......... .......... .......... .......... .......... .......... .......... ..........
talienw3  .......... .......... .......... .......... .......... .......... .......... ..........
talienw4  .......... .......... .......... ....C..... .......... .......... ..G....... ..........
talienw5  .......... .......... .......... ....C..... .......... .......... .......... ..........
plica1    .....A..A. .......... .......... .T.....G.. ...GG..... .......... .......... ...C.....
ariaken1  .....A..A. .......... .......... .T.....G.. ...GG..... .......... .......... ...C.....
plica2    .....A..A. .......... .......... .T.....G.. ...GG..... .......... .......... ..........
plica3    .....A..A. .......... .......... .T.....G.. ...GG..... .......... .......... ..........
plica4    .....A..A. .......... .......... .T.....G.. ...GG..... .......... .......... ...C.....
ariaken2  .....A..A. .......... .......... .T.....G.. ...GG..... .......... .......... ...C.....
plica5    .....A..A. .......... .......... .T.....G.. ...GG..... .......... .......... ..........
ariaken3  .....A..A. .......... .......... .T.....G.. ...GG..... .......... .......... ..........
plica6    .....A..A. .......... .......... .T.....G.. ...GG..... .......... .......... ..........
plica7    .......A.. .......... .......... .T.....G.. ...GG..... .......... .......... ..........
ariakenO  .....C..A. .......... .......... .TC....... GT..G.. C. ........A .......... ..........
virg1     .......AT .GCTG..A.. AT.G.....A .T....CT. .G.GA..... ....T...A .....C.... .......GC.
```

Figure 3. Alignment of 17 oyster haplotypes of a 579-bp fragment of the mtCOI gene obtained in this study (*C. virginica* as outgroup) with published sequences for *C. gigas* and *C. ariakensis* (O'Foighil et al. 1995, 1998). gigasO and ariakenO designate the sequences of *C. gigas* and *C. ariakensis* from O'Foighil's study, respectively. Haplotype names are abbreviated as: gigas for *C. gigas*, talienw for *C. talienwhanensis*, plica for *C. plicatula*, ariaken for *C. ariakensis* and virg for *C. virginica*. Additional haplotypes per species are numbered consecutively. Dots indicate nucleotide identity to the first sequence presented, gigas1. The number of individuals observed for each haplotype is indicated in parentheses at the end of sequence.

```
                                                                              400
gigas1     ATCAACTTAC TCTTATCATG GAGTTTGTAT AGACCTTGCA ATTCTAAGCC TTCACCTTGC TGGTATTAGC TCTATTTTCA
talienw1   .......... .......... .......... .......... .......... .......... .......... ..........
gigas0     .......... .......... .......... .......... .......... .......... .......... ..........
talienw2   .......... .......... .......... .......... .......... .......... .......... ..........
talienw3   .......... .......... .......... .......... .......... .......... .......... ..........
talienw4   .......... .......... ......C.. .......... ...T...... .......... .C........ ..........
talienw5   .......... .......... ......C.. .......... ...T...... .......... .C........ ..........
plica1     G..G...... .......... ......C.. G......... ..T....TT .A.....A.. .......... ..........
ariaken1   G..G...... .......... ......C.. G......... ..T....TT .A.....A.. .......... ..........
plica2     G..G...... .......... ......C.. G......... ..T....TT .A.....A.. .......... ..........
plica3     G..G...... .......... ......C.. G......... ..T....TT .A.....A.. .......... ..........
plica4     G..G...... .......... ......C.. G......... ..T....TT .A.....A.. .......... ..........
ariaken2   G..G...... .......... ......C.. G......... ..T....TT .A.....A.. .......... ..........
plica5     G..G...... .......... ......C.. G......... ..T....TT .A.....A.. .......... ..........
ariaken3   G..G...... .......... ......C.. G......G.. ..T....TT .A.....A.. .......... ..........
plica6     G..G...... .......... ......C.. G......... ..T....TT .A.....A.. .......... ..........
plica7     G..G...... .......... ......C.. G......... ..T....TT .A.....A.. .......... ..........
ariaken0   .......... .......... .G..C..C. .......... .......... ...TT.A.. ...A.....G ........T.
virg1      G.......TT ......C... ......C.. G..TT....C ...T....GT .A..T.A.. A........G ..........

                                                                              480
gigas1     GGTCAATTAA TTTCATAGTA ACGATTAGAA ATATGCGATC TGTTGGGGGC CATTTACTAG CACTATTCCC TTGATCTATT
talienw1   .......... .......... .......... .......... .......... .......... .......... ..........
gigas0     .......... .......... .......... .......... .......... .......... .......... ..........
talienw2   .......... .......... .......... .......... .......... .......... .......... ..........
talienw3   .......... .......... .......... .......... .......... .......... .......... ...G......
talienw4   .......... ...T...... .......... .......... .......... .......... T.. C..... ..........
talienw5   .......... ...T...... .......... .......... .......... .......... T.. C..... ..........
plica1     .......... ...T...... ..A....... ...A...... .......... ...T.G. .G..G..T. C.........
ariaken1   .......... ...T...... ..A....... ...A...... .......... ...T.G. .G..G..T. C.........
plica2     .......... ...T...... ..A....... ...A...... .......... ...T.G. .G..G..T. C.........
plica3     .......... ...T...... ..A....... ...A...... .......... ...T.G. .G..G..T. C.........
plica4     .......... ...T...... ..A....... ...A...... .......T .. ...T.G. .G..G..T. C.........
ariaken2   .......... ...T...... ..A....... ...A......T .......... ...T.G. .G..G..T. C.........
plica5     .......... ...T...... ..A....... ...A...... .......... ...T.G. .G..G..T. C.........
ariaken3   .......... ...T...... ..A....... ...A...... .......... ...T.G. .G..G..T. C.........
plica6     .......... ...T...... ..A....... ...A...... .......... ...T.G. .G..G..T. C.........
plica7     .......... ...T...... ..A....... ...A...... .......... ...T.G. .G..G..T. C.........
ariaken0   .......... .....T.... .......... .......... ...C..T..G .....GT.G. .G.....T. A..G.....C
virg1      ....T..... ...T...... ..C....... .C........ .......... ..T ..CA..T... ....T..... ...G..A...

                                                                              560
gigas1     AAGGTTACTT CATTCTTGCT TTTGACTACT CTCCCAGTGT TAGCTGGAGG TCTTACTATA CTTTTGACTG ATCGTCATTT
talienw1   .......... .......... .......... .......... .......... .......... .......... ..........
gigas0     .......... .......... .......... .......... .......... .......... .......... ..........
talienw2   .......... .......... .......... .......... .......... .......... .......... ..........
talienw3   .......... .......... .......... .......... .......... .......... .......... ..........
talienw4   .......... .......... .......... .......... .......... ......G... .......... ..........
talienw5   .......... .......... .......... .......... .......... ......G... .......... ..........
plica1     .......... ....TC.A.. ...A...... ..T....... .......G. C.........G .C........
ariaken1   .......... ....TC.A.. ...A...... ..T....... .......G. C.........G .C........
plica2     .......... ....TC.A.. ...A...... ..T....... .......G. C.........G .C........
plica3     .......... ....TC.A.. ...A...... ..T....... .......G. C.........G .C........
plica4     .......... ....TC.A.. ...A...... ..T....... .......G. C.........G .C........
ariaken2   .......... ....TC.A.. ...A...... ..T....... .......G. C.........G ..........
plica5     .......... ....TC.A.. ...A...... ..T....... .......G. C.........G ..........
ariaken3   .......... ....TC.A.. ...A...... ..T....... .......G. C.........G ..........
plica6     .......... ....TC.A.. ...A...... ..T....... .......G. C.........G ..........
plica7     .......... ....TC.A.. ...A...... ..T....... .......G. C.........G ..........
ariaken0   ..A..C..A. .....T..A. ...A..A..C ..T..G..AC .......... .....C..G .......... .C..G.....
virg1      ..A..G..A. .....C.... GC.T..C..G ..A..T..TC .......C.. G........G ..CC.T.... ....A.....

           579
gigas1     TAATACCTCT TTTTTTGAC (8)
talienw1   .......... ......... (1)
gigas0     .......... ......... (20)
talienw2   .......... ......... (1)
talienw3   .......... ......... (1)
talienw4   .......... ....C.... (4)
talienw5   .......... ....C.... (3)
plica1     ...C..G... ........T (1)
ariaken1   ...C..G... ........T (4)
plica2     ...C..G... ........T (1)
plica3     ...C..G... ........T (1)
plica4     ...C..G... ........T (3)
ariaken2   ...C..G... ........T (2)
plica5     ...C..G... ........T (2)
ariaken3   ...C..G... ........T (1)
plica6     ...C..G... ........T (1)
plica7     ...C..G... ........T (1)
ariaken0   ...C..G... ........T (5)
virg1      ......A..G ......... (2)
```

Figure 3. (Continued)

Harry (1985). The inability to clearly classify closely-related oysters has created problems for classification and species identification worldwide.

Although morphologic identification of oysters often turned out to be unreliable or ambiguous, mtDNA sequence analysis has proven to be a powerful tool for oyster identification and discrimination between closely related species or between native and non-native species. Banks et al. (1993) discriminated closely related oyster species, *C. gigas* and *C. sikamea*, via mitochondrial 16S rRNA gene sequencing and PCR/RFLP analysis. O'Foighil et al.

TABLE 1.

Pair-wise sequence divergence (mean genetic distances) according to Kimura's two-parameter model (Kimura 1980) among the five species based on 443-nucleotide 16S rDNA and 579-nucleotide COI sequences.

Species	16S						COI					
	1	2	3	4	5	6	1	2	3	4	5	6
C. gigas	0						0					
C. taliemwhanensis	**0.0081**	0					**0.0108**	0				
C. plicatula	0.0233	0.0174	0				0.1113	0.1072	0			
C. ariakensis	0.0245	0.0185	**0.0013**	0			0.1143	0.1100	**0.0059**	0		
C. ariakensisO	0.0450	0.0487	0.0449	0.0462	0		0.1619	0.1639	0.1652	0.1691	0	
C. virginica	0.1636	0.1605	0.1659	0.1673	0.1937	0	0.2569	0.2573	0.2510	0.2513	0.2849	0

C. ariakensisO indicates S. ariakensis sequence from O'Foighil's studies (1995, 1998).
Pair-wise comparisons yielding low genetic distances estimates are showed in boldface.

(1995) succeeded in distinguishing C. virginica from two closely related oysters, C. gigas and C. ariakensis, and C. gigas from C. ariakensis by employing sequencing and PCR/RFLP analysis of part of a fragment (443 bp) of the 16S rRNA gene. Sequence data revealed that C. gigas and C. ariakensis showed higher levels of similarity to each other (95%) than to C. virginica (84–86%). Comparison of a 579-nucleotide fragment of the COI between the Portuguese oyster, C. angulata, and several Japanese oysters were made by O'Foighil et al. (1998), showing that Portuguese oyster haplotypes clustered firmly within a clade of Asian congeners and were closely related to C. gigas (but not identical). This result supports an Asian origin for the Portuguese oyster.

Reportedly, there are over 20 recorded species of oysters occurring along the coast in China (Zhang et al. 1956, Qi 1989), and for some of them classification and identification have been problematic or uncertain. Based upon extensive anatomic studies of almost all oyster species in China, Li and Qi (1994) concluded that there were 15 species of oysters, and claimed that identification of a few oyster species was clarified. Most of the species are rare and found in South China Sea. However, Even for the four common species (the Zhe oyster, Pacific oyster, Suminoe oyster, and Dalianwan oyster), it is often not empirically easy even for marine zoologists sometimes, to distinguish them clearly. This has caused inconveniences and difficulties in broodstock management and aquaculture practices. If the Dalianwan oyster is a discrete species,

separate stock conservation and management should be applied. Accordingly, clarification of the Zhe oyster's status would also help oyster aquaculture practices. These are widespread concerns for the oyster fishery along the coast of China.

The molecular data provide some clarification on the species status and phylogenetic relationships of these four species. For Dalianwan and Pacific oysters, the 16S data show close similarity between the samples of these two species, and the haplotypes of Dalianwan and Pacific oyster formed a clear clade in the phylogenetic tree. This relationship is strongly supported by the COI data set, in which all five haplotypes of the Dalianwan oyster and the only haplotype of the Pacific oyster clustered closely. This is also supported by the evident similarity in morphology between these two species. The Dalianwan oyster samples were collected from typical distribution areas, identified carefully according to the
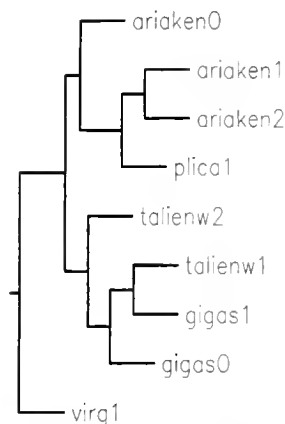
Figure 4. A consensus phylogenetic tree based on parsimony analysis of 443-nucleotide mt 16S rDNA fragment according to Kimura's model with C. virginica as an outgroup.
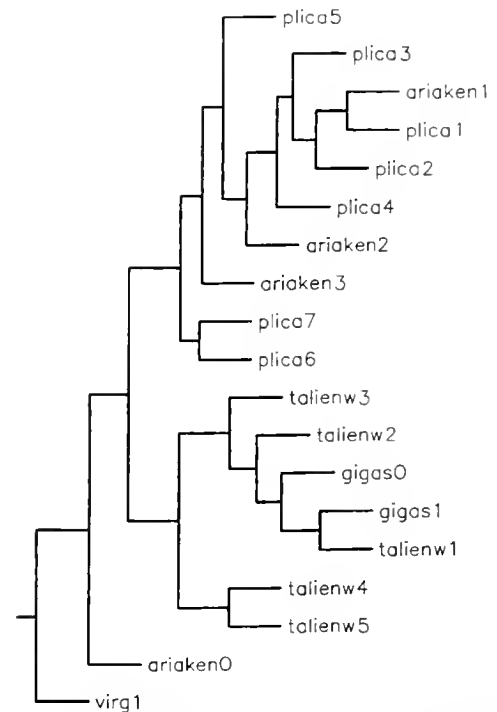
Figure 5. A consensus phylogenetic tree based on parsimony analysis of 579-nucleotide mt COI gene fragment according to Kimura's model with C. virginica as an outgroup.

descriptions of Zhang et al. (1956) and Qi (1989). Although there are some morphologic differences compared with the Pacific oyster, Dalianwan oysters share some morphologic characteristics with Pacific oysters as described by Zhang et al. (1956) and Qi (1989). A similar situation exists in scallops *Pecten maximus* and *P. jacobeus*, where they share highly similar morphologic features but have a surprisingly close genetic distance based on 16S sequences (Canapa et al. 2000). Our molecular data suggest that Dalianwan and Pacific oysters belong to the same species, which supports Li and Qi's (1994) conclusion based on anatomy studies.

Results for the Zhe and Suminoe oysters are rather surprising. The divergence between the two is much less than expected. The genetic distances between them are as low as 0.13% (for 16S) and 0.59% (for COI), even lower than that between the Dalianwan and Pacific oysters (0.81 and 1.08%). They share a high degree of similarity in these two gene fragments. In contrast, they showed higher divergence from the Pacific and Dalianwan oysters in both the 16S and the COI sequence data, though more strongly in the latter. Also, haplotypes of the Zhe and Suminoe oysters clustered in a single clade in both trees. This result is different from that generally concluded from morphologic data. Morphologically, the Zhe and Suminoe oysters are easy to distinguish in most cases. Therefore, caution should be taken for the concern of status of these two species. A possible explanation could be as follow. the "Zhe oysters" we sampled could actually be a morph of Suminoe oysters living in high salinity habitats. Because ecologically the Suminoe oyster has a wide distribution and can tolerate a wide range of salinities, morphologies could vary in different habitats. Samples collected from the habitats other than an estuary may look different from the Suminoe oysters from a typical habitat. It is possible that Suminoe oysters from high salinity area and on rocky shores are mistakenly classified as Zhe oysters because of morphologic plasticity. It has been shown that the Zhe-like small oysters found in the rocky intertidal zones of northern coast, once removed to more productive waters, could grow to a bigger size, which resemble the Suminoe oysters from an estuary habitat (R. Wang, personal comm.). To confirm either of these possibilities, a more extensive sampling and sequence analysis throughout their natural range are needed.

An interesting finding from this study is that O'Foighil's COI sequence of the Suminoe oyster showed a significant divergence not only from that of the Dalianwan-Pacific oysters, but also of the Suminoe-Zhe oysters. The divergence may be due to the fact that mt protein-coding genes like COI are usually more variable than rDNA (Hixson & Brown 1986) and the fact that O'Foighil's Suminoe oyster samples, which came from a hatchery stock originated from Japan, may represent a different population that is genetically isolated from the Chinese population (our samples). However, analysis of more specimens from Japan or other parts of their natural range is needed for confirmation.

Li and Qi (1994) suggested that the Zhe-like oysters most commonly found in the rocky intertidal zone were Pacific oysters instead of Zhe oysters as most people assumed. If so, the mtDNA sequences of these (Zhe) oysters should have higher similarity to (or low divergence with) those of the Pacific oysters or Dalianwan oysters we presented here and that of O'Foighil's. Actually this is not the case. Our sequence data show that these smaller oysters from rocky shores could be Suminoe oysters, rather than Pacific oysters.

Additionally, in this study the COI sequences showed more variations, as expected, than the 16S sequences. For instance, in the 16S data, we detected only one haplotype for Zhe oyster, two for each of the Dalianwan and Suminoe oysters; but in COI data, the numbers of haplotype are seven, five and three for these three species, respectively. Also, the divergence between *C. gigas* and *C. plicatula* or *C. ariakensis* is three times as high as that between *C. gigas* and *C. talienwhanensis* in the 16S data, whereas the divergence is eleven times higher in the COI data. The COI sequence is more sensitive in discriminating closely related species, supporting the observation by Boudry et al. (1998) where no variability was detected with nine endonucleases among 253 individuals of *C. gigas* and *C. angulata* with 16S rDNA, but reasonable polymorphism was detected with four enzymes with COI. Other works have also proved that COI sequence is a good choice for similar purposes (Meyran et al. 1997, O'Foighil et al. 1998).

In summary, the mtDNA sequence data strongly suggest that *C. talienwhanensis* is not a discrete species and should be considered as synonymous with *C. gigas*. Our data also indicate that the "Zhe oyster" is different from the Pacific and Dalianwan oysters, but is genetically very close to Suminoe oyster, at least for the ones we sampled.

ACKNOWLEDGMENTS

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INCREASED BIOMASS YIELD FROM DELAWARE BAY OYSTERS (*CRASSOSTREA VIRGINICA*) BY ALTERNATION OF PLANTING SEASON

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ABSTRACT The practice of moving oysters from low-salinity to high-salinity areas for improving growth and meat quality has been practiced for well over a century. In the Delaware Bay, the practice was abruptly changed when MSX (*Haplosporidium nelsoni*) caused large-scale oyster mortality in the higher salinity portions of the bay. Similar disruptions occurred in Chesapeake Bay and other areas. In time the Delaware Bay, the oyster industry learned how to operate around the disease, but in early 1990s, Dermo (*Perkinsus marinus*) began to cause serious mortality on transplanted oysters. Despite the historic and continuing movement of oysters within and between estuaries, there is little published scientific literature indicating optimum conditions for transplantation. We investigated the effects of transplantation from a low-salinity seed bed to a typical higher salinity leased ground. The transplants were designed to evaluate an early, the traditional spring, and two fall transplant dates on the subsequent disease levels, growth, and survival of the oysters in three size classes: market, submarket, and small. Environmental and oyster disease data suggest we conducted the experiment under nearly worse-case conditions, high Dermo, and low food (chlorophyll). There were no significant differences associated with the timing of transplant. We did not record significant growth on any size oyster and disease caused mortality exceeded 50% for early transplants. Smaller oysters experienced greater mortality than market size individuals. Despite these conditions, meat dry weight nearly doubled within 1 to 2 mo after transplant in all but the March transplant. Under these disease and environmental conditions the only economic gain would be from the doubling of the meat weight and associated better meat quality. No gain can be expected from submarket oysters growing into the market size classes.

KEY WORDS: oyster, *Crassostrea*, Delaware Bay, season, disease, growth

INTRODUCTION

In the Delaware Bay oysters have been transplanted from upper bay low-salinity seed producing areas to lower bay higher-salinity growing beds for more than 150 years (Ford 1997; Fig. 1). Similar transplantation strategies have been used by oyster growers in Chesapeake Bay (Andrews & McHugh 1957) and New England (Ingersoll 1881, Goode 1887). Further, to increase production and/ or to supplement local seed as resources became depleted, oysters were imported from distant sources. Despite these historic and continuing large scale movement of oysters within and between systems, there is little scientific literature indicating the optimum conditions for transplantation.

Hopkins and Menzel (1952) developed a framework for studying the transplantation of oysters based on the biomass yield of the product, and Andrews and McHugh (1957) used biomass yield estimates from trays of oysters to evaluate the effectiveness of transplantation strategies. Reliance on biomass as a means of assessment in both of these studies was based on the assumption that the majority of oysters were destined to be shucked, and thus meat yield was the most important aspect of production. This may not be the case for those oysters that are grown to be sold for the half-shell trade. In this latter case, assuming adequate meat quality, numbers at market size are more important than total biomass.

Haskin et al. (1983) and Hargis and Haven (1988) both indicate that the oyster planting industry in the Delaware Bay and the Virginia portion of Chesapeake Bay, respectively, operated under the assumption that transplanting was profitable if one bushel of seed oysters yielded one bushel of market oysters. In the late 1950s, the parasite MSX, *Haplosporidium nelsoni*, caused epizootic mortalities in both estuaries and forced major changes in oyster industry practices. In the Virginia portion of Chesapeake Bay, growers abandoned higher salinity grounds and concentrated efforts in areas that historically produced higher than the 1:1 yield (Hargis & Haven 1988). Despite *H. nelsoni*-caused losses, the

Delaware Bay oyster industry continued to transplant oysters based on the system developed in the 1800s. Oysters were left on the planted grounds, where high salinity favored the *H. nelsoni* parasite, but for no more than 1 y (Ford 1997), and yields continued to be about 1:1 (Haskin & Ford 1983). After the 1950s *H. nelsoni* epizootic, the importation of seed from out of state into the New Jersey portion of Delaware Bay was banned.

In 1990, an outbreak of Dermo disease caused by *Perkinsus marinus* prompted a further change in strategy by the Delaware Bay oyster industry. After 1990, *P. marinus* infected most of the oysters in the seed bed areas (Ford 1997), and oysters planted in the spring of 1991 suffered high mortality in the late summer. The oyster industry and the State of New Jersey responded by developing a program to market oysters directly from the seed beds. This strategy produced oysters that had poorer meat quality and a lower value than those from higher salinity waters.

At the same time, it was realized that although Powell et al. (1997) modeled the effect of transplant time, disease, and predation on market oyster populations, there were no real data on which to base transplantation decisions in the presence of this new parasite. The model predicted that fall (November) transplants left for 1 y yielded the best survival of market oysters compared with transplants in January, March, or May that were harvested in November. In all cases the number of market oysters declined from July to November. The model did not include an August transplant with immediate harvest that fall, a strategy that would minimize disease-caused mortalities while still taking advantage of typically good fall "fattening" conditions. The industry requested data on the following: 1) the best time of the year to transplant oysters; 2) the survival of transplanted oysters at various times after transplant; 3) the numbers of market oysters expected from the net result of growth and mortality; and 4) the gains that could be made in meat quality and the length of time after transplant this gain might take.

The industry, through a nonprofit foundation, collaborated with
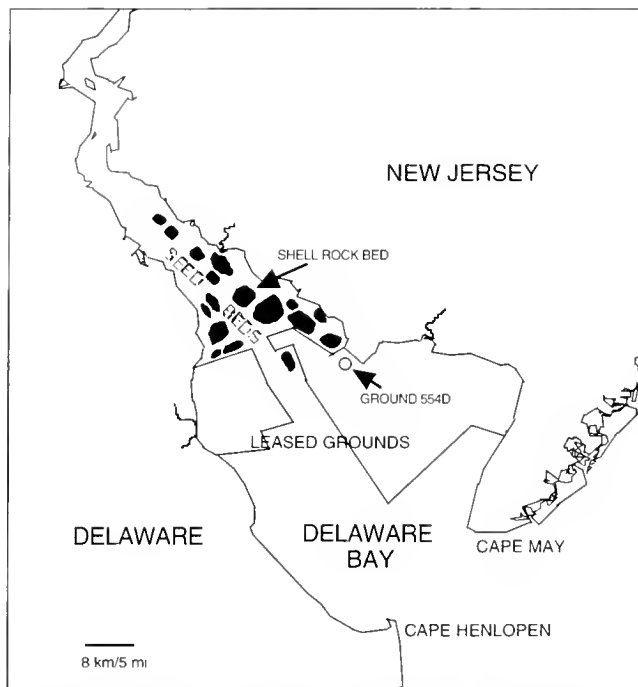
Figure 1. Delaware Bay showing locations of the seed beds and Shell Rock bed, leased grounds, and the ground used for transplant studies.

state New Jersey Department of Environmental Protection (NJDEP) and Haskin Shellfish Research Laboratory (HSRL) personnel to conduct an initial test of alternative planting dates. This study (Canzonier 1998) moved oysters from the Shell Rock seed bed to higher salinity grounds (527 D) in December, February, May, and August. The effort clearly established that transplanting in months different from the historical spring period was economically feasible, but cautioned that a single year's result could not provide sufficient background for assessing year-to-year variation. In addition, all months but the traditional spring transplant period, represented by the May transplant, gave nearly identical results. The May transplant had significantly less market oysters produced than the other months (Canzonier 1998).

The information at the onset of the current study suggested that transplantation strategy would depend on several factors: oyster population size frequency distribution, source stock disease level, seed bed used as a source, environment of the planted ground, disease pressure, and harvest timing. In addition to biological variables, market factors, and industry seasonal work cycles affect the economic impact of alternative planting seasons. The present study builds upon earlier efforts and evaluates the effects of varying the timing of transplanting oysters from one seed bed to a lower bay planting ground.

MATERIALS AND METHODS

Experimental Design

Oysters from Shell Rock Bed were transplanted to ground 554 D (Fig. 1) in March, May, September, and October of 1999. Shell Rock was selected because it represented a central seed bed source, had a significant number nearly market size oysters, and provided the oysters for the Canzonier (1998) study.

The transplant ground was subdivided into experimental plots,

each marked with navigation coordinates. A preliminary sampling indicated that only a small number of large residual oysters (mean 99 mm) were present (mean 2.4 oysters bu^{-1} from 8 one-bushel samples). Approximately 1800 US Standard bushels (36.4 L; herein after referred to as bushels or abbreviated as bu.) of oysters were planted on each 24.4×91.4 m plot each transplant time (3,200 bu.$acre^{-1}$ or 90,000 oysters $hectare^{-1}$).

At each transplant time, triplicate bushels of oysters were removed from the deck load of the boat and analyzed in a manner similar to the techniques used for the subsequent monthly samples (see below). In addition, oysters were processed for disease diagnosis.

After planting, at least three dredge samples were collected each month from each planting. All material was placed in the bushels so that triplicate composite bushel samples of material were examined from each planting each month. These were examined in the same manner as the source oysters, but with special attention to growth, meat condition, *P. marinus* level, and mortality (apportioned by oyster size). In the latter months, additional oysters were set aside after the samples had been collected to be sure enough material was available in all size classes to process *P. marinus* and condition index samples. *H. nelsoni* levels were not determined on the monthly samples, but were evaluated on the final samples from each plot in November, as well as on the initial transplants.

Sample Processing

All live oysters >20 mm, old, new boxes, and gapers in the entire sample were counted. All oysters >20 mm were measured and divided into market (>76 mm) and submarket (55–75 mm) and small (<55 mm) classes. All parameters were normalized to a standard bushel for comparison with other samples. Mortality was estimated by calculating the percentage of new boxes and gapers in each sample. This was considered recent mortality. Recent mortalities were accumulated to provide an estimated cumulative mortality at the end of the study (Ford & Haskin, 1982).

Twenty oysters (six or seven from each of the 3 bu.) of each size class were set aside for evaluation of condition index and an additional group of similar size was examined for *P. marinus* infection. Condition index was derived from the ratio of meat dried at 50°C, and greatest shell dimension (height). *P. marinus* was diagnosed after incubation of the rectum and a piece of mantle in Ray's fluid thioglycollate medium. Infection intensity was scored from 0 to 5 (Ray 1954) and a weighted prevalence calculated as the mean intensity, including zeros, of all oysters in a sample. Oysters in the initial planting and final sampling were diagnosed for *H. nelsoni* by tissue section histology. Infection intensities were rated from 0 to 4 (Ford 1985) and a weighted prevalence calculated as for *P. marinus*.

Individual Oyster Growth and Mortality Study

To evaluate production requires size–class-specific growth and mortality data. This was approximated from the bushel samples, but a second method was utilized to provide a more precise evaluation of individual oysters. A group of experimental oysters representative of the source bed was deployed at the time of transplant. This group consisted of five replicates of 20 oysters from each of three size classes (63.5 to 69.9 mm, 70 to 75.9 mm, and >76 mm) for a total of 300 oysters. Fishing leader tethers were glued to the top valve of each oyster with Marine Tex. The tethers

were then attached with cable ties along the side of a square reinforcing rod frame square (~1 m on each side) that was held approximately 5 cm above the bottom by a centrally located cement anchor. The entire array was attached to a surface float. Each individually identified oyster was measured (height) and the array deployed so that the oysters would lie on the bottom. Each month each oyster was measured and mortality or loss noted. In this instance, mortality was calculated directly because the history of each oyster was known.

Environmental Data

The following environmental data were collected on bottom water on at least an every other week basis: temperature, salinity, dissolved oxygen, pH, total suspended solids, Chlorophyll a, and suspended organic material. In addition, temperature was monitored continuously with an electronic recorder. Salinity was obtained with a refractometer. All grab sample temperature and dissolved oxygen data were measured with a YSI oxygen meter, and pH data were obtained with an electronic pH meter. Suspended solids, chlorophyll and particulate nitrogen samples were obtained from at least 500 ml of water filtered through Whatman GF/C glass fiber filters, which were stored on ice until they were returned to the laboratory. Chlorophyll samples were immediately placed in buffered acetone and refrigerated. Particulate samples were dried at 50°C. All environmental data were analyzed according to Strickland and Parsons (1968).

Data Analysis

Size frequency data were normalized by adjusting the base live and recent dead (gapers and new boxes) frequency distributions from all individuals collected in the three bushel samples (in 5-mm increments) to 100 individuals. These frequencies were then adjusted to the number of live or dead bu.$^{-1}$ by multiplying the frequency of occurrence in all sizes by the average number of live or dead bu.$^{-1}$ Data were summarized and significant tests were run using one-way analysis of variance, t tests, or other descriptive techniques. Percentages were transformed using an arc-sine transformation before performing analysis.

RESULTS

Environmental Data

Temperature on the transplant ground was 3.5°C in March, at the beginning of the study, and peaked in August at 27.5°C. Salinity was generally between 21 and 23 ppt., with a low of 19 ppt in April and a high of 26 ppt in October and December. pH remained relatively stable, ranging from 7.8 to 8.6 with the exception of a low value of 6.9 on September 1. Dissolved oxygen ranged from a high of 13.5 mg L^{-1} in March to a low of 5.6 mg L^{-1} on July 14. In general dissolved oxygen levels remained near or above saturation at temperatures below 20°C and near or slightly below saturation above those temperatures. Total suspended solids were typically between 30 and 55 mg L^{-1}, with highest and lowest values of 86 and 18 mg L^{-1} on August 18 and May 5, respectively. Chlorophyll a showed a typical spring (late March to early April) bloom followed by generally lower vales in summer (Fig. 2). There was an increase in Chlorophyll a in fall (October to early November). Highest Chlorophyll a levels were found March 25, April 1, May 18 and November 5 with values of 54, 46, 38 and 39 mg m^{-3} respectively.
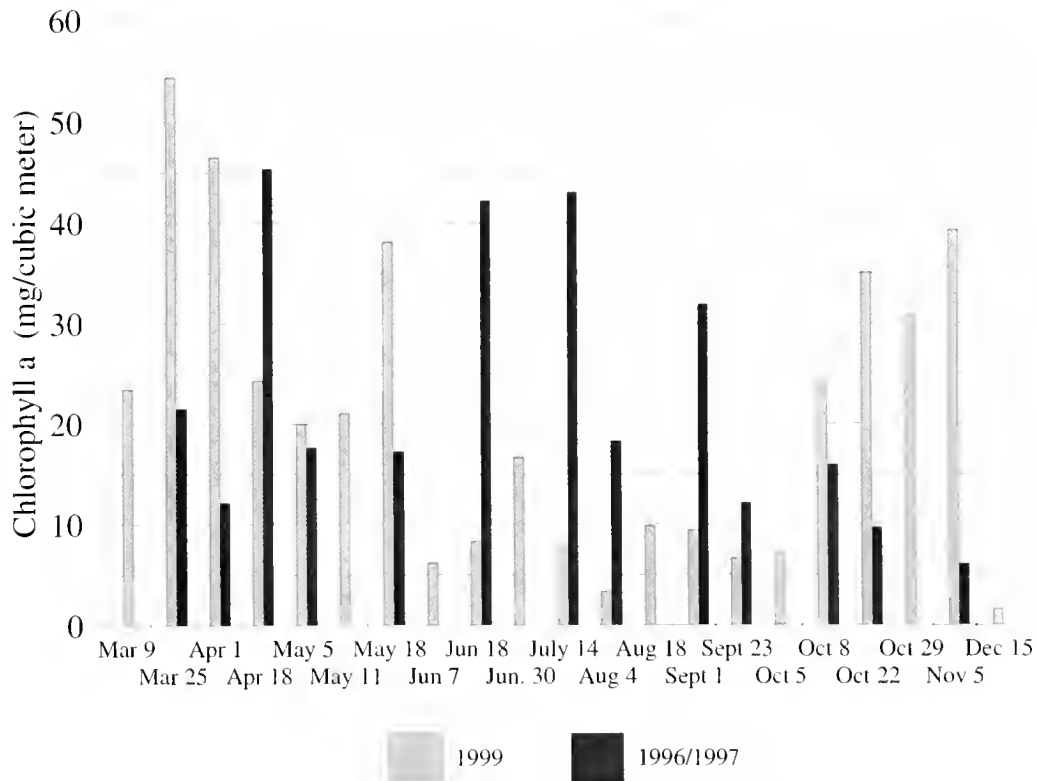
Figure 2. Bottom water chlorophyll a in samples taken from bottom water over ground 554 D in Delaware Bay in 1999 compared with similar data taken over ground 527 D in Delaware Bay in 1997. Data are in mg per m^3. 1997 data from Canzonier (1998).

Oyster Data

Because the samples taken at the time of transplant represented the source bed and culling machinery on the boat, not the ground to which the oysters were transplanted and monitored, time 0 (T_0) for subsequent analyses was the first sample after transplant. The samples taken from the deck at the time of transplant were utilized to estimate the size, condition and numbers of oysters transplanted.

Numbers of Live and Dead Oysters

The numbers of oysters being transplanted, based on the initial samples for each transplant period, suggests that all groups, with the exception of the October transplant, received approximately the same number of individuals per unit volume of material moved. The October samples had fewer oysters than those groups transplanted in March and September, but was equivalent to the May transplant (Table 1). It seems likely that more live oysters were moved in the May transplant than in October, but the high variance in May precludes making a definite statement.

The total numbers of live oysters significantly decreased from T_0 to the final samples (T_f) in November. The numbers in the March and May transplants fell approximately 50% from 200 in initial post-planting samples to <100 bu.$^{-1}$ in the final November sample (Table 1). The mean oysters bu.$^{-1}$ in October and November, traditional harvest months, were greatest for the September transplants, but the difference was statistically significant only in October. The decrease in oysters from planting to November was least in the September transplants, but the time between T_0 and T_f was only one month. No calculation can be made for the October planting because $T_0 = T_f$ (Table 1).

Live oyster numbers were also analyzed by size (Table 2). Data from dredged samples show that numbers of marketable oysters declined about 50% for March transplants, but that subsequent transplants experienced little or no loss. Submarket and small oyster numbers also declined, and, with the exception of the September transplant, these declines were usually greater than for market size oysters and often more than 50%. Despite large losses of oysters, there were no statistically significant differences in November in the number of market size, or submarket size oysters in any transplant period. Numbers of small oysters in the March and

May transplants had declined appreciably by November and there were about half as many small oysters per bushel as in the other two size classes, even though small oysters were most abundant at the time of transplant. Numbers of small oysters remained high in the final sample of the September transplant, but not in the October group.

Recent mortality, for all size classes, was greatest in the fall (Fig. 3). These losses occurred across all size classes, but, with the exception of the October transplant, losses were greatest in the smallest size classes. Estimated cumulative mortality from transplanting to the final sample of all size oysters was 54, 55, 15, and 9% for March, May, September and October transplants, respectively. Total losses of small oysters were greater than those of market or submarket oysters for the March and May transplants (Table 3). There were no differences between the market and submarket oyster losses in any transplant group.

Disease Levels

H. nelsoni was detected, in initial and final samples, only at very low levels. There was no association with size or transplant time. The highest infection level (prevalence) was 30%, but most infections averaged <15%. The highest weighted prevalence (0.4) was found in the fall samples.

In contrast, *P. marinus* levels were high in all plantings and all size classes (Fig. 4). Infections were nearly as heavy and abundant on the source bed as they were in oysters already transplanted to the higher salinity experimental site. Percent infection (prevalence) for the March and May transplants exceeded 80% by July and was usually 90 to 100% until it dropped below 80% in November. For the later transplants, *P. marinus* levels usually increased to 90 to 100% within 1 mo after transplant. Weighted prevalence was relatively high in the March transplants, but underwent a typical drop in April/May (Bushek et al. 1994). The same drop occurred on the source bed as the May transplants had a weighted prevalence similar that of the March transplant at the same time. Intensities in both groups then increased over the summer until September, when levels in all size categories decreased concurrent with an increase in mortality (compare Fig. 3 to 4). Levels increased again in the October sample and then dropped by nearly 50% in November. At

TABLE 1.

Mean numbers of live oysters >20 mm bu.$^{-1}$ by month with 95% confidence limits ($n = 3$ for each monthly sample).

	March			May			September			October		
	Mean	95% Conf. Limits		Mean	95% Conf. Limits		Mean	95% Conf. Limits		Mean	95% Conf. Limits	
M	323	363	283									
A	212	230	195									
M	**124**	**189**	**59**	296	403	188						
J	156	209	103	188	239	137						
J	105	155	56	**121**	**169**	**73**						
A	119	144	93	76	154	0						
S	80	120	40	90	143	38	307	355	259			
O	108	131	84	92	104	79	**169***	**203**	**136**	243	254	232
N	76	87	65	79	113	45	146	205	87	**78**	**101**	**56**

Bold numbers indicate a significant difference from the prior month. The area in gray indicates samples removed from the deck of the transplant vessel. These were not used in subsequent calculations.

* Significantly more oysters than in other transplants during the sample period.

TABLE 2.

Mean number of live market (>76 mm), submarket (75–55 mm), and small (55–20 mm) oysters bu.$^{-1}$ of dredged material from transplants in March, May, September, and October 1999.

	March 1999			May 1999			September 1999			October 1999		
	Market	Submark	Small	Market	Submark	Small	Market	Submark	Small	Market	Submark	Small
M	58	115	150									
A	63	76	75									
M	48	32	45	78	104	114						
J	61	42	54	34	69	87						
J	29	37	40	38	34	49						
A	40	41	38	23	25	28						
S	30	30	21	25	37	39	56	86	167			
O	32	38	38	38	33	21	29	47	94	54	70	120
N	32	29	15	34	33	16	27	35	84	25	26	28

Oysters were transplanted from Shell Rock to Ground 554D on the Delaware Bay leased grounds. Areas of gray indicate samples from deck loads of transplanted oysters. All other samples were dredged from transplant plots. Submark = submarket.

this time, heavy mortality was observed in the March transplants only (Fig. 3) and the drop was probably the beginning of the overwinter loss of infections (Bushek et al. 1994). Oysters transplanted in September and October had weighted prevalence similar to those transplanted earlier, but, unlike the former, infections remained at very high levels in these oysters into November. The persistence of high infection levels was associated with low mortality in both fall groups.
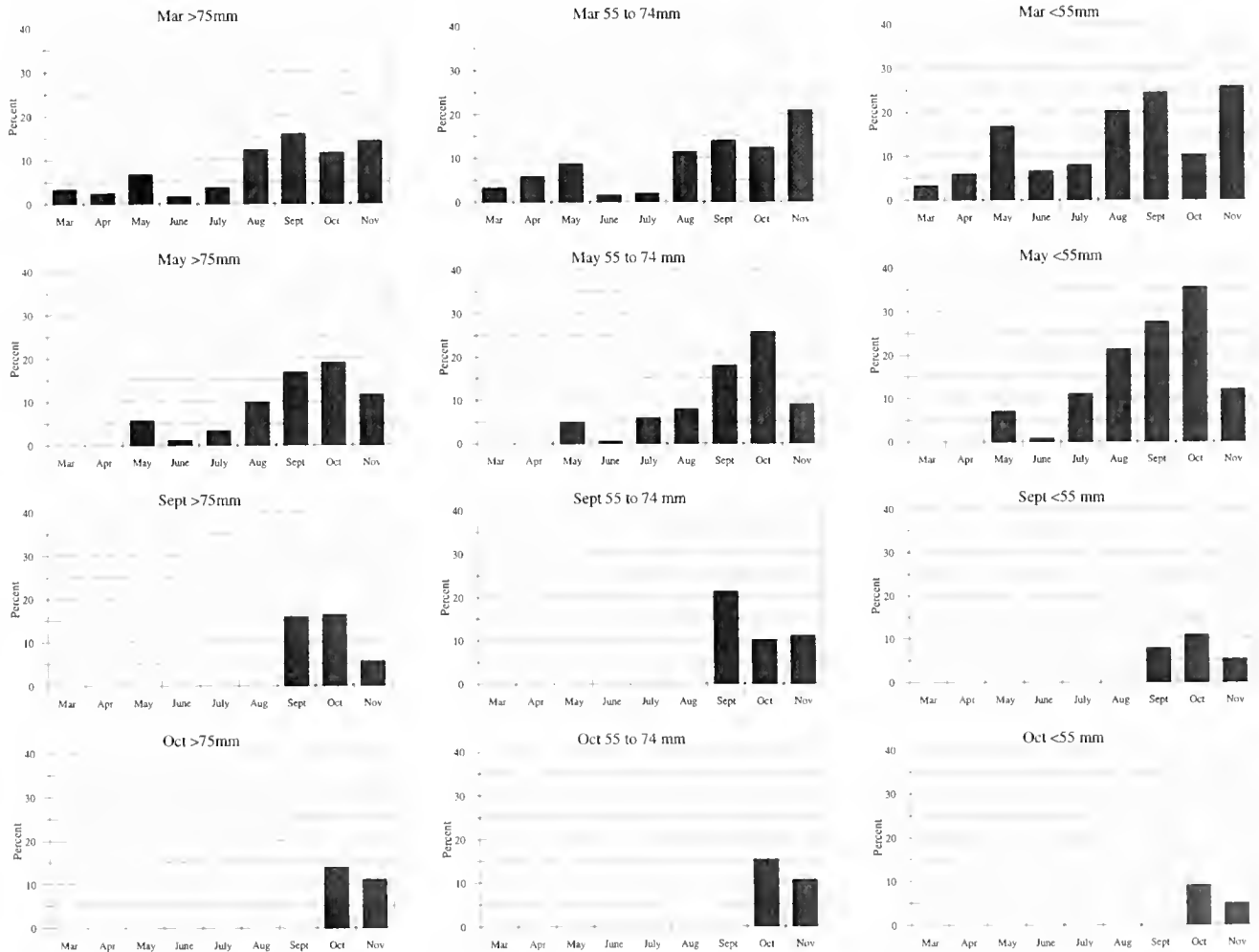
Figure 3. Interval percent mortality by month of market (>75 mm), submarket (55 to 74 mm), and small (<55 mm) oysters transplanted from Shell Rock to Delaware Bay ground 554 D in 1999. Transplant months were March (top graphs), May (middle top graphs), September (middle bottom graphs), and October (bottom graphs).

Estimated cumulative percent mortality, from planting to November 1999, by size category of dredged oyster samples collected in Delaware Bay by transplant month.

March 1999			May 1999			September 1999			October 1999		
Market	Submark	Small	Market	Submark	Small	Market	Submark	Small	Market	Submark	Small
46	48	65	45	48	88	19	16	14	11	11	5

Market (>76 mm), submarket (75–55 mm), and small (55–20 mm).

Growth and Condition

With the exception of the March transplants, there were no differences in the sizes of oysters in the submarket and small categories through time. Mean dry meat weight of market oysters for the March and May transplants increased significantly in June, after 3 and 1 mo, respectively (Table 4). That of market-size September and October transplants rose in November after 2 and 1 mo, respectively. There were no significant differences in meat weight among any of the transplanted groups by November. While not statistically significant, there was a consistent increase in meat weight in all transplants of market-size oysters between October and November. In general, meat weight increases of submarket and small oysters mirrored those of the market-size individuals.

Reflecting the increase in meat weight without increased shell size in market oysters, the condition index increased during the study period. With the exception of the March transplants, oysters required one month after transplant to the lower bay to improve condition, and they typically retained this condition throughout the summer and into the fall. While not statistically significant, there
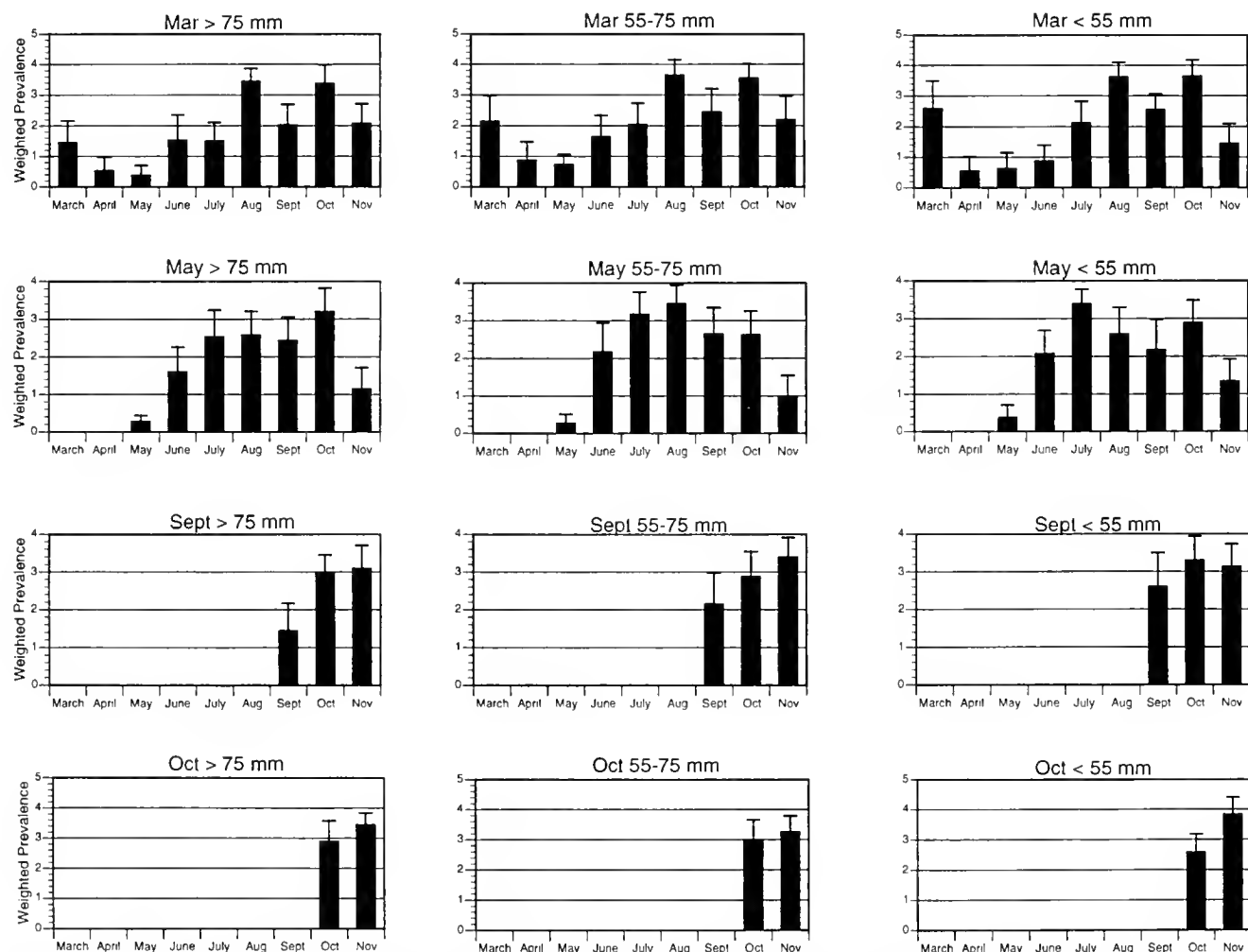
Figure 4. Monthly weighted prevalence of Dermo (*P. marinus*) infections in market (>75 mm), submarket (55 to 74 mm), and small (<55 mm) oysters transplanted from Shell Rock to Delaware Bay ground 554 D in 1999. Transplant groups were March (top graphs), May (middle top graphs), September (middle bottom graphs) and October (bottom graphs). For each transplant group, the first sample represents that on Shell Rock bed when the oysters were moved. All subsequent samples represent infection levels on ground 554 D. Error bars represent 95% confidence interval.

TABLE 4.

Mean dry meat weight (g) of market-size oysters by month with 95% confidence limits.

	March			May			September			October		
	Mean	95% Conf. Limits		Mean	95% Conf. Limits		Mean	95% Conf. Limits		Mean	95% Conf. Limits	
M	1.1	1.2	0.9									
A	1.3	1.5	1.2									
M	1.5	1.8	1.2	1.5	1.7	1.3						
J	**2.4**	**2.8**	**2.0**	**2.3**	**2.6**	**1.9**						
J	2.5	2.8	2.2	2.3	2.7	2.0						
A	2.2	2.4	2.0	2.2	2.4	2.0						
S	2.4	2.7	2.1	2.5	2.8	2.2	1.6	1.8	1.4			
O	2.2	2.6	1.8	2.3	2.7	2.0	1.9	2.3	1.6	1.1	1.3	1.0
N	2.9	3.4	2.3	3.1	3.6	2.6	2.5	2.9	2.1	**2.7**	**3.2**	**2.2**

Bold numbers indicate a significant difference from the previous month.

was a general trend for market oysters to improve in condition from October to November.

Condition index for submarket and small oysters generally followed the same trends as for the market oysters with no significant change from June to November. In general, there was a significant increase in condition within 1 mo after transplant for all submarket and small oysters with the exception of the March transplants and small oysters transplanted in October.

By November, the meat condition index of all size classes in the March and September transplants was statistically the same. Among the October transplants, condition of submarket and market oysters was the statistically similar, and greater than that of small oysters, while the condition of market oysters in the May transplants was greater than that of either submarket or small oysters.

Growth and Mortality of Individually Marked Oysters

For calculations of mortality, the data from the tethered oysters were corrected for oysters lost during the experiment by reducing the numbers of oysters present from the initial counts. A few oysters were lost because of detachment of the adhesive, but one entire rack was lost.

Mortality of tethered oysters mirrored that of oysters transplanted at similar times, with a few notable exceptions (Fig. 3). It is evident from the cumulative mortality data (Table 5) that the tethered oysters (particularly those put out in May and September) had substantially more mortality than that estimated from examination of boxes and gapers in dredged samples. At times, shells on one section of an array were observed to have become blackened. This suggests that some silting had taken place around these oysters and may have elevated the mortality above that experienced by the planted oysters, but we have no independent measure to evaluate if some planted oysters were silted in and not adequately

TABLE 5.

Cumulative percent mortality of tethered oysters, and oysters in dredged samples as a function of transplant time.

	Month of Transplant			
Method	March	May	September	October
Tethered	76	93	59	38
Dredged	54	55	15	9

sampled with the dredge. There were no significant differences in recent or cumulative mortality based on size of the tethered oysters.

Because all tethered oysters were large and the growth increment was small relative to the potential error, the monthly growth increment of tethered oysters was difficult to measure. This difficulty is evident in the fluctuations in increment growth for the various size classes (Fig. 5) and the negative growth measured for some months. Growth, as indicated by new shell being accreted to the oysters, was observed on some oysters in all but the coldest months.

Because individual oysters were followed, cumulative growth is the difference between the initial measurement and the measurement of surviving oysters at any time period (Fig. 5). Because not all oysters survived through all time periods, cumulative growth reflects both survival and growth of individuals.

By November there were no differences in growth of surviving tethered oysters classed as market-sized in March and May, but individuals in both groups had grown more than those tethered in September and October. There was no statistically significant growth for either of these latter two periods. Growth of submarket size oysters was also at the limits of detection. The 70- to 75-mm size class showed >0 growth only for the May and September groups when the mean were 4.8 and 1.8 mm, respectively. With the exception of the March tethered individual (only one oyster survived to October) oyster classed as small did not show measurable growth.

DISCUSSION

Hopkins and Menzel (1952) indicated that the major difficulty in deriving estimates of production was not related to measurement of growth, but to measurement of losses due to mortality. In our case, where only large oysters were being evaluated and growth was poor; it was also difficult to assess growth.

The dominant themes of Delaware Bay oyster transplantation in 1999 were related to high Dermo (*P. marinus*) levels and the associated high mortality and low chlorophyll and the associated poor growth. There is a general hypothesis that mortality of transplanted, market-sized oysters, due to disease or other factors, can be made up for by oysters growing from smaller sizes to the market classes during the year Powell et al. (1997). This can happen in some years (Canzonier 1998), but in periods such as 1999 with high *P. marinus* levels and relatively low food, growth may
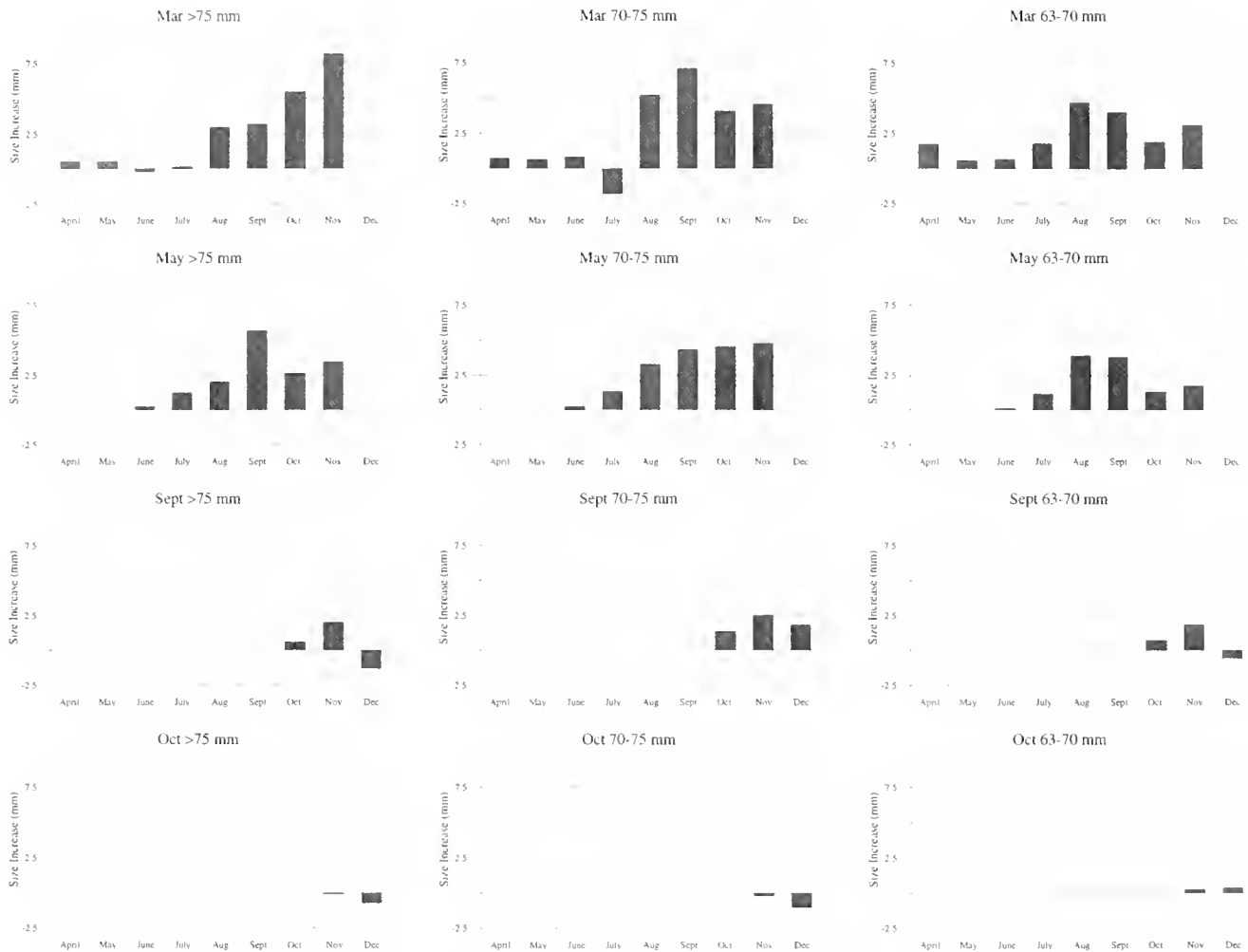
Figure 5. Cumulative growth of >75 mm, 70–75 mm, and 63- to 70-mm tethered oysters transplanted from Shell Rock to Delaware Bay ground 554 D in 1999. Transplant months were March (top graphs), May (middle top graphs), September (middle bottom graphs), and October (bottom graphs). Negative growth is due to measurement error. All oysters were followed as individuals and growth is the summation of all oysters alive in that size class at the time of measurement.

be reduced to the point that this hypothesis is not valid. Neither the tethered oysters nor the transplanted oysters in the dredged samples, of any size class, in the present study showed statistically significant growth.

The data did not show statistically significant differences in numbers, based on month of transplant, of market, submarket, or total oysters per bushel in final sampling in November. This suggests that in periods of high *P. marinus*, high mortality, and low food the timing of transplantation is not a major consideration from the point of view of the numerical yield of market oysters. In addition to the nearly 50% losses of submarket and market oysters, losses of small oysters exceeding 65% suggest that transplantation of small oysters with the expectation that they will grow into the market-size category is not an efficient use of the resource under high *P. marinus* conditions.

In view of lack of significant differences in the numbers of marketable oysters associated with transplant month, possible differences in meat quantity need to be considered. In all cases (except the March transplants when water temperatures were low) total meat weight improved within one month following transplantation (Table 4). Beyond this initial improvement in there was no change during the summer months, but in all cases there was a

trend (not statistically significant) toward further improvement in between the October and the November samples. Clearly the improvement in meat weight in the May to June period could be due to the increase in gonadal tissue, but the weight did not decrease in the summer or fall, after the spawning period, indicating that some of this weight gain was more than gonadal production. The improvement in meat quality occurred in 1999 despite the high disease levels, high mortality and lack of shell growth.

Comparison with Previous Studies

Powell et al. (1997) modeled the effect of transplanting Delaware Bay seed bed oysters in November, January, March, April, and May on the number of market size oysters available the following July to November. The model predicted that a November transplant with a November harvest provided the best yields, and that growth of submarket sized oysters compensated for the losses of market sized individuals. Mortality of submarket oysters was less than for larger ones because the added scope-for-growth offers these individuals some disease protection. Simulated *P. marinus* levels peaked slightly above four weighted prevalence a level nearly reached in the present study. The model simulated that

TABLE 6.

Comparison of numbers of market and submarket oysters bu.$^{-1}$ planted on leased grounds in 1996 to 1997 and 1999.

Year of Transplant	Market	95% Confidence Limit	Submarket	95% Confidence Limit
1999	62	±18	232	±55
1996/97	56	±13	576	±105

Data from 1996 to 1997 are from Canzonier (1998). Data are from samples removed from the deck of the transplant vessels.

submarket size oysters were less susceptible to mortality from *P. marinus* than the market-sized oysters, which allowed them to grow to market size and replace larger, individuals with lethal infections. This simulation was not verified in the present studies. One reason is that, in contrast with the model simulation, the smaller oysters did not grow. Thus, they did not increase in biomass fast enough to "outgrow" the parasite and maintain parasite burdens below lethal levels. It is important to emphasize that the food present in 1999, as indicated by Chlorophyll *a*, was lower than that used in the model of Powell et al. (1997). It seems likely that the low food concentrations in 1999 reduced the potential for compensatory growth of submarket oysters to replace market oysters that died during the study period. The lack of growth may also have been a consequence of high disease levels (Menzel & Hopkins 1955, Paynter 1996). Further, many of the assumptions of the Powell et al. (1997) simulations were based on age/size relationships observed in the Gulf of Mexico, which do not apply to Delaware Bay. In Delaware Bay, for instance, submarket-sized oysters (55–75 mm) obtained from seed beds are at least 3 years old and many of the small oysters (<55 mm) are at least 2 y old. All sampling of oysters in the Bay indicate that by age 2, oysters have *P. marinus* infection levels that are equal to that of older oysters. Thus, it is not surprising that cumulative mortality for our submarket and small oysters was equal to, or greater than, that of market-sized oysters. A second major difference between our study and the model simulations is that significant numbers of submarket oysters did not grow into market individuals in 1999.

Canzonier et al. (1998) reported on a similar transplant. He moved oysters from the same seed bed (Shell Rock) in December 1996, and February, May and late August 1997, and sampled them until November 1997. Growth of oysters into the market size cat-

egory was clearly evident in the 1996 to 1997 period (Canzonier 1998). The number of oysters bu.$^{-1}$ transplanted differed significantly between this study and the present one (Table 6). There were no differences ($P = 0.43$) in the numbers of market oysters bu.$^{-1}$ from the deck loads of the two studies, but there were nearly twice as many submarket oysters in the earlier trial (Table 6). In 1996 to 1997, the percentage of market oysters bu.$^{-1}$ ranged from 8 to 10% whereas in 1999 market oysters were between 18 to 26% of the total. Canzonier (1998) found the number of market oysters from dredge samples remained relatively constant throughout the test period in spite of the substantial mortality. Thus despite twice as many submarket size oysters and growing conditions that were better than in 1999, there were no changes in the number of market size oysters in any month of transplant in 1996 to 1997. Growth of submarket oysters made up for the loss of older oysters.

As opposed to the 1999 results, in which a 21% decrease in the numbers of market oysters was observed in all transplants, Canzonier (1998) reported an insignificant 4% decrease in the number of market-size oysters at the end of the experiment in November. *P. marinus* levels were generally lower in 1996/97 when compared to both the model and the 1999 data (Table 7). Cumulative mortality was less for December and February transplants but apparently higher for May and August transplants in 1996/97 when compared with roughly similar transplant months in 1999 (Table 8). Chlorophyll *a* in 1997 showed a slight peak in the spring, a second peak in June and continued high levels (relative to 1999) throughout the summer, but a general decline from late August to November (Fig. 2). In this latter condition, Chlorophyll *a* in the earlier period was similar to those in the Powell et al. (1997) model. The presence in 1996 to 1997 of high summer food concentrations, lower *P. marinus*, and consequently lower mortality than in 1999 suggests that the 1999 conditions may be nearly a worst-case representation. The only exception would be the presence of the fall bloom in 1999 that would have allowed the oysters to enter the winter in better condition. This may or may not be important because there was no difference between the dry meat weights in 1996 to 1997 when there was no fall bloom and 1999.

Canzonier (1998) reported that market oysters moved from Shell Rock in December, February, May, and August averaged the same dry meat weight (1.2 to 1.3 g) as those at the time of transplant in the present study. His final product in November had a meat weight of 2.8 g, the same weight as oysters in 1999.

How the increase in meat quality in transplanted oysters, vs. those marketed directly from the seed beds, would affect profit-

TABLE 7.

Initial and selected months.

	December		February		May		August	
	Market	Submark	Market	Submark	Market	Submark	Market	Submark
D	1.8	1.3						
F			0.8	0.7				
A	0.2	0.1	0.1	0.1				
M					0.1	0.1		
A	2.1	1.4	1.2	1.3	0.7	1.1	1.0	0.6
S	1.2	1.3	1.9	1.3	1.3	2.3	1.7	1.8
N	0.4	0.6	0.2	1.2	0.5	1.2	0.5	0.9

Weighted prevalence of *P. marinus* (Dermo) in oysters transplanted from Shell Rock to 527D in 1996 to 1997. Market >75 mm, Submark = Submarket (55–75 mm). (From Canzonier 1998).

TABLE 8.

Cumulative percent mortality from planting to November of oysters from Canzonier (1998) and present study.

Study	Month of Transplant			
	March	May	September	October
Present study	54	55	15	9
	December	February	May	August
Canzonier (1998)	43	45	30	15

ability is dependent on the relationship among the following parameters: 1) the number of market oysters bu.$^{-1}$ and/or the amount of meat bu.$^{-1}$ that could have been harvested directly from the seed beds; 2) the number of market oysters and/or the amount of meat bu.$^{-1}$ that could have been harvested from the transplanted oysters; 3) the cost of re-harvesting the transplanted oysters; 4) the added value that is derived from post-shucking processing (washing with fresh water and blowing with air to help remove shell materials) a higher salinity oyster; and 5) the value of the bushel of oysters to the market. The latter value is dependent on the season of harvest, competing product and whether the oysters are shucked or sold in the shell.

If oysters are used as shell stock, there would be little gain in value to the harvester from an increase in meat yield, because in current conditions, there is little chance that additional price would be paid (S. Fleetwood, Bivalve Packing, pers. comm.). The best that could be expected would be a longer term value increase because of better market acceptance. Before the disease infestations, Delaware Bay oysters received a premium price because of their high meat yields. Thus for shell stock oysters, in years of high or moderately high *P. marinus* disease-caused mortality, there would be little to gain from transplantation.

For oysters that are to be shucked, results of both the 1996 to 1997 and 1999 studies indicate a significant increase in meat yield after transplantation. It is important to note that the meat yield increase, during months with warm water, can be obtained in one or at most two months. In 1999 the average meat yield increase by

November was about 115%, and in 1996 to 1997 the meat yield increased by about 133% (Table 9).

Given that there was no difference in the number of oysters available for market in November (Table 9) associated with transplantation time, it would appear that there was no value added from transplantation in any month or for the average of all months. It should be emphasized that under current conditions, market oysters are culled on board. This means that nearly equal numbers of oysters bu.$^{-1}$ would be delivered to the packing house from both the seed beds and the planted grounds. Under these conditions the meat from oysters harvested from the planted grounds in both trial periods would weigh approximately 124% more that of oysters from the seed beds. In both cases the use of oysters for shucking stock would result in increased yields. The higher salinity on the planted grounds and the added meat weight, will provide additional gains during the washing and blowing of the meats during processing.

CONCLUSIONS

When combined with the Canzonier (1998) study the data cover two of a myriad of possible cases. In 1996 to 1997 there were slightly elevated summer chlorophyll levels, moderate growth and moderate *P. marinus*, whereas in 1999 there were low or typical Delaware Bay summer chlorophyll levels, no growth and high *P. marinus*. The month of transplant did not have a significant effect on the numbers of market oysters available at the end of the year. When *P. marinus* levels were elevated and food supply was low, transplanted small oysters were lost at a higher rate than market or submarket oysters. The data from both studies suggest that food levels on the planted grounds in the warmer part of the year are generally sufficient to support increases in meat yield 1 to 2 mo after transplant, but may not be sufficiently high to support shell growth in all years. Under high to moderate *P. marinus* conditions, exclusive of market timing, meat weight or shucked meat volume gain were the most important factors for economic comparison of market oysters between the seed beds and the planted grounds.

TABLE 9.

Estimated dry meat yield (g) of market oysters (>76 mm) bu.$^{-1}$ of dredged material at time of transplant (Shell Rock) and in November 1997 and 1999.

	Shell Rock			Transplants		
Transplant Month	Oyster/bu.	Dry Meat Wt	Dry Meat/bu.	Oyster/bu.	Dry Meat Wt	Dry Meat/bu.
1999						
March 99	63	1.1	69	32	2.9	92
May 99	34	1.5	51	34	3.1	105
September 99	29	1.6	44	27	2.5	68
October 99	25	1.1	28	25	2.7	68
Average	38	1.3	49	30	2.8	84
1996/1997						
December 96	110	1.1	121	108	2.8	302
February 97	92	1.2	110	110	2.5	275
May 97	95	1.5	143	93	2.7	251
August 97	133	1.3	174	106	3.0	318
Average	108	1.2	130	104	2.8	291

Oyster numbers for Shell Rock have been adjusted by using data from the first month of post transplant sampling to accommodate for differences culled deck load samples and dredge samples. Oysters transplanted from Shell Rock by month of transplant.

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U.S. CONSUMERS: EXAMINING THE DECISION TO CONSUME OYSTERS AND THE DECISION OF HOW FREQUENTLY TO CONSUME OYSTERS

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ABSTRACT Oyster consumption has been decreasing in the United States. Investigating consumer attitudes and preferences can help identify factors involved in this decrease. This study used data obtained through a nationwide survey in a double-hurdle regression model to determine factors that influence both the decision to consume oysters and frequency of consumption. Results indicate there is a significant difference in the reasons people choose to eat oysters or not and the reasons oyster consumers choose how frequently to eat oysters. Concern for product safety significantly influenced the decision of how frequently to consume but not whether to consume oysters. Consumers also indicated a potential willingness to pay for measures that would increase product safety.

KEY WORDS: consumer preference, double-hurdle model, food safety, marketing, oyster industry

INTRODUCTION

Overall per capita fresh shellfish consumption in the United States has increased from 2.5 pounds in 1980 to a high of 4.7 pounds in 2000 (Fig. 1). Per capita consumption of oysters, however, has decreased from an average of 0.35 pounds per year (average of 1980–1989) to 0.25 pounds in 1990 to 0.20 pounds in 1999 and 2001 (USDOC 2001; Fig. 2).

Food safety is a factor often blamed for decreases in consumption of oysters. In a 1993 news release, a multi-state outbreak of viral gastroenteritis related to consumption of oysters occurred in Louisiana, Maryland, Mississippi, and North Carolina (Centers for Disease Control and Prevention 1993). In 1998, bacteria-tainted oysters from Texas were identified as the cause of sickness for 368 people, and in the preceding summer, 209 laboratory-confirmed cases of illnesses were linked to consumption of raw oysters harvested in the Pacific Northwest (ABC News 1998). The Center for Science in the Public Interest has asked FDA "to take immediate action to protect consumers from raw oysters contaminated with deadly bacteria" (Center for Science in the Public Interest 2000). They cite 36 deaths in the previous 2 years and 119 deaths since 1989 associated with *Vibrio vulnificus*—contaminated raw oysters and other shellfish. In 1990, Billups (2001) showed only 9% of respondents considered oysters "not at all safe" compared with 31% rate in a similar survey conducted 5 years later.

Although food safety is suspected to be a major factor in the decision to consume oysters, other factors may be involved. Regional and national oyster consumption can be affected by many determinants that may vary across geographical region, ethnicity, income levels, and perceptions of nutrition (Wessells et al. 1994, Gempesaw et al. 1995, Wessells & Anderson 1995, Manalo & Gempesaw 1997, Wessells & Holland 1998, Holland & Wessells 1998). The goal of this study was to investigate the decision to consume oysters and the decision of frequency of oyster consumption.

METHODS

The data for this study was obtained through a mail survey. After conducting a number of focus groups of seafood consumers and nonconsumers (in three locations in the United States), and conducting survey pretests, a questionnaire designed to elicit information on seafood consumption, specifically consumption of oysters, shrimp, tuna, and catfish, was mailed to a sample of 9000 households in the United States, with 1000 mailed to each of the nine major census regions (shown in Fig. 3; Hanson et al. 2002). The stratified sample was chosen as the region is expected to be a significant determinant of both the choice to consume and the choice of how often to consume oysters. The surveys were mailed in late 2000 and early 2001, with households receiving a second copy of the survey if they did not return the first. This approach resulted in a return of 1790 surveys or a response rate of 20.1% (after accounting for "return-to-sender" surveys). Because of the length and complexity of the survey, a large number of respondents did not answer all of the questions in the survey, therefore, a total of 874 observations are included in this study.

Table 1 shows descriptive statistics for the responses used in this study. Compared with U.S. Census data (United States Census Bureau 2000), the results showed a larger percent of Caucasians responded to the survey (89% in the survey compared with 75% in the 2000 US Census). The survey results also contained a sample slightly older than the US population, with 69% of survey respondents over the age of 45, compared with 53% of the US adult (over 25) population. The mean response for income in the survey was in the $50,000–$59,999 category, compared with a US mean of $42,148. Religious composition of the survey respondents corresponds to that presented in the World Almanac and Book of Facts (1999), i.e., 85% of the US population practices Christianity, including 23% Catholic, and approximately 2% and 1% of the US population practices Judaism and Islam, respectively. Our survey results indicated 83% Christianity with 25% Catholic, and 3% practicing Judaism.

In a series of six questions, respondents were asked to indicate how often they consumed oysters for breakfast, lunch, and dinner, both at home and away from home. This differs from most previous studies (including Cheng & Capps 1988, Yen & Huang 1996)

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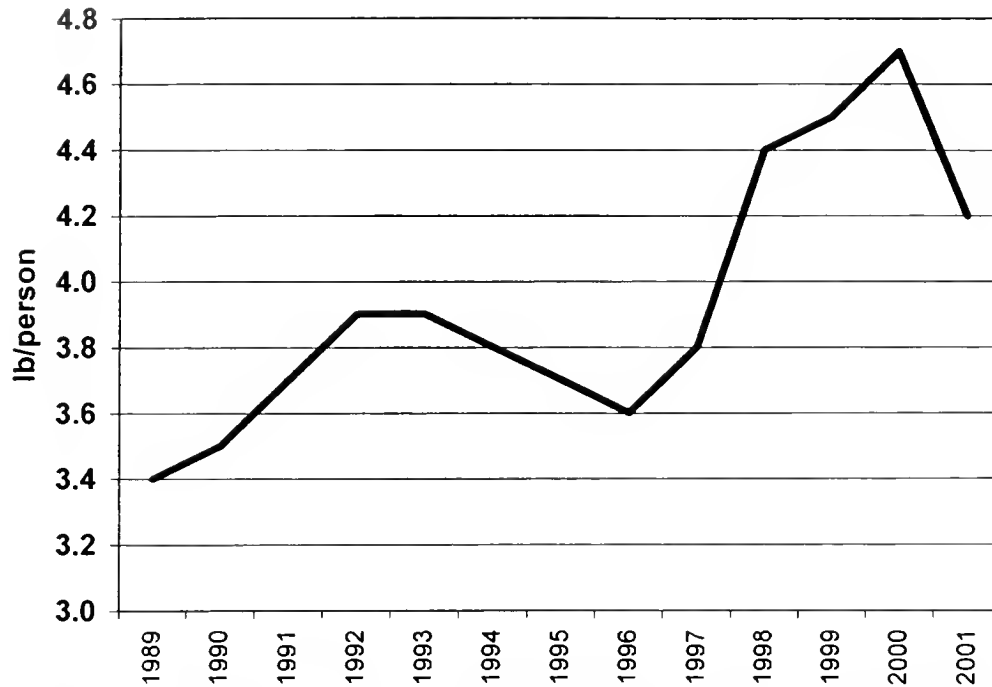
Figure 1. United States per capita fresh and frozen shellfish consumption (Source: USDA, ERS, 1999).

that analyze at-home consumption only. Overall, 56.9% of the respondents indicated that they never ate oysters. The means and ranges of the responses are shown in Table 2. As expected, consumption of oysters, as well as other seafood products, differed by region of the respondent's residence (Fig. 3).

Additionally, respondents were asked to identify and rank the top three reasons they consumed and did not consume oysters. Results from the question on reasons nonconsumers do not consume oysters and why consumers do not consume more oysters provide an interesting insight into the data (Fig. 4). Visual inspection of the results from this question may provide support for a double-hurdle regression model because it appears nonconsumers have different reasons for not consuming compared with consumers decision on frequency of consumption.

A number of factors were hypothesized to be relevant to the consumption and frequency of consumption decisions. The same set of variables was used as regressors in both equations as theory provides no guidance for differences and to allow for a specification test. The dependent variable was constructed from responses to a set of six questions regarding frequency of consumption of oysters for breakfast, lunch, and dinner at-home and away-from-home. If a respondent indicated they never consumed oysters for each of the six questions, the value of the dependent variable was set to zero. For the sample, 56.9% of the responses were zero. For the remainder of the sample, the responses were summed to determine the frequency of consumption in one month. For example, if a respondent answered they consumed oysters once per month for dinner at home and once per month for dinner away from home, but never for lunches and breakfasts, their frequency of consumption for the month was two. Those who did eat oysters consumed oysters on an average of 2.2 times per month. Quantity of oyster consumption was not obtained in this survey because respondents were not asked how much was consumed (or by how many in the household) because of time and space limitations of the survey. Additionally, because the survey was asking for all consumption, including away from home and recreational catch, it

was determined from the focus groups and test surveys that respondents were having difficulty answering in terms of quantity (i.e., pounds or ounces—other quantities, such as number of oysters, were not considered because of the fact other species were considered and did not have comparable measures).

Independent variables included demographic variables (age, gender, ethnicity, religion, household income), variables relating to the respondents geographic location and variables relating to stated preference. For geographic location, a dummy variable was included representing the census region the respondent belonged to, as well as one variable that represented how close the respondent currently lives to a coast. It was hypothesized that persons living closer to the coast would have a higher probability of consuming shellfish. Other expected explanatory variables included perceptions of safety and top reasons for eating and not eating oysters as indicated by the respondent. Descriptive statistics for all variables are shown in Tables 1 (demographic) and 3 (other).

Model

Cheng and Capps (1988) and Yen and Huang (1996) recognized the restrictions of using a tobit model in demand analysis for finfish and shellfish. The tobit model assumes the factors that affect level of consumption are the same as those that determine the probability of consumption. Cheng and Capps (1988) used a Heckman two-step procedure and Yen and Huang (1996) used a generalized double hurdle model to analyze household demand for finfish. As a result of information obtained in focus groups and the preliminary visual appearance of the data, we have chosen to use Cragg's (1971) double-hurdle model, similar to the model used by Yen and Huang (1996).

The double-hurdle model has separate participation and consumption equations that are related in the following manner:

$$y_i = y_i^* \qquad \text{if } y_i^* > 0 \text{ and } d_i > 0 \qquad (1)$$

$$= 0 \qquad \text{otherwise} \qquad (2)$$
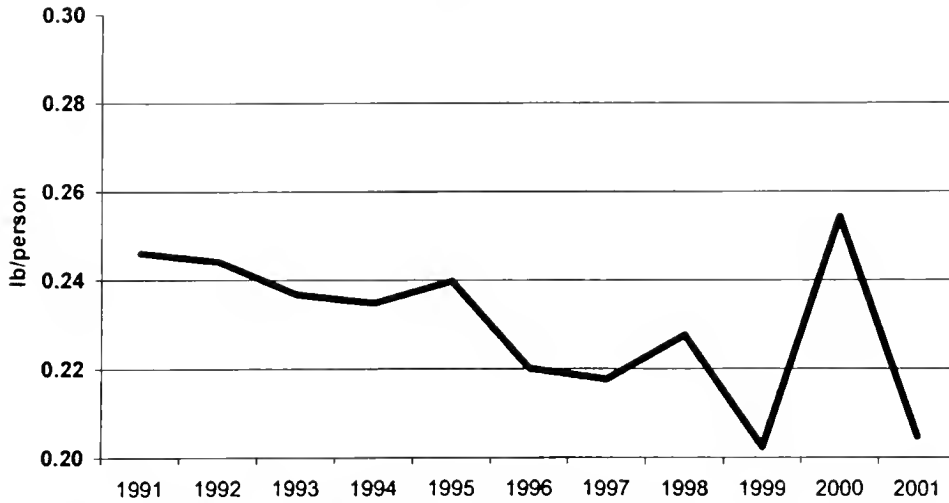
Figure 2. United States per capita consumption of oysters (Source: USDOC/NOAA/NMFS, 'Fisheries of the U.S., 2001,' September 2002).

where y_i^* represents the consumption decision and d_i is a latent variable describing participation as shown below:

$$y_i^* = x_i'\beta + \varepsilon_1 \tag{3}$$

$$d_i^* = z_i'\alpha + \eta_1 \tag{4}$$

where x_i and z_i are vectors of explanatory variables and β and α are vectors of parameters. Estimation of the double-hurdle model is straightforward. Maximum likelihood estimation of a probit equation is used to evaluate the censoring rule ($z_i'\alpha$), whereas maximum likelihood estimates that account for a truncated normal distribution are used for the subsample of uncensored observations. A specification test that evaluates the restrictions imposed by the tobit specification (assumption that the decisions are based on the same parameters) is obtained through a comparison of the log-likelihood function values of the tobit, probit, and truncated normal regression models (Greene 1995). Specifically, assuming that

the same explanatory variables appear in all three equations, the following value will be distributed as a χ^2 random variable with degrees of freedom equal to the number of explanatory variables under the null hypothesis that the Tobit specification is correct:

$$\lambda = -2(f_{\text{Tobit}} - f_{\text{Probit}} - f_{\text{Truncated}}), \tag{5}$$

where the f_is represent the respective log-likelihood function values.

RESULTS

Using the double-hurdle model with frequency of oyster consumption as the dependent variable, the model was estimated with the variables described in Table 4. The coefficients from the probit and truncated tobit equations, as well as the marginal effects (calculated at the means) are reported in Table 5. The probit model correctly predicted a consumer's likelihood to consume or not
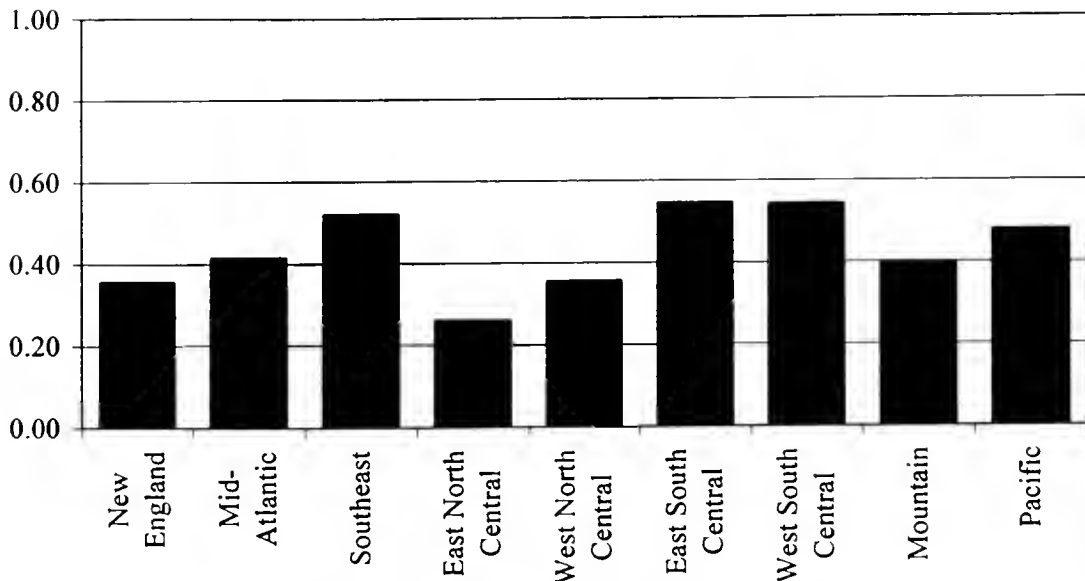
Figure 3. Percent consumption of oysters by region.

TABLE 1.

Summary of demographics.

	Oyster Nonconsumers (%)	Oyster Consumers (%)	Overall Sample (%)
Age of Respondent			
Greater than 65	17.3	19.6	18.3
Between 50 and 65	34.0	39.3	37.0
Between 35 and 50	39.4	33.7	36.3
Under 35	9.3	7.4	8.5
Gender			
Percent female	52.7	67.4	59.0
Household Income			
Less than $29,999	16.3	11.4	14.2
Between $30,000 and $59,999	37.2	34.0	35.8
Between $60,000 and $99,999	29.2	28.1	28.7
100,000 or greater	17.3	26.5	21.3
Region of Residence			
New England	13.1	9.8	11.7
Mid-Atlantic	10.7	8.8	9.8
Southeast Atlantic	9.3	14.6	11.6
East North Central	14.7	8.0	11.8
East South Central	8.2	12.2	10.0
West North Central	13.9	9.3	11.9
West South Central	7.0	13.8	10.0
Mountain	13.5	13.0	13.3
Pacific	9.7	10.6	10.1
Lives within 50 miles of Coast	29.4	30.0	29.6
Religion			
Catholic	26.4	23.6	25.2
Christian	56.1	59.4	57.6
Other	17.5	17.0	17.3
Ethnicity			
Caucasian	90.5	87.3	89.1
Noncaucasian	9.5	12.7	10.9
Education			
High school or less	17.1	14.9	16.1
Some College	32.2	30.0	31.2
College degree(s)	50.7	55.2	52.6

consume oysters 87% of the time (incorrectly predicted consumption 4% of the time and no consumption 9% of the time). The results of the test shown in equation (5) indicate the double-hurdle model is a better specification than the traditional tobit (λ = 264.9, df = 43). The results indicated that different variables affected the decision to consume versus the decision of frequency of consumption, as expected. A set of variables was included to determine if the location of purchase of seafood affected either decision. Results indicated that if a person bought seafood (any seafood, not just oysters) at grocery stores (GRSOURCE) or specialty stores (OTHERCS; such as fish markets or gourmet stores), they were more likely to be oyster consumers. However, these variables did not significantly influence frequency of consumption. The variables indicating if a person consumed seafood purchased from restaurants (RESTSC) or obtained through recreational catch (RECCATCH) were not significant in determining if a person would consume oysters, but significantly decreased the frequency of consumption. A potential explanation for these results is that if a person purchases seafood (again, any seafood) from grocery stores or specialty stores, they are a different type of seafood consumer than someone who purchases from a restaurant or eats recreational catch. Perhaps they are more "dedicated" seafood con-

sumers than those who eat at restaurants, hence more likely to eat oysters, as well as consume different types of seafood than those who eat recreational catch (unlikely to be oysters). Following this line, a person who does eat oysters, but is a restaurant or recreational catch consumer is likely to consume oysters less frequently. Our results indicate the average oyster consumer consumes oysters 2.21 times per month. Respondents who purchased seafood from restaurants were likely to consume oysters 1.16 times per month and those who indicated recreational catch as a source of seafood were likely to consume 1.84 times per month.

Respondents were asked to identify the top three reasons they consumed oysters. These reasons give insight to the type of person that both consumes oysters and what influences a person to consume more or less frequently. If the person indicated they enjoyed the flavor (FLAVOR) of oysters, as expected, they were both more likely to consume oysters (66.5% more likely) and consume oysters more frequently (0.46 more times per month). Tradition (TRAD) plays a part in determining how frequently a consumer eats oysters, but did not influence whether the person was a consumer. In other words, those who indicated they eat oysters out of tradition, or habit, were likely to eat oysters 0.62 times more often per month. Importance of availability was shown in the probit, but

TABLE 2.

Statistics on frequency of oyster consumption ($n = 1067$).

	Mean (Times Consumed/Month)	Mode (% Frequency)	Range
Breakfast at home	0.05	Never (93.0%)	Never to less than weekly
Breakfast away from home	0.01	Never (97.1%)	Never to less than 1/month
Lunch at home	0.14	Never (84.0%)	Never to 1/week
Lunch away from home	0.20	Never (74.8%)	Never to 1/week
Dinner at home	0.21	Never (73.8%)	Never to 1/week
Dinner away from home	0.34	Never (63.0%)	Never to 1/week

Respondents used a scale of 0 to 6 to indicate frequency where 0 = Never; 1 = Infrequently (<1/month); 2 = 1/month, 3 = 1/week ... 6 = Daily.

not truncated tobit equation. Consumers who believed availability was an important reason for consumption were 22.4% more likely to consume oysters. This may be reinforced by the results from the regional variables. Additionally, those who indicated variety in diet (VDIET) was an important factor were 30.3% more likely to consume oysters. Although insignificant, it is interesting to note the sign on the coefficient for VDIET in the results from the truncated tobit equation was negative. Intuitively this is attractive, as someone interested in adding variety might eat oysters, but not that frequently. Factors that were indicated as a reason for consuming oysters, but were not significant, included health reasons (HEALTH), price (PRICE), convenience (CONV), preparation knowledge (KNOWHOW), and aphrodisiac properties (APHROD).

Respondents were also asked to identify the top three reasons they did not consume oysters, or did not consume oysters more frequently. Three of these reasons significantly influenced the de-

cision to consume oysters: price (NOPRICE), allergic reaction (ALLERGY), and taste (TASTE). Consumers who indicated they did not like the taste of oysters or were allergic to oysters were significantly less likely, 16.3% and 38.7%, respectively, to consume oysters. Those who indicated price was a reason for not consuming oysters were 17.9% more likely to be oyster consumers, but were likely to consume 0.39 times less frequently than the average oyster consumer. Oyster consumers who lacked preparation knowledge (LPKLDGE) were likely to consume 0.62 times less frequently per month than average.

Perhaps the most interesting result is that "concerns about product safety" (PRODSAFE) did not influence a person's decision whether to eat oysters. Additionally, a variable that indicated the respondent believed oysters were the least safe of all seafood products (UNSAFE) was not significant in the decision to consume. Concern about product safety did, however, decrease frequency of
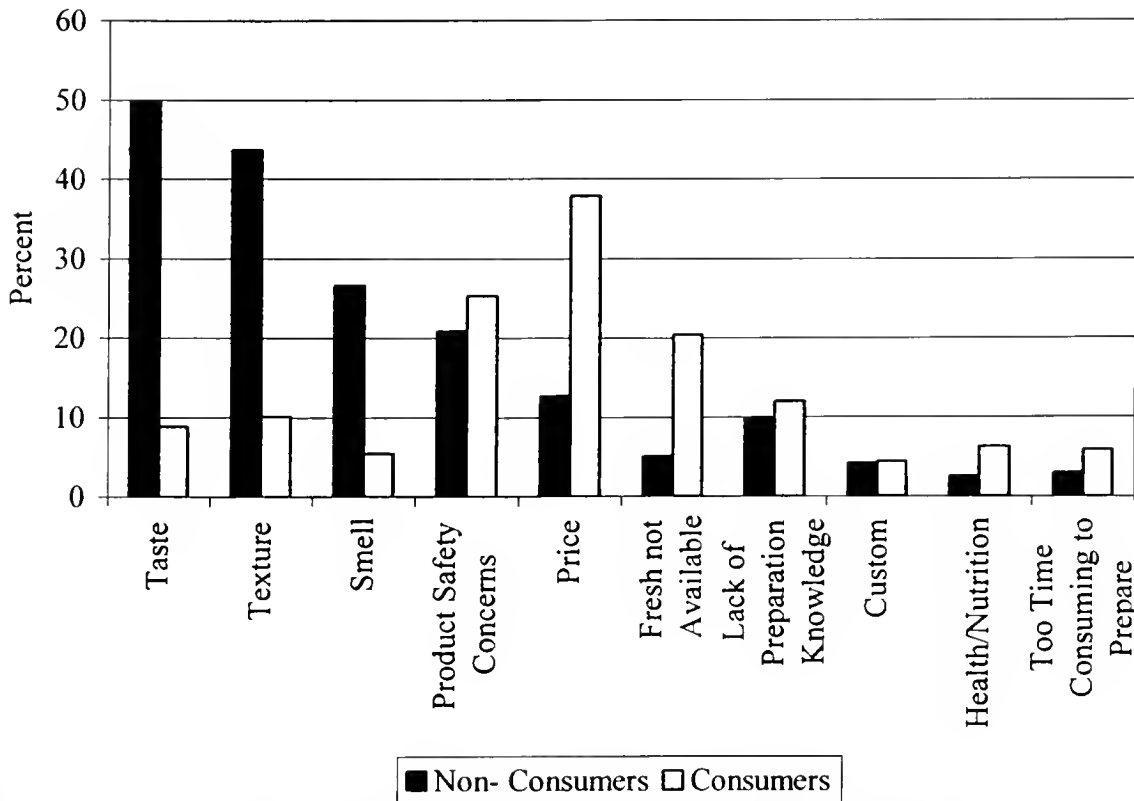
Figure 4. Reasons given for not consuming oysters or not consuming more oysters.

TABLE 3.

Statistics on factors included in the double-hurdle model.

	Mean, Nonconsumers	Mean, Consumers	Overall Mean
Frequency of oyster consumption (dependent variable)	0/month (497 observations)	2.21/month (377 observations)	0.95/month
Indicated oysters were the least safe of all shellfish and finfish products	34.6%	44.5%	39.0%
Indicated the following was a source of seafood for consumption:			
Grocery store	86.1%	89.4%	87.5%
Restaurant	86.3%	90.7%	88.2%
Recreational catch or fish farms	15.7%	27.1%	20.6%
Fish market or gourmet store	17.5%	37.1%	26.0%
Indicated the following was one of the top three reasons for consuming oysters			
Enjoy flavor	4.4%	65.6%	31.8%
Variety in diet	2.2%	31.6%	15.3%
Availability	1.5%	21.9%	10.6%
Tradition/habit	2.2%	16.6%	8.6%
Health/nutrition	1.0%	16.4%	7.9%
Know how to prepare	0.5%	8.2%	3.9%
Convenience	0.5%	7.2%	3.5%
Price	1.0%	5.9%	3.2%
Aphrodisiac properties	0.3%	4.8%	2.3%
Other	0.3%	4.0%	2.0%
Indicated the following was one of the top three reasons for not consuming oysters			
Taste	49.7%	8.8%	31.5%
Texture	43.8%	10.1%	29.1%
Smell	26.7%	5.5%	17.2%
product safety concerns	20.9%	25.3%	22.9%
Price	12.7%	37.9%	23.9%
Fresh not available	5.1%	20.4%	11.9%
Lack of preparation knowledge	9.8%	12.0%	10.8%
Custom	4.2%	4.4%	4.3%
Health/nutrition	2.5%	6.3%	4.2%
Too time consuming to prepare	3.0%	5.9%	4.3%
Other	8.2%	3.2%	5.8%

consumption for oyster consumers, from the average of 2.21 to 1.63, a 0.58 per month decrease.

Demographics did have an effect on both the choice to consume and the frequency decision. Persons living in the Southeast Atlantic (SEATL) and West South Central (WSC) regions of the country were more likely (17.8% and 33.2% respectively) to consume oysters than persons living in New England. Other regions did not significantly differ from the New England region. Persons in the East South Central (ESC), West South Central (WSC), and Pacific (PACIFIC) regions were likely to consume more frequently (0.90, 1.08, and 0.80 times per month, respectively) than those in the New England region. In the United States, 67% of oyster landings come from the Gulf of Mexico and 23% from the Pacific region (USDOC 2002). Given the three regions that consumed oysters significantly more frequently are closest to oyster production, these results make intuitive sense.

All income categories above the base category of $30,000 or less consumed oysters significantly more frequently. However, income was not a factor in the decision to consume. Birthdate (BD) was a factor in both decisions, with younger ages significantly less likely to consume oysters, or if they were oyster consumers, significantly likely to consume less frequently. Education levels, religion, gender, and ethnicity did not significantly influence either

the participation or consumption decisions in this study. However, the sample did not include a representative portion of the nonCaucasian population in the United States. Future studies might benefit from specifically targeting these populations for information on seafood consumption.

DISCUSSION

The two main goals of this study were to determine whether the factors that influenced the decision to consume oysters differed from the factors that influenced the decision of how often to consume oyster and to see what factors were significant that could be used to develop marketing strategies for the oyster industry. Results showed that the two decisions were based on significantly different factors, as suspected. Though food safety is often credited as a reason why people do not consume oysters, this was not, in fact, the case. Concerns about food safety did influence how often oyster consumers ate oysters, but did not significantly influence whether a person was an oyster consumer. In fact, the belief that oysters are the least safe of all fish and seafood products did not influence this decision either. Somewhat surprisingly, nearly 45% of oyster consumers identified oysters as the least safe of all seafood products, while only 35% of nonconsumers identified oysters.

TABLE 4.

Description of independent variables.

Variate	Variable Name	Description
Source of purchase	GRSOURCE	1 if seafood is purchased at a grocery store
	RESTSC	1 if seafood is purchased at a restaurant
	RECCATCH	1 if seafood is from recreational catch
	OTHERSC	1 if seafood is purchased at specialty fish markets or gourmet stores
Reasons for eating oysters		The following variables are 1 if this reason was listed as one of the top three reasons for consuming oysters:
	FLAVOR	Enjoy flavor
	HEALTH	Health/nutrition
	TRAD	Tradition
	PRICE	Price
	AVAIL	Availability
	CONV	Convenience
	VDIET	Variety in diet
	KNOWHOW	Knowledge of how to prepare
	APHROD	Aphrodisiac properties
Reasons for not eating oysters, or not consuming oysters more frequently		The following variables are 1 if this reason was listed as one of the top three reasons for NOT consuming oysters, or not consuming MORE oysters:
	NOPRICE	Price
	NOFPAVAI	Lack of availability of fresh products
	NOCUSTOM	Custom
	LPKLDGE	Lack of preparation knowledge
	TOOTIME	Too time consuming to prepare
	TEXTURE	Dislike texture
	SMELL	Dislike smell
	TASTE	Dislike taste
	TRAUMA	Traumatic experience
	PRODSAFE	Product safety concerns
	ALLERGY	Allergic reaction
Safety perception	UNSAFE	1 if respondent believes oysters are the least safe of all seafood products
Region of residence (U.S. Census regions)		
	NEWENG	New England (omitted category)
	MIDATL	Mid-Atlantic
	SEATL	Southeast Atlantic
	ENC	East North Central
	ESC	East South Central
	WNC	West North Central
	WSC	West South Central
	MOUNTAIN	Mountain
	PACIFIC	Pacific
Religion	CHRISTIA	Christian (omitted category)
	CATHOLIC	Catholic
	OTHERREL	Other religions
Race/Ethnicity	CAUC	1 if Caucasian, 0 otherwise
Income	INC1	<$30,000 (omitted category)
	INC2	$30,000–$59,999
	INC3	$60,000–$99,999
	INC4	$100,000 or above
Education	EDUCAT1	High School degree or less
	EDUCAT2	Some College
	EDUCAT3	At least one degree from College
Proximity to Coast	PROXCST	1 if currently lives within 50 miles of a coast
Age	BD	Birth date
Gender	GENDER	1 if female

However, 25% of oyster consumers indicated they ate oysters less frequently due to product safety concerns.

Results indicated that people did not consume oysters, and did not consume oysters as frequently, if they indicated price was an inhibiting factor. Future studies are needed to address the issue of willingness to pay for safer oyster products. Consumers who indicated price was a reason they did not consume oysters more frequently were likely to consume oysters 0.39 times per month

TABLE 5.

Empirical results from double-hurdle model.

Variable Name	Probit Coefficient	F(z)/X	Truncated Coefficient	E(Y*)/X
Source of seafood for consumption				
GRSOURCE	0.391***[a] (0.197)[b]	0.155	1.949 (2.054)	0.263
RESTSC	0.005 (0.196)	0.002	−7.783* (2.142)	−1.050
RECCATCH	0.249 (0.164)	0.099	−2.711*** (1.509)	−0.366
OTHERSC	0.699* (0.155)	0.277	2.039 (1.362)	0.275
Top three reasons for consuming oysters				
FLAVOR	1.682* (0.181)	0.665	3.434*** (2.016)	0.463
HEALTH	0.155 (0.324)	0.061	−2.771 (2.968)	−0.374
TRAD	−0.223 (0.241)	−0.088	4.579* (1.746)	0.618
PRICE	−0.201 (0.340)	−0.080	3.124 (2.134)	0.422
AVAIL	0.566** (0.261)	0.224	−0.821 (1.445)	−0.111
CONV	0.411 (0.467)	0.163	1.210 (2.034)	0.163
VDIET	0.766* (0.223)	0.303	−1.576 (1.361)	−0.213
KNOWHOW	0.164 (0.417)	0.065	1.819 (2.147)	0.245
APHROD	0.569 (0.517)	0.225	−2.500 (3.286)	−0.337
Top three reasons for not consuming oysters, or not consuming more oysters				
NOPRICE	0.454* (0.155)	0.179	−2.852** (1.473)	−0.385
NOFPAVAI	0.172 (0.209)	0.068	0.402 (1.749)	0.054
NOCUSTOM	−0.217 (0.296)	−0.086	−3.530 (3.464)	−0.476
LPKLDGE	0.065 (0.184)	0.026	−4.618** (2.170)	−0.623
TOOTIME	−0.314 (0.307)	−0.124	0.008 (2.556)	0.001
TEXTURE	−0.030 (0.175)	−0.012	3.312 (2.523)	0.447
SMELL	−0.215 (0.192)	−0.085	−3.531 (3.511)	−0.477
TASTE	−0.412** (0.169)	−0.163	−5.850** (3.054)	−0.790
TRAUMA	−0.727 (0.519)	−0.288	14.509 (9.523)	1.958
PRODSAFE	−0.145 (0.152)	−0.057	−4.311* (1.708)	−0.582
ALLERGY	−0.977** (0.589)	−0.387	−4.596 (7.728)	−0.620
Believed oysters to be least safe of all seafood products				
UNSAFE	−0.048 (0.136)	−0.190	1.889 (1.354)	0.255
Demographics				
MIDATL	0.152 (0.279)	0.060	3.535 (3.207)	0.477
SEATL	0.450** (0.270)	0.178	2.263 (2.918)	0.305
ENC	−0.118 (0.290)	−0.047	4.071 (3.470)	0.549
ESC	0.480 (0.299)	0.190	6.632** (3.211)	0.895
WNC	0.040 (0.297)	0.016	3.991 (3.438)	0.539
WSC	0.840* (0.308)	0.332	8.017* (3.151)	1.082
MOUNTAIN	0.246 (0.290)	0.097	3.851 (3.367)	0.520
PACIFIC	0.139 (0.274)	0.055	5.927** (3.044)	0.800
CATHOLIC	0.039 (0.150)	0.015	−1.259 (1.538)	−0.170
OTHERREL	−0.008 (0.168)	−0.003	1.183 (1.688)	0.160
CAUC	−0.266 (0.190)	−0.105	−0.944 (1.796)	−0.127
INC2	0.151 (0.193)	0.060	7.973* (2.601)	1.076
INC3	0.099 (0.210)	0.039	6.859* (2.634)	0.926
INC4	0.224 (0.229)	0.089	6.105** (2.701)	0.824
EDUCAT2	0.081 (0.190)	0.032	2.545 (2.070)	0.343
EDUCAT3	−0.077 (0.191)	−0.031	−0.831 (2.014)	−0.112
PROXCST	−0.220 (0.185)	−0.087	2.112 (1.705)	0.285
BD	−0.008* (0.0002)	−0.003	−0.007* (0.003)	−0.001
GENDER	0.106 (0.130)	0.042	1.461 (1.453)	0.197
Log-likelihood function	−281.04		−635.67	
Percent of correct predictions in probit model		87.1%		

[a] One, two, and three asterisks indicate significance at the 0.01, 0.05, and 0.10 levels, respectively.

[b] Standard errors of the coefficients are reported in parentheses.

less frequently than the average oyster consumer. However, consumers who indicated concern for product safety was a reason for not consuming were likely to consume oysters 0.58 times per month less frequently. The tradeoff between an increased price due to increases in costs of implementing safety programs and in-

creases in consumption if consumers believe oysters to be safer is an area for future investigation.

Overall, this study does identify characteristics that the oyster industry can use to segment consumers for marketing purposes. As expected, people living in regions nearest to oyster production are

more likely to consume oysters and more likely to consume more oysters. Availability of fresh products also significantly increased the likelihood of the respondent to consume oysters. Consumers who purchase seafood products at grocery stores or specialty stores may be a segment that could be targeted, as they are more likely to consume oysters.

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REHABILITATION OF THE NORTHERN QUAHOG (HARD CLAM) (*MERCENARIA MERCENARIA*) HABITATS BY SHELLING—11 YEARS IN BARNEGAT BAY, NEW JERSEY

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ABSTRACT The use of shell or other coarse material to enhance survival of newly set hard clams (*Mercenaria mercenaria*) has been suggested as a management strategy to increase clam stocks. Barnegat Bay, New Jersey and surrounding areas supported a large clam fishery throughout the 1950s and 1960s, but this resource has declined in recent years. We established replicate 20×70 m plots of high shell density, low shell density, and no shell (control) in a Latin Square design in 1990 and have obtained periodic samples since that time. The shell, obtained from ocean quahog processing plants, had been broken into a variety of sizes. High-density shell received 900 bu per plot, and low-density shell received 300 bu per plot. Plots with high shell density had significantly more clams after 10 years than those with low-density shell or controls. High shell density significantly increased hard clam recruitment, but this exceeded 1 m^{-2} in only one year, from the years 1990 to 2000. In plots with low shell or in controls, recruitment never exceeded 0.4 m^{-2}, and in half or more of the years no recruitment was found. Some individual plots with shell did not enhance recruitment, indicating that factors not investigated must be important as well. In spite of the low recruitment density, there appears to be an increase in survivorship when the shell content is greater than 8000 gm^{-2}.

KEY WORDS: Mercenaria mercenaria, shelling, hard clam recruitment, quahog

INTRODUCTION

Methods of increasing natural abundance of hard clams (*Mercenaria mercenaria*) are important to state resource managers and the shellfish industry. There are several approaches a manager can use to improve shellfish stock abundance: (1) increasing the numbers of spawners (spawner sanctuary); (2) reducing harvests or providing alternate areas in some cycle so the stocks last longer; (3) adding hatchery produced clam seed to a selected area; and (4) protecting naturally set clams (shelling or other substrate modification and use of chemicals to eliminate predators).

The theoretical concept underlying a "spawner sanctuary" is that increasing the number or density of clams in an area will increase the number of eggs, larvae, and, set clams. The potential for an increased number or greater concentration of clams to produce more larvae when conditions are favorable is suspect because it depends on the existence of a spawner-recruit relationship (more spawners = more recruits) over a wide range of clam densities. In addition, there are large numbers of clams in most bays even at low densities, and thus the numbers of clams that must be transplanted to have even a small probability of significantly increasing the number of active spawners in the region is extremely large. Finally, of those sanctuaries that have been created in New Jersey and New York, preliminary evidence indicates that little detectable enhancement of natural hard clam stocks may be expected (Kassner & Malouf 1982, Barber et al. 1988).

Reducing harvests allows clams to be harvested over a longer period of time while waiting for the next surviving set. While this appears to be attractive, hard clams are different than most species harvested from the wild. Smaller sizes of hard clams (littleneck) command a premium price. Economic considerations suggest that most of the clams should be harvested in the smaller sizes and that larger clams should only be taken as a last resort. Growth rates in most areas are such that clams remain in these premium size classes only a few years. This suggests that the best economic returns would be from intense harvest on these sizes. The only way to manage the fishery for maximizing economic benefit would be through an extensive monitoring program to delineate areas with maximum concentrations of appropriate sizes (McHugh 1991).

The third option, the use of hatchery seed to enhance hard clam production is well established in aquaculture (Manzi & Castagna 1989). In general, predation rates on high-density plantings of seed without protection devices are too high to recommend this option (Kraeuter & Castagna 1989). Preliminary experiments using low density seeding of hard clams suggest this may yield higher survival rates than would be expected from dense plantings [Macfarlane (Orleans, MA), and Relyea (F. M. Flowers and Sons, pers. comm.)]. These observations are supported by the work of Paulsen and Murray (1987). They conducted a number of short-term (less than one year) experiments using three seed sizes, at high and low density, planted both on and below the sediment surface. They reported that survival (58 days) of clams planted below the sediment surface at high densities was no greater than if seed were broadcast. Low-density plantings of hard clams below the surface significantly increased long-term survivorship when compared with similar high-density plantings. Peterson et al. (1995) have provided additional evidence indicating that low-density plantings of large (>20 mm) seed may be an economically viable means of increasing hard clam stocks in isolated basins.

The fourth option, modifying the substrate to increase post settlement survival of juvenile hard clams, has been shown to work. MacKenzie (1977, 1979) demonstrated that treating areas of bay bottom with various pesticides significantly increased juvenile hard clam survivorship by eliminating arthropod predators such as, shrimp and crabs. Similar techniques provided additional protection to seed clams planted in mesh and gravel protected aquaculture plots (Kraeuter & Castagna 1985). The use of this technique

is considered to be unacceptable because it requires introducing toxic chemicals into the environment, and these may produce long-term detrimental effects. Parenthetically, it is plausible that the massive use of pesticides during the 1950s and 1960s, to control insects in the coastal marshes of New Jersey, was the proximal cause of the high abundance of hard clams in some of these shallow, poorly flushed systems.

An alternative of the fourth option, that has also been shown to increase survival of juvenile hard clams in different habitats, is "shelling" the bottom (Parker 1975, Kraeuter & Castagna 1977, Kraeuter & Castagna 1989, Kassner et al. 1991). This practice involves broadcasting pieces of broken shell or stone aggregate over the bottom to increase the percent composition of larger particles (stone or shell) in the sediments. This technique was developed from the many studies revealing that hard clams are more abundant in areas with a higher percentage of shell in the bottom (Pratt 1953, Wells 1957, Saila et al. 1967, Walker & Tenore 1984, Craig & Bright 1986, Papa 1994). The larger particles have two mechanisms by which they can affect hard clam abundance. Wells (1957) suggested that shell might create areas of low current speed in which small clams either collect (*sensu* Carriker 1961) or at least are not swept away. He also proposed that the hard substrates provide a byssal attachment point for newly set clams. A large body of evidence indicates that coarser material can interfere with the ability of many hard clam predators to detect or manipulate small clams (Arnold 1984, Kraeuter 2001). Any or all of these mechanisms can have a positive effect on natural set resulting in greater numbers of clams surviving to market size.

The shelling option can be used, but it cannot be used with confidence. Kassner et al. (1991) found no significant enhancement of a clam area with low abundance in Great South Bay, New York, one year after placing 12.5L shell m^{-2} on a mud bottom. Several important variables associated with construction of shelled plots are unknown. For example, the amount of shell added must fall within a bounded region; too little shell may not effectively deter predators while too much shell may serve as a haven for the same predators. There is uncertainty regarding the amount of shell needed to afford protection. For example, most studies and surveys of natural populations indicate a positive effect of larger particles, but Day (1987) has observed in the laboratory that mud crab predation was greater in gravel and gravel and sand mixtures than in sand alone. She suggests that the gravel substrates offer hiding places for these small predators and thus increase the predation rate. Further, little information exists on: density of shelling, shell size, plot size, substrate type (grain size, percentage of organic matter, redox discontinuity level, water content, etc.) and their interactions (density of shell × shell size, density of shell × plot size, density of shell × substrate type, etc.) relative to clam survival. This information is essential to allow some predictive capability concerning whether the increased numbers of clams available for harvest will justify the cost of the original shelling. In addition to the effects of shelling on the clams, information concerning the shell size, shelling density, substrate type, and their interactions is required to evaluate the increased effort that might be required to harvest the potentially increased numbers of clams (shell fragments could interfere with the harvest).

METHODS

This study was designed to determine whether shelling the bottom, at a spatial scale large enough to be meaningful to habitat

management, produces significant increases in hard clam abundance. A subset of the design examined two densities of shell cover: low density and high density. The major uncontrolled variables were the sporadic nature of hard clam spat set and predator populations.

The experimental design was a Latin Square matrix of 20 × 70 m plots (slightly more than 0.15 ha). A rectangular shape was chosen because a boat was used to place the shell into the plots. Plots were arrayed in a 3 × 3 Latin Square design with 30 m buffer zones between each of the separate plots. The entire matrix was surveyed using sextants; corners were marked with stakes and buoys. Three treatments were arrayed within the plots: (1) 10 high-density shelling—900 bushels per plot (15 L/m^2); (2) 20 low-density shelling—300 bushels per plot (5 L/m^2); and (3) control—no shell added. Most of the shell consisted of broken pieces (2–8 cm^2) of *Arctica islandica*, although some *Spisula solidissima* shell and the shell debris of other offshore species could be seen. This shell is available in large quantity from several local clam-processing plants.

Shell Spreading

The experiment was located approximately 200 m east by northeast from Gulf Point in Barnegat Bay, New Jersey. The co-ordinates of the matrix are: NE corner—39°44.23′N by 74°9.05′W, NW corner—39°44.23′N by 74°9.12′W, SE corner —39°44.04′N by 74°9.05′W, and SW corner—39°44′N by 74°9.12′W. The site, characterized by sandy sediments with relatively low silt-clay content and few naturally occurring shells, experienced only moderate tidal currents. It had a fairly uniform bottom composition and water depth (4 m), was protected from the longest fetches that occur in the bay, and had long been a hard clam habitat. The latter was determined through discussions with several local watermen who aided in the site selection process and designated this area as the best location for our project and least disruptive to their activities.

Shell was spread onto the experimental plots during the week of April 23 to April 27, 1990 using the Ocean County Bridge Department's LCM, the *Benjamin H. Mabie*. The shelling required 2 days to complete.

The shell was stored in the middle of the ship and transferred to a hopper with a small catloader. The volume of the scoop of the catloader was calibrated previously so that the shell volume going overboard could be estimated. The shell moved from the hopper via a conveyor belt to a highway salt spreader located in the bow approximately 4 m above the water. This procedure produced an evenly dispersed spread of shell on the bay bottom. SCUBA observation subsequent to spreading confirmed the even nature of the shell on the bottom.

During the second day, it became apparent that the volume of shells delivered was short. To accommodate this, we reduced the size of the last high-density plot to 20 × 50 m to maintain the same density of shell. To ensure that all plots received as nearly identical disturbance as possible, the LCM was powered over each control plot as if it was being shelled.

Sampling

Samples were retrieved from each plot using a diver-operated suction sampler. Each plot was located with sextant coordinates (or later GPS); the center was marked, and a diver was deployed

approximately 9 m from the center mark. During the first year of sampling (May 1991), a ring made from a bottomless galvanized bucket was used to mark the area to be sampled. Samples were collected approximately 1 m apart by removing all material from the ring to a depth of 10 cm with a suction sampler. All materials were collected in a 3 mm mesh bag, brought to the surface, and preserved. During the first year of sampling, 9 samples were collected from each plot each sample covering 0.043 m². Samples were returned to the laboratory and numbers of clams removed and the volume of the material was recorded. All hard clams were measured in length, height and width.

Subsequent sampling followed the same protocol except that the ring was modified and size of the area sampled was increased to 0.25 m². The number of samples was reduced to five or six during 1996 and increased to 10 during partial sampling in 1998 (3 plots) and in 2001. The procedure in the laboratory remained the same except that that the weight of the dried shell material was measured rather than its volume. A factor of approximately 850 g dry weight is equivalent to 1L of this material. We chose <15 mm as the size limit for seed clams (0 y class). For the 1996, 1998, and 2001 samples, we sectioned one of the valves of each clam that was older than seed to determine approximate age. We counted the annual growth rings in the valves to determine if the clams were those that might have set since the 1990 shelling. We could not accurately age animals older than10 years; therefore, we considered these individuals to have been the residual population, even though by 2001 they may have recruited after the experiment started.

RESULTS

Samples were retrieved from plots on May 21 to May 28, 1991; September 30 to October 2, 1992; November 24, 1993; June 23 to June 25, 1996; August 10 to August 12, 1998; and November 14 to November 15, 2001. During the first year of sampling, only one hard clam was found in the 72 samples that were sorted in the laboratory. Because of insufficient numbers of hard clams in the samples, these data were not analyzed further.

During the second year, we increased the sample size to 0.25 m² and reduced the numbers of replicate samples per plot (Table 1). In general, setting was sparse. Data from the second year indicated that on one heavy shelling treatment there was enhanced setting. Shell weight data indicated that the other heavily shelled plots were not sampled, and control plots were over represented. None of the control plots had seed clams, and there were seed clams on two of the three low-density treatment plots. Because of the sampling difficulties, no Latin Square analysis was attempted on the 1992 data. A linear regression of the effect of shell mass on total clams and seed clams collected showed that shell density had a significant positive effect on the presence of both total clams and seed clams (Table 2).

Considerable effort was directed toward surveying the plots for the third year, and weights of shell indicate we were successful in sampling the stations in all but one case. Even with this effort, the low-density plot (2–3), based on shell weight data (Table 1) appears to have had high-density shell. We conducted an ANOVA with that plot characterized both "as-sampled" and "corrected". In

TABLE 1.

Numbers of replicate samples removed from Barnegat Bay, NY shell plots by year.

Year	Sample Grid / Shell Density	1-1 H	2-2 H	3-3 H	1-2 L	2-3 L	3-1 L	1-3 C	2-1 C	3-2 C
1991	# replicates	9	9	9	9	9	9	9	9	9
	Mean Shell DW									
	Total Clams	1	0	0	0	0	0	0	0	
	Recruits	0	0	0	0	0	0	0	0	
1992	# replicates	6	6	6	5	5	8	5	5	4
	Mean Shell DW	4994	1676	59.9	1059	0.2	1315	409	2.5	14.6
	Total Clams	13	1	0	3	0	3	1	0	1
	Recruits	11	1	0	2	0	0	0	0	0
1993	# replicates	5	5	5	5	5	5	5	5	5
	Mean Shell DW	5892	5305	3904	1463	4256	1538	89	27	23
	Total Clams	10	6	2	9	3	5	0	1	1
	Recruits	8	6	2	7	2	3	0	0	0
1996	# replicates	5	5	5	5	5	5	5	5	5
	Mean Shell DW	6279	3966	4264	312	2986	1893	10	56	154
	Total Clams	5	4	7	2	11	2	1	0	3
	Recruits	5	4	6	1	5	2	0	0	2
1998	# replicates	13			10			10		
	Mean Shell DW	4031			288			1004		
	Total Clams	11			5			4		
	Recruits	10			1			4		
2001	# replicates	10	10	10	10	10	10	10	10	10
	Mean Shell DW	3285	2358	5095	639	1104	1039	24	22	280
	Total Clams	15	12	20	5	1	4	2	1	4
	Recruits	12	11	18	3	0	3	2	0	1

H = High density shell, L = low density shell, and C = control. In 1991 replicates were 0.043 m² all subsequent samples were 0.25 m². Shell dry weight is in grams. Clams and recruits are the totals for all samples.

TABLE 2.

Intercept, regression coefficient and correlation coefficient for the effects of shell density (g) on total clams and those that have recruited since 1990 (clams 0.25 m^{-2}).

Year		Intercept	Regression	r^2
1992	Total Clams	0.005 NS	4.028 E-04***	0.47
	Recruited Clams	−0.082 NS	3.338 E-04***	0.48
1993	Total Clams	0.297 NS	2.152 E-04**	0.18
	Recruited Clams	0.110 NS	2.080 E-04***	0.26
1996	Total Clams	0.363 NS	1.827 E-04**	0.19
	Recruited Clams	0.118 NS	1.876 E-04***	0.31
1998	Total Clams	0.314 NS	1.476 E-04*	0.14
	Recruited Clams	0.073 NS	1.624 E-04**	0.26
2001	Total Clams	0.129 NS	3.713 E-04***	0.45
	Recruited Clams	−0.003 NS	3.703 E-04***	0.53

NS = not significant. *0.05. **0.01. ***0.001.

both cases the results with respect to treatments were similar, and we have presented the as-sampled data (Table 3). High and low shell density plots had similar numbers of total clams and seed; both had significantly more total clams and seed than the controls. We arrayed the data according to shell density and used linear regression. Both total numbers of clams and seed clams (Table 2) were significantly correlated with shell density.

Latin Square analysis of the 1996 data on shell weight indicated that column 2 had significantly less shell than the other two. These differences negated further use of the Latin Square. We evaluated the total clams and recruited clams with ANOVA based on the three treatments (high density shell, low density shell, and control); a linear regression for all samples (total clams and recruited clams vs. shell weight) was then computed. There were no significant differences in total clams with treatment (Table 3); however, the regression line showed a significant positive effect of shell density (Table 2). In contrast, the ANOVA analyzing the effect of shell on clams that had recruited since 1990 was significant. A Tukey (HSD) test found that clam density in high-density shell and low density shell were not significantly different, and low

TABLE 3.

Tukey (HSD) results (number 0.25 m^{-2} for total number (Total) of hard clams (*Mercenaria mercenaria*) and those that had recruited to the population (Recruit) since the beginning of the experiment (1990).

1993	High	Low	Control	1996	High	Low	Control
Total	1.29	1.13	0.07	Total	1.07	1.00	0.27
Recruit	1.14	0.73	0.00	Recruit	1.00	0.53	0.67
1998	High	Low	Control	2001	High	Low	Control
Total	0.85	0.50	0.40	Total	1.53	0.33	0.23
Recruit	0.69	0.30	0.10	Recruit	1.40	0.20	0.10

High = those areas covered with high density of shell, low = those areas covered with low density shell, control = those areas that did not receive shell. Underlines indicate those treatments that were not significantly different a (α = 0.05).

density shell and control areas had similar clam density (Table 3). High density shelling increased clam recruitment over that observed in the control areas.

In 1998, only 3 plots were sampled, and ANOVA results were similar to 1996. There was no difference in total clams between treatments, but the clams that had recruited since 1990 were more abundant in high shell plots. There were no significant differences between low shell and control (Table 3). Again, linear regression indicated a positive effect of shell density on total and recruited clams (Table 2).

In 2001, as with previous sampling, Latin Square analysis of the shell distribution revealed significant differences between all columns and some rows. The total numbers of clams and clam recruitment were evaluated relative to shell weight and treatment type with general ANOVA and linear regression techniques. After 11+ years, most plots remained intact, but the increasing differences between rows and columns suggest that the shell is gradually being dispersed. In contrast to 1996, when total clams were not significantly different by treatment, both the total and recruiting clams since 1990 exhibited significant differences by treatment. In both the total clams and recruited clams, the Tukey (HSD) test found that high shell density plots had significantly more clams than either the low-density shell or the control. The latter two treatments were not significantly different from each other. The similarity between total and recruiting clams after 11+ years may have been greater than indicated by the base data. We were unable to distinguish ages of clams >10 y. Thus, some of the clams in this class may have recruited to the area since the shell was placed on the bottom. In 2001, 20.7% of the sampled clams were in the age 10 or older category. As a comparison in 1996, 31.4% of the clams were from classes that had recruited before the shell was placed on the bottom).

Reeruitment

We considered clams <15 mm in shell length to be seed clams. Relatively few of these clams were found (Table 4), and never in the control areas. In some years, seed can be as large as 20 mm. We found only one clam of this size in a control plot (Table 4).

We have attempted to evaluate annual recruitment (long-term survival) of clams at this site by back calculating from the age data to determine when particular clams had set (Fig. 1). We have averaged the data from the 1996, 1998, and 2001 samples, but, because so few animals were obtained by sampling, have not attempted to place error bars around these estimates. With the exception of 1993, there is a relatively good correspondence between the back calculated data and that from animals recovered. The

TABLE 4.

Mean number of seed clams m^{-2} by treatment.

	Seed <15.1 mm			Seed <20.1 mm		
Year	High	Low	Control	High	Low	Control
1992	2.00	0.50	0	2.25	0.75	0
1993	2.67	0.53	0	2.67	0.80	0
1996	0	0.53	0	0.27	0.53	0.27
1998	0	0	0	0	0	0
2001	0	0	0	0	0	0

Seed = <15.1 mm or <20.1 mm Shell Length. Number of 0.25 m^2 samples is given in Table 1.
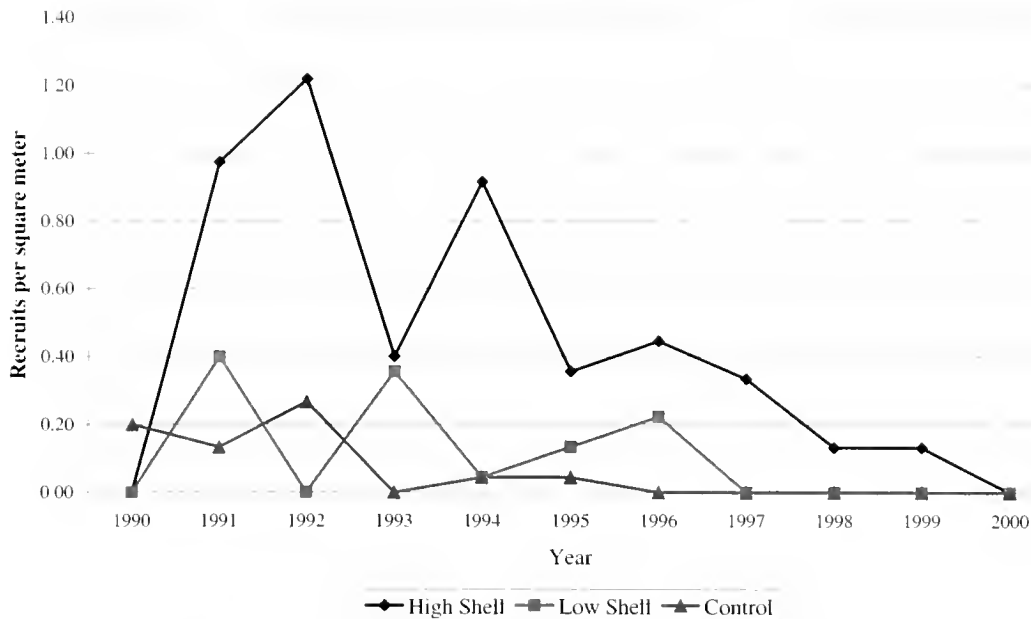
Figure 1. Recruitment of hard clams (*Mercenaria mercenaria*) into high-density shell, low-density shell and control plots in Barnegat Bay, New Jersey. Data represent the average estimated recruitment, based on live animals collected in 1996, 1998, and 2001.

scarcity of animals precluded meaningful statistical analysis of these data. For both estimates, areas with high shell density had more recruiting clams than areas of low shell, and these in turn receive more recruits than control areas. Based on these estimates, clam recruitment to this area has generally been very low for the past decade. Annual average recruitment, based on aged shells, exceeded 1 m^{-2} only on the high shell plots sampled in 1992. These same plots approached 1 m^{-2} again in 1994. Recruitment in the low shell density and control plots was below 0.5 m^{-2} in all years and has been 0 since 1997. Average annual recruitment in the high shell plots shows a general trend toward less recruitment from 1998 to present when it reached 0. Data from clams <20.1 mm also suggest there has been little or no recruitment since 1996.

Figure 2. Growth of hard clams based on average size-at-age of live clams collected in all experimental plots in 1996, 1998, and 2001, and all clams <20.1 mm collected in all plots from 1992, 1993, 1996, 1998, and 2001. All plots were in Barnegat Bay, New Jersey. Data are mean length (mm) and the 95% confidence limits. Bars lacking confidence limits are based on one individual. Animals older than 9 could not be aged, thus all data for ages 10 and 11 come from animals collected in 1996. Animals >10 y are based on the average of all data from all animals aged in all years.

Growth

Size-at-age was computed for clams from the 1996, 1998, and 2001 samples. These data were compiled and averaged to yield an estimate of growth (Fig. 2). Although we have few clams of age 1 and 2, the data indicate that growth is rapid until age 3, and then abruptly slows. Growth is sporadic after age 5. The largest clam found at this site was 82.8 mm shell length. In addition, when the clam meat was being removed to prepare the shell for sectioning it appeared very dark brown, black, or gray in color, except in small clams. This condition has existed in Barnegat Bay and Little Egg Harbor clams for a number of years.

DISCUSSION

We have demonstrated that shelling increased the number of hard clams on the bottom at an experimental site in lower Barnegat Bay. These data are consistent with observations about the effects of shell on the bottom and wild hard clam populations. At this site, the shell has persisted for 11 years and appears to continue to support hard clam recruitment. After 11 years, linear regression of both total and recruited clams showed the positive effect of shell density, but the effect of shell on clam recruitment was not significant until shell exceeded 8 kg m^{-2} (Fig. 3). The larger numbers of recruits between 1992 and 1994, as well as the lack of difference between clam abundance in high and low density shell during this period, suggests that the shell continued to enhance recruitment. Beginning with the 1996 samples, there was no statistical difference in clam abundance between the high and low shell density plots. There was also no significant difference between the low shell density and the control sites, and by 2001 the high-density plots were significantly different from the low-density shell and the control. This appeared to be coupled with a general loss in overall recruitment at the sites. The low density shelling may have started to lose its effectiveness, but we cannot determine whether this reflects a drop in actual recruitment or some loss of effective-

Figure 3. Numbers of surviving clams m^{-2} based on survival in high-density shell, low density shell, and control plots placed in Barnegat Bay New Jersey in 1990. Data represent back calculated (from regression equations) mean and 95% confidence limits of the number of live clams and clams <10 y of age. Numbers of the latter clams are based on shell sections and ages of animals. These represent the animals recruited since 1990.

ness of the shell caused by its protracted residence time on the bottom.

Our study indicates that in areas experiencing low recruitment, several years of data may be required to thoroughly evaluate the effectiveness of shelling on the survivorship of hard clam seed. Similar experiments, perhaps of significantly smaller scale, should be conducted on different types of bottom to ascertain how much shell is required.

Economics is one of the many important factors to consider before any large-scale shelling program commences. It clearly costs more to add more shell to the bottom, but we do not have sufficient data to determine full costs per unit of shell spread. The cost of the shell is a direct multiple of the amount to be spread (3 × more shell will cost 3 × more), but the cost of spreading the higher density shell will be somewhat less per unit on the bottom than will the lower density shelling. Shell costs are not insignificant, and transportation adds to these costs. In New Jersey there are large quantities of shell produced by the surf clam and ocean quahog processing plants and these can be purchased for about $0.50 bu^{-1}. The logistics of handling the shell on a regular basis have precluded it being available free for repletion. Private contractors remove the shell and store it for roads and other purposes. Oyster shell repletion, utilizing large boats (3,000 + bu load) cost about $1,000 day^{-1}, for the boat. Smaller boats (1,000 bu. load) cost about $600 day^{-1}. Extrapolating from these basic data, it would cost between $2,300 and $3,100 acre^{-1} to spread shell at the highest density used in this experiment, but boat availability, transport of shell to the sites, and other logistical costs may make these data unreliable. We know that this particular shelling lasted at least 11 years without substantial loss of shell. Figure 2 also makes it clear that high-density shell increased the clam population from a mean of 0.7 m^{-2}–7.6 m^{-2}, nearly a factor of 10 increase, during the first few years. This population generally persisted throughout the course of the experiment. It is impossible to know whether the sporadic nature of the recruitment was due to changes in recruitment, shell effectiveness, or a combination of the two.

It is also unclear how long a plot can continue to enhance clam set. It is certain that high shell density continued to support more clam, even after 11 years, but there has been a noticeable decline in the number of clam seed (those <15 mm) through time. This is true in both the shelled and unshelled areas. As noted above, whether this is due to loss of effectiveness of the shell or lack of recruiting individuals cannot be determined, but there was a general tendency for low-density shell to be somewhat effective at the beginning. By 2001, low-density shell had clearly reduced capacity to sustain clam recruitment, but high density shelling continued to retain recruited animals. The different rates of loss of effectiveness make it tempting to conclude this is a function of the shell density; however, under conditions of low recruitment, other factors may be operative and the interpretation remains uncertain. It will require placing shell out for a number of consecutive years on different bottom types to allow evaluation of the length of time shell remains effective. This requires differentiation of recruitment processes on freshly planted shell and shell placed out for a number of years.

Disturbance of the shell either by natural physical forces, such as burial by sediments, or human activities, such as clam harvesters working within an area, could alter the effectiveness of the shell. We have no data regarding the effects of increased clam harvesting on the enhancement capability of each shell density. The density of marketable hard clams was low in this area; therefore, we do not believe disruption of the shell or sediment by harvesting was high during the study. Pieces of shell were covered with fouling organisms so at least some of the material remained near the sediment surface for the duration of the study.

A 2002 survey of hard clam populations by the New Jersey Department of Environmental Protection in Little Egg Harbor Bay stopped just south of our experimental area, but it reported a nearly two thirds reduction in hard clam standing stocks since the last survey in the middle 1980s (Joseph pers. Comm.). Commercial clam harvesters working throughout the area also indicated that they believe that clam populations have declined significantly in recent years.

Low levels of recruitment made it difficult to detect statistically significant effects, even with 0.25 m^2 samples. It was only through time and repeated sampling that we were able to evaluate the effectiveness of the shell in this low clam density, low recruitment area. It is also clear that in the 11 years of this experiment that the control areas had just sufficient recruitment to maintain the population at the 1990 levels. This study only covered one type of substrate and the results could be very different under different substrate, depth, and current regimes.

While the relationship between shell in the bottom and increased hard clam density occurs wherever studies of natural populations have been conducted (Gulf of Mexico to New England), the types of predators and their effects are substantially different. During 1996, we enumerated other organisms in the samples. There was an increase in species, mainly epifauna, on the shelled areas relative to the controls. This clearly indicates that other species are enhanced as well. The nature of the sampling (suction sampler and a 3-mm mesh collection bag) precluded examination of the effects on infauna. Many of the epifauna we found are known to prey on hard clam seed (Kraeuter 2001). The "reef effect" from mounds of shell may cause an increase in epifaunal predators. It is important to spread the shell evenly and not allow mounds to form that would attract and retain these organisms. The best combination is for shell to become an integral part of the bottom with only a small portion protruding above the sediment surface. In other areas, particularly where oyster setting is high, the effect of shelling on the establishment of oyster populations needs to be carefully evaluated. Extrapolation of shell density recommendations to different

environments should be examined carefully before large-scale attempts are made.

The slow growth rate of clams after 3 to 5 years and the small size of clams >10 years old, the small size of the largest clam collected (82.8 mm shell length), and the dark color of the meat on most clams suggests that conditions at this site are not optimal for hard clam production at present.

CONCLUSIONS

Shelling the bottom of Barnegat Bay, New Jersey increased the abundance of hard clam seed by nearly a factor of 10. The shell remained on the plots for at least 11 years and continued to enhance the set throughout that period. Settlement was 0.5 clams m^{-2} on the control plots and exceeded 1 m^{-2} only once in the high shell areas. Clams <15 mm in shell length were never found in control plots. This method presents a potentially viable protocol for increasing survivorship of small clams from natural set, but more thorough evaluation is needed before it can be used on a variety of bottom types.

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SPATIAL VARIATION IN THE BODY MASS OF THE STOUT RAZOR CLAM, *TAGELUS PLEBEIUS*: DOES THE DENSITY OF BURROWING CRABS, *CHASMAGNATHUS GRANULATA*, MATTER?

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ABSTRACT A series of functional-group hypotheses proposed for marine soft-sediment systems predict that either deposit-feeders or highly mobile bioturbators exclude low-mobile suspension feeders because of their sediment reworking activity. However, a low-mobile suspension-feeder—the stout razor clam *Tagelus plebeius*—coexists with highly mobile deposit-feeding burrowing crabs, *Chasmagnathus granulata*, in several Southwestern Atlantic estuaries. In this study, we compared the body mass (as relationship between shell length and dry weight of flesh) of the stout razor clam between replicated patches showing contrasting densities of burrowing crabs. Spatial variation was observed in the slope of the relationship between shell length and dry weight of flesh of *T. plebeius* in the three samplings dates (July 1999, January 2000, and April 2000). However, the pattern of spatial variation in the slope of this relationship was not consistent with the pattern of spatial variation in crab density. In addition, the pattern of spatial variation in the slope of the relationship between shell length and dry weight of flesh of the stout razor clams was not consistent between the three sampling dates. These results suggest either that (1) body mass of the stout razor clam is affected by habitat features other than crab density, or (2) effects of burrowing crabs on body mass of the stout razor clam are masked by spatial variation in other habitat features that affect body mass of stout razor clams or the extent to which crabs are able to affect clams.

KEY WORDS: bioturbation, body mass, *Chasmagnathus granulata*, spatial variation, *Tagelus plebeius*

INTRODUCTION

The stout razor clam *Tagelus plebeius* Solander (Veneroida: Solecurtidae) is an euryhaline species that occurs in estuarine environments from North Carolina (34°N, United States) to the San Matias Gulf (41°S, Argentina; see Holland & Dean 1977a, 1977b, Viegas 1981, Gutiérrez & Iribarne 1998, 1999, Gutiérrez & Valero 2001). This is a suspension-feeding species that construct permanent burrows (up to 50 cm deep) lacking lateral mobility (Holland & Dean 1977a, 1977b, Gutiérrez & Valero 2001). In several Southwestern Atlantic estuaries, this species coexists with the burrowing grapsid crab *Chasmagnathus granulata* Dana (Gutiérrez & Iribarne 1998, Gutiérrez & Valero 2001). *C. granulata* is one of the dominant macroinvertebrates in tidal flats and salt marshes of Southwestern Atlantic estuaries from Rio de Janeiro (23°S, Brazil) to the San Matías Gulf (41°S, Argentina; Boschi 1964, Spivak et al. 1994, Iribarne et al. 1997). This is a gregarious species that excavate and maintain semipermanent open burrows in the intertidal, from soft bare sediment flats to areas vegetated by the cordgrass *Spartina densiflora* (Spivak et al. 1994, Iribarne et al. 1997). At sediment flat areas, individuals of *C. granulata* behave as deposit-feeders, showing large (up to 1.4 l volume) and mobile burrows (up to 5 cm day^{-1}: Iribarne et al. 1997).

Coexistence between these two species, however, must not be expected according to any of the functional-group hypotheses that were proposed to predict species assembly in soft-substrate environments. For instance, the trophic-group amensalism hypothesis (Rhoads & Young 1970) predicts that deposit-feeders, such as *Chasmagnathus granulata*, exclude suspension feeders, such as *Tagelus plebeius*, by increasing the amount of sediment resuspended in the water column, which clogs the filtering appendages of suspension-feeders. The adult-larval interaction hypothesis (Woodin 1976) predicts that sediment reworking by deposit-feeders kill the larvae of recently settled suspension-feeders be-

cause of direct damage or burial to unsuitable depths. The mobility-mode hypothesis (Brenchley 1981, 1982) proposes that mobile benthic species, such as *C. granulata*, exclude more sedentary forms, such as *T. plebeius*, by continually burrowing trough the sediment. The coexistence between *C. granulata* and *T. plebeius*, however, illustrates that sedentary suspension-feeders are not always excluded from areas inhabited by mobile burrowing deposit-feeders. In fact, the latter is not a novelty: much evidence supporting the occurrence of the mechanisms predicted by the functional-group hypotheses often refer to negative but non-lethal effects (see Posey 1989 for a review). Therefore, regardless the lack of exclusion between both species, we are still in conditions to expect for negative, but nonlethal effects of *C. granulata* on stout razor clams.

The patchy distribution of burrowing crabs in the tidal flats of several Southwestern Atlantic estuaries (see Botto & Iribarne 1999, 2000) provides a good opportunity to explore this possibility at a realistic scale. In this study, we compare the body mass (the relationship between dry weight of flesh and shell length) of the stout razor clam in patches with high and low density of burrowing crabs. We recognize that this comparative approach does not allow to address cause-effect relationships between the presence of crabs and the body mass of stout razor clams, but comparing the body mass of stout razor clams among replicated areas with high and low density of burrowing crabs allow to discern between the following logical possibilities:

(1) The body mass of the stout razor clam vary between habitats depending on crab density, which may indicate (a) that burrowing crabs affect body mass of the stout razor clam or, (b) that the habitat features that affect crab density also affect body mass of stout razor clams.

(2) The body mass of the stout razor clam vary between habitats but irrespective of crab density, which may indicate (a) that body mass of the stout razor clam is affected by habitat features other than crab density, or (b) that effects of burrowing crabs on body mass of the stout razor clam are

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TABLE 1.

Mean (SD) density (burrows m^{-2}) of burrowing crabs *Chasmagnathus granulata* in the locations under study and results of one way ANOVA (df = 114) evaluating differences in crab density between locations.

Sampling Date	Location						ANOVA	
	1	2	3	4	5	6	MS	F
July 1999	1.35 (0.81)	1.30 (0.86)	1.40 (0.75)	0.15 (0.37)	0.25 (0.44)	0.20 (0.52)	2.64	24.36*
January 2000	3.15 (1.35)	3.35 (1.50)	3.60 (1.43)	0.45 (0.51)	0.50 (0.61)	0.60 (0.50)	6.85	53.87*
April 2000	1.70 (0.86)	1.60 (0.99)	1.80 (0.83)	0.60 (0.60)	0.45 (0.51)	0.55 (0.51)	1.97	14.68*

* $P < 0.01$. Tukey tests: $(1 = 2 = 3) \neq (4 = 5 = 6)$ in all sampling dates.

overwhelmed by spatial variation on other habitat features that affect body mass of stout razor clams or the ability of crabs to affect clams.

(3) The body mass of the stout razor clam did not vary between habitats, which may indicate (a) that burrowing crabs does not affect body mass of stout razor clams, or (b) that effects of burrowing crabs are being compensated by spatial variation in other habitat features that affect body mass of the stout razor clam.

MATERIALS AND METHODS

This study was conducted at the Mar Chiquita coastal lagoon (37°S, Argentina), which is a 46-km^2 body of brackish water affected by semidiurnal low amplitude (<1 m) tides and characterized by mudflats and large surrounding marshes dominated by the halophyte *Spartina densiflora* (Spivak et al. 1994, Iribarne et al. 1997). Samplings for crab density and collections of stout razor clams were conducted in July 1999 and January and April 2000, in an area approximately located 2.5 km upstream from the lagoon inlet, which comprises about 700 m of shoreline. At this area, six locations were selected; three of them characterized by high burrow densities of *Chasmagnathus granulata* (locations 1, 2, and 3), and the others by very low burrow densities (locations 4, 5, and 6). Crab density at each location was estimated by random sampling using a 1×1 m sampling unit ($n = 20$). Single factor analysis of variance followed by Tukey test (Zar 1984) was used to test for differences between locations in the density of burrowing crabs. Locations grouped under the same level of crab density did not differed significantly in the density of crab burrows in all sampling dates (see Results and Table 1). Sixty clams per location were collected at each sampling date by excavating the sediment using hand shovels. The length of the clams was measured along the anterior-posterior axis to the nearest 0.01 mm and their flesh was

removed from the gaping shell after a short immersion in boiling water. The flesh was dried separately at 70°C for 48 h before their dry weight was determined. Correlation analysis (Zar 1984) was used to evaluate the existence of a significant relationship between shell length and dry weight of flesh in clams at each location and sampling date. Once significant relationships between the shell length and the dry weight of flesh of the clams were observed at all locations and sampling dates (see Results and Table 2), parallelism tests followed by Tukey tests (Zar 1984) were used to compare the slope of this relationship between locations at each sampling date. Given that clams smaller than 50 mm occurred in low numbers and not in all locations, we excluded these data from the analysis of correlation and parallelism to cover the same range of sizes in all locations. After removing these data, we also randomly discarded some data from clams larger than 50 mm to attain an equal sample size between locations (July 1999: $n = 57$; January 2000, $n = 56$, April 2000, $n = 52$).

RESULTS

Single-factor analysis of variance indicated that the density of burrowing crabs significantly differed between locations in the three sampling dates (Table 1). Tukey tests revealed that the six locations can be subdivided in two clearly defined groups: locations with relatively high crab density (locations 1, 2, and 3) and locations with low crab density (locations 4, 5, and 6); being this pattern consistent in the three sampling dates irrespective of temporal variations in the density of crab burrows (Table 1). Correlation analysis indicated a significant linear relationship between the dry weight of flesh of stout razor clams larger than 50 mm and their shell length in all locations and sampling dates (Table 2). Parallelism tests indicated that the slope of the relationship between shell length and dry weight of flesh of *T. plebeius* differed

TABLE 2.

Site-specific regression equations and determination coefficients (between brackets) observed for the relationship between dry weight of flesh and shell length of the stout razor clam *Tagelus plebeius* in the three sampling dates.

Location	July 1999	January 2000	April 2000
1	y = 0.019x–0.467 (0.386)	y = 0.026x–0.739 (0.383)	y = 0.021x–0.509 (0.383)
2	y = 0.030x–1.135 (0.562)	y = 0.034x–1.191 (0.282)	y = 0.019x–0.427 (0.244)
3	y = 0.027x–0.897 (0.361)	y = 0.014x–0.126 (0.182)	y = 0.028x–1.024 (0.540)
4	y = 0.026x–0.927 (0.446)	y = 0.014x–0.082 (0.086)	y = 0.016x–0.307 (0.333)
5	y = 0.013x–0.286 (0.285)	y = 0.033x–1.088 (0.331)	y = 0.033x–1.144 (0.317)
6	y = 0.032x–1.169 (0.548)	y = 0.018x–0.458 (0.265)	y = 0.026x–0.870 (0.416)

$P < 0.05$ in all cases.

significantly between locations in the three sampling dates (Table 3, Fig. 1). Tukey multiple comparison of slopes indicated that the patterns of spatial variation in the slope of the relationship between shell length and dry weight of flesh of *T. plebeius* was not consistent between sampling dates. In addition, spatial variation in the slope of the dry weight-shell length relationship did not match the

pattern of spatial variation in crab density in any of the sampling dates (Table 3).

DISCUSSION

Recalling the logical possibilities established in the introduction, our results suggest either (1) that body mass of the stout razor
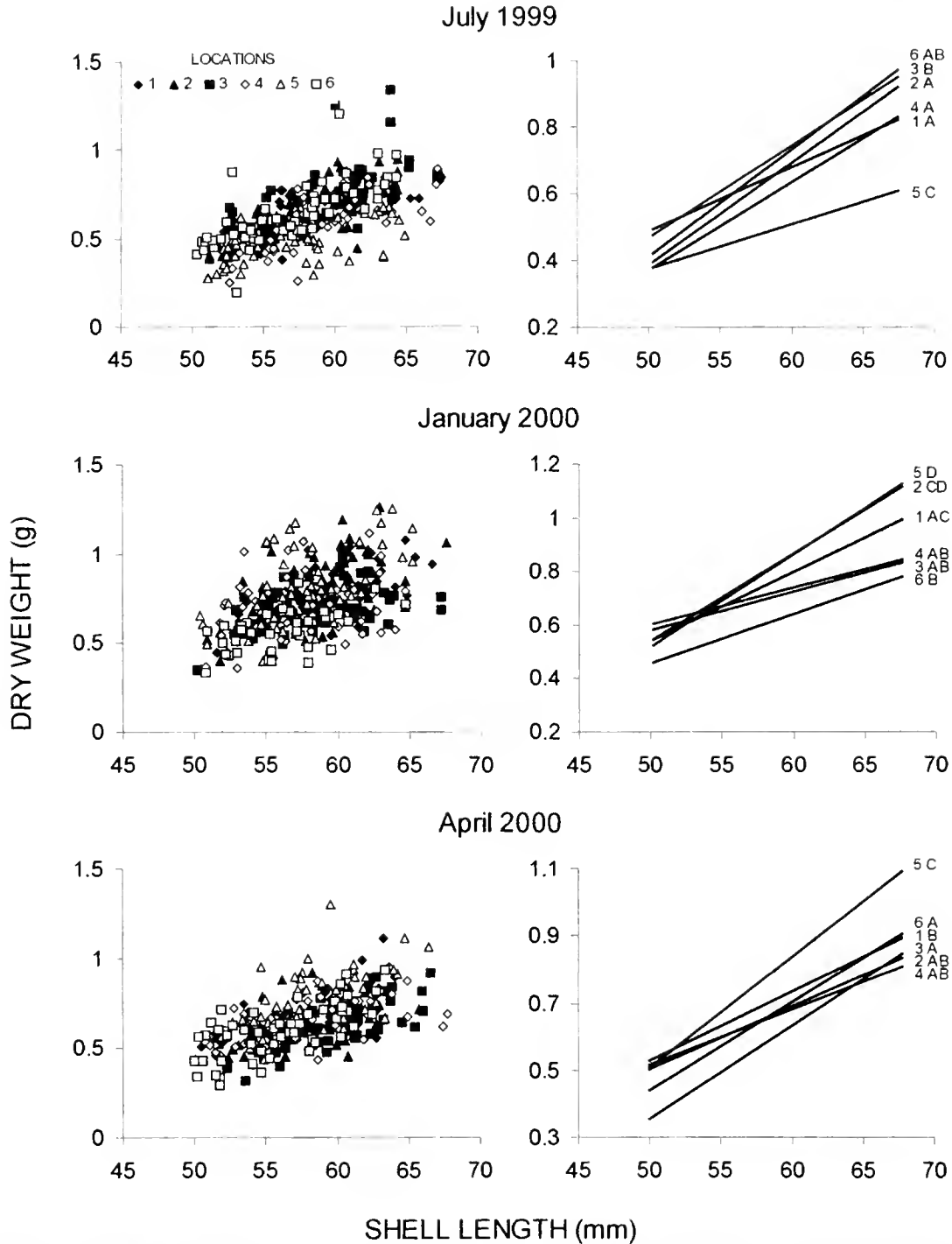
Figure 1. Relationship between dry weight of flesh and shell length of the stout razor clam *Tagelus plebeius* at each location and sampling date. Left: Dry weight data points plotted against shell length. Right: Curves corresponding to the linear fit of data points in the figures on the left. Locations are denoted by numbers beside each curve (locations 1, 2, and 3: High crab density; locations: 4, 5, and 6: low crab density). Curves showing the same letter beside their respective location numbers have slopes that are not significant different (*P* > 0.05) after Parallelism tests followed by Tukey tests.

TABLE 3.

Results of tests for parallelism and Tukey tests used to evaluate differences between locations in the slope of the relationship between dry weight of flesh and shell length of *Tagelus plebeius*

Sampling Date	Parallelism Test		Tukey Test
	MS	F	
July 1999	0.015	24.126*	(1,2,4,6) (3,6) (5)
January 2000	0.021	20.418*	(1,3,4) (3,4,6) (1,2) (2,5)
April 2000	0.014	14.194*	(2,3,4,6) (1,2,4) (5)

Numbers between brackets indicate locations that did not significantly differed in the slope of the dry weight–shell length relationship after Tukey tests.
* $P < 0.01$.

clam is affected by habitat features other than crab density, or (2) that effects of burrowing crabs on body mass of the stout razor clam are masked by spatial variation in other habitat features that affect body mass of stout razor clams or the extent to which crabs are able to affect clams. This is reasonable to occur because the locations encompassed in this study differ in many features -as sediment characteristics and orientation-irrespective of the presence of crabs (personal observation). Sediment characteristics may directly affect clam body mass (e.g., by determining the costs of burrowing; see Swan 1952, Newell & Hidu 1982) as well as the nature and extent of habitat modifications derived from crab burrowing that may be detrimental for stout razor clams (e.g., sediment resuspension; see Turner & Miller 1991). Differences in the orientation of the locations in relation to winds determine, for example, the degree to which clams are exposed to events of environmental disturbance by waves and currents (see Turner & Miller 1991, Bock & Miller 1995) as well as the degree to which sediment reworking by crabs might be overwhelmed or not by physical reworking (see Grant 1983).

The overall conclusion of this study is that crabs alone do not promote a spatial pattern in body mass of the stout razor clam at the scale of crab patches. It is uncertain, however, whether effects of crabs on the body mass of stout razor clams are occurring at locations with high density of crabs but overwhelmed by other sources of spatial variation that affect clams. Several lines of evidence suggest that crabs might have important local effects on the body mass of stout razor clams. For instance, organisms that are known to exclude low-mobile suspension-feeders, such as callianassid shrimps (see Posey 1989) excavate sediments at rates of 2.7–3.5 kg (dry) m^{-2} d^{-1} (Vaugelas 1984, Swinbanks & Luternauer 1987, Witbaard & Duineveld 1989), whereas burrowing crabs excavate sediments even at higher rates [5.9 kg (dry) m^{-2} d^{-1}; Iribarne et al. 1997]. Consequently, the detrimental effects of sediment reworking by crabs on the stout razor clam predicted by the functional-group hypotheses are still possible.

However, considering the rates at which burrowing crabs and callianassid shrimps remove sediments, the question at this point is why burrowing crabs does not exclude stout razor clams as callianassid shrimps do with a variety of suspension-feeders. The answer is, perhaps, in the different modes by which callianassid shrimps and burrowing crabs rework sediments. Callianassid shrimps burrow and sift the sediments continuously for food, destabilizing them and increasing water turbidity (Aller & Dodge 1974, Murphy 1985). However, *C. granulata* reworks sediments mostly during low tide eventually depositing mounds of fine, cohesive sediment above the surface, which are not likely to be easily resuspended by tidal currents (e.g., Iribarne et al. 1997, Botto & Iribarne 2000). This implies that some mechanisms predicted to exclude suspension-feeders from areas dominated by deposit-feeders, such as sediment resuspension (see Rhoads & Young 1970) might not take place in the case of burrowing crabs. Further, the latter suggests that sediment reworking is not a good predictor of the actual effect of burrowing deposit-feeders on suspension feeders.

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MARICULTURE SITING—TIDAL CURRENTS AND GROWTH OF *MYA ARENARIA*

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ABSTRACT Mariculture of the soft-shell clam *Mya arenaria* L. involves seeding juvenile shellfish on intertidal mudflats for grow-out. Laboratory studies have shown that constant current velocity affects shellfish growth. Few studies have determined the effect of tidal currents on shellfish growth *in situ*. Spot estimates of tidal currents can be generated with portable current meters and by measuring the erosion of Plaster of Paris hemispheres called clod cards placed in the current. Current velocities for Geographical Information System (GIS) coverages for entire estuaries can be estimated using numerical flow models. Although these different types of measurement have different relative advantages of cost, ease of describing large areas, and accuracy, each can be potentially used in evaluating sites for shellfish grow-out. Current velocities averaged over the flood tide were estimated by a numerical flow model and by clod cards for 16 locations at the same elevation in a bay in Eastern Maine and were compared with the annual shell increment of clams collected at the same locations. Statistical models included main effects and interactions between initial shell size, year of sample, and high-low current category estimated by clod cards or a numerical model. Models explained 57–58% of the variability in growth increment with initial shell size and year affecting growth more than current. Faster tidal currents resulted in 22–24% greater shell growth. Sites categorized as low flow had means for tidal currents ($\pm$SD) of 4.35 $\pm$ 0.37 cm/s and 2.99 $\pm$ 0.43 cm/s using the numerical model and clod cards, respectively. Least squares means ($\pm$SE) for the annual increment in shell length increment was 9.56 $\pm$ 0.247 mm for the low flow sites identified using the numerical model and 9.51 $\pm$ 0.274 mm for the low flow sites identified using clod cards. Sites categorized as high flow had current means ($\pm$SD) of 5.86 $\pm$ .62 cm/s using clod cards and 5.84 $\pm$ 0.46 cm/s using the numerical model and least squares means ($\pm$SE) for growth increment of 11.90 $\pm$ 0.32 and 11.70 $\pm$ 0.33 mm, respectively. The stimulatory effect of tidal currents on clam growth could be used in mariculture siting. Placing clod cards at specific intertidal locations at the same elevation could be used to estimate relative current velocities. Current velocities estimated using numerical models and displayed as GIS grids of entire regions will not have the same resolution as spot estimates from current meters or clod cards. However, grids can be used for siting if the grid cells are comparable in size to area to be seeded.

KEY WORDS: numerical model, Geographical Information System (GIS), current, growth, *Mya arenaria*

INTRODUCTION

Seed planting and transplanting has been an integral part of the hard clam and oyster industries (Malouf 1989). With hundreds of miles of mudflats in Northeastern Maine and a 45% decline in state landings over the past 15 y (DMR, 1997), mudflats with low densities of *Mya arenaria* L. are being seeded with juvenile clams. Site-specific characteristics must be evaluated in selecting sites for shellfish seeding (Beal et al. 2001, Peterson et al. 1995, Newell 1996), but determining environmental parameters capable of sustaining populations of bivalve seed is difficult in most cases (Malouf 1989).

Among a variety of biologic and environmental that influence growth of bivalves *in situ*, sufficient current speed is recognized as an important factor. Water velocity, horizontal advection, and vertical mixing in the water column influence the availability of phytoplankton to mussels (Fréchette et al. 1989). Currents are needed to avoid depletion of oxygen and food particles to suspension feeders, especially at high-density levels (Jørgensen 1990). Newell (1990) suggested a minimum current speed (about 3 cm/s) below which bottom culture of mussels may not be cost effective. An actual reduction in food intake of bivalves was found when current rates are not kept high enough (Bayne et al. 1976). Faster flow results in a greater flux of organic particles (Peterson & Skilleter 1994). Shell growth rates for hard clams over a 15 wk period increased by 10.7% in fast relative to slow current sites in coastal lagoon in New Jersey (Grizzle & Morin 1989). Soft-shell clams were found to orient perpendicular to the principal component of current direction potentially to optimization energy acquisition during an entire tidal cycle (Vincent et al. 1988).

The effect of water flow on growth varies with species of bivalve. For infauna, northern quahogs displayed a consistent in-

crease in shell growth with higher flow speed in the range of stream velocities between 0 to 4 cm/sec (Grizzle et al. 1994). Growth response in the soft-shell clams was similar to that observed in hard clams with a proportional increase in shell length for 4 y old, 40 mm clams with flow and no evidence of growth inhibition between free-stream velocities of 0.1 to 5.8 cm/sec (Emerson 1990).

For epifauna species, it has been speculated that growth is maximized at water flows that match inhalant pumping speed. Mussels grown in multiple flume trials at flow velocities of 0, 1, 2, 4, and 8 cm/sec had a statistically nonsignificant increase in shell growth at a flow of 2 cm/sec, which matched the approximate inhalant pumping speed (Grizzle et al. 1994). Eastern oysters increased growth at a flow of 1 cm/sec relative to flows of 0 and >1 cm (Grizzle et al. 1992). The constant flow in flume studies, however, is different from tidal currents, which vary in magnitude and direction. Flume experiments with ascending and descending flows have found clearing or grazing rates of scallops differed by 30% (Pilditch & Grant 1999).

Currents may affect shellfish growth, but estimating current velocities can be difficult. A device commonly used to determine flow rates is a current meter. However, collecting time series velocity profiles with current meters over large areas is time consuming with conventional instrumentation, particularly in intertidal waters. When current rates and flow patterns are needed for large regions being considered as potential shellfish grow-out sites, the use of current meters becomes impractical.

Two- and three-dimensional numerical computer models can be used to describe the direction and magnitude of currents for individual cells in grids covering coastal areas. The output data from numerical models can then be used to create thematic maps for Geographical Information System (GIS) coverages (Congleton

et al. 1999). Numerical models are supported by data for bottom elevations for each cell in the grid and tidal amplitude at the ocean boundaries of the models. They simulate time series estimates of velocity vectors for grid cells covering the model domain. Velocities may be estimated for discrete layers in individual grid cells or may be vertically averaged, as in this study. Model output can be analyzed in the GIS to identify sites with optimum conditions for shellfish growth. The major drawback, however, is the difficulty of initializing and running a numerical model.

An alternative method for estimating currents is by measuring a process, which is affected by the current magnitude. A physical analog measurement of current velocity is the dissolution of calcium sulfate (Plaster of Paris or gypsum) blocks or hemispheres, called clod cards, placed in moving water (Muus 1968, Doty 1971, Peterson & Skilleter 1994). Thompson and Glenn (1994) developed an equation for calculating mean water speed from field deployed clod cards using clod cards from the same batch for laboratory calibration in quiescent water of the same salinity temperature as in the field. They concluded that proper execution of field and calibration tests result in a simple and practical method for measuring water motion over a wide range of temperatures, salinities, and current speeds. Clod cards are inexpensive and simple to construct, but the difficulty of deploying large numbers limits their usefulness for estimating current magnitudes over large areas.

The objective of this study is to evaluate the relationship between (1) field measurements of tidal currents made with clod cards; (2) average current estimates generated by a numerical flow model; and (3) growth of soft-shell clams on a mudflat in Eastern Maine. The appropriateness of incorporating current estimates from a numerical model into a GIS for the selection of sites for grow-out of juvenile shellfish will then be considered.

METHODS

The study was conducted in Mason Bay in Eastern Maine on the western side of Englishman Bay, which bounds the Gulf of Maine. The bay (Fig. 1A) is 2.39 km long by 1.03 km wide, oriented in an east-west direction, and is located 9.7 km north of Jonesport, Maine (44°61.80'N, 67°56.23'W). At low tide (mean low water = −1.875 m msl), mudflats are exposed along the entire length of the bay with two channel inlets from Englishman Bay joining on the west side of Spar Island and running the length of the bay (Fig. 1B). Water temperatures vary from 5°C in April to 16°C in September (Beal et al. 2001).

Soft-shell clams were collected at 15 sites at the same water line spaced 40 m apart to the south of Spar Island and west of Flake Point Bar (Fig. 1B) at an elevation of −2.0 m msl in Spring, 1996. These sample sites were close to one of the inlets of the bay with a maximum separation of 485 m from the most easterly site to the most westerly site. A sixteenth site between the tip of Flake Point Bar and Spar Island was sampled during spring of 2000 to increase the range of water velocities sampled. One of the low flow sites in the center of the earlier sampling array was also sampled the second year. Sites were relocated in the second year using their global positioning system (GPS) coordinates.

Location of the 16 sites was determined by carrier-phase GPS measurements made with a Trimble GeoExplorer™ GPS receiver, and post-processed. Carrier-phase GPS is commonly used for surveying with sub-decimeter accuracy for measurements in the horizon plane. Measurements in the vertical plane are less accurate.

The range in the elevation measurements for the 10-min carrier-phase GPS readings at the 16 sites was −1.5 to −2.7 m msl with 95% confidence range of ±0.55 m for individual measurements (Congleton et al. 1999). Because inaccuracy in GPS measurements alone could have resulted in a difference in elevation between sites, locations were selected with simultaneous flooding and drying times.

Sample site coordinates were then imported into the MapInfo™ GIS creating a layer of sampling site locations (Fig. 1B). Sediment cores from four of the sites were analyzed for composition by the Analytical Laboratory of the Maine Soil Testing Service using the hydrometer method for particle size and 1050°C combustion analyzer for total carbon. Fifty clams were dug with a clam rake at the 15 sites South of Spar Island at the end of the first growing season. A single low flow site (sixth site counting from the most easterly) and the high flow site SE of Spar Island were sampled in the second growing season.

External annual rings were used to determine the increase in shell length during the preceding summer. Brousseau (1979) found winter rings to be a reliable method of determining age in soft-shell clams from Gloucester, Massachusetts. However, MacDonald and Thomas (1980) found external growth rings to be less reliable for age determination than thin shell sections, and Lewis and Cerrato (1997) found shell increment might be temporarily decoupled from soft-tissue growth by high temperature or starvation. However, external growth rings have been used for long-term estimation of growth (Kube et al. 1996) and growth and age (Jacques et al. 1984, Evans & Tallmark 1977) of *in situ Mya arenaria*.

Because of limitations of using growth rings for measuring age, length between the last shell check marks were used to measure the size at the beginning of last growing season. Initial size was then used as a covariate in the statistical analysis instead of age. Annual growth increment was then calculated by subtracting the final shell length from the initial size. The problem of lengthy shell abrasion limiting the usefulness of external rings in aging was minimized by taking measurements of growth only in the last growing season.

NUMERICAL MODEL OF TIDAL CURRENTS

Estimated currents for Mason Bay were obtained from the Mason Bay Model (MBM), which is an adaptation of Princeton Ocean Model (Mellor 1992, Blumberg & Mellor 1987) modified to describe intertidal areas (Congleton et al. 1999). Input bathymetry data for the model were processed in the MapInfo GIS including subtidal depths from NOAA nautical chart no. 13325, the shoreline boundary traced from an aerial photograph and 27 high accuracy carrier phase GPS measurements made at the waterline near low water on a single Spring tide. To increase the accuracy of the description of the bottom in the study area, fourteen of the GPS measurements were in the region, which is enlarged in Figure 1b. These data were used to generate a 100 by 76 grid covering the bay composed of square cells with 36.125 m sides. The 7600 cells in the grid gave increased resolution of depths between points with known elevations without unnecessarily increasing computing time for a run describing a tidal cycle. Grid cells (36 m sides) were smaller than the distance between the clam sampling locations (40 m) resulting in a different estimate of current velocity at each sample site.

The model generated estimates of vertically averaged current velocities for each grid cell flooded by the tide at one-second
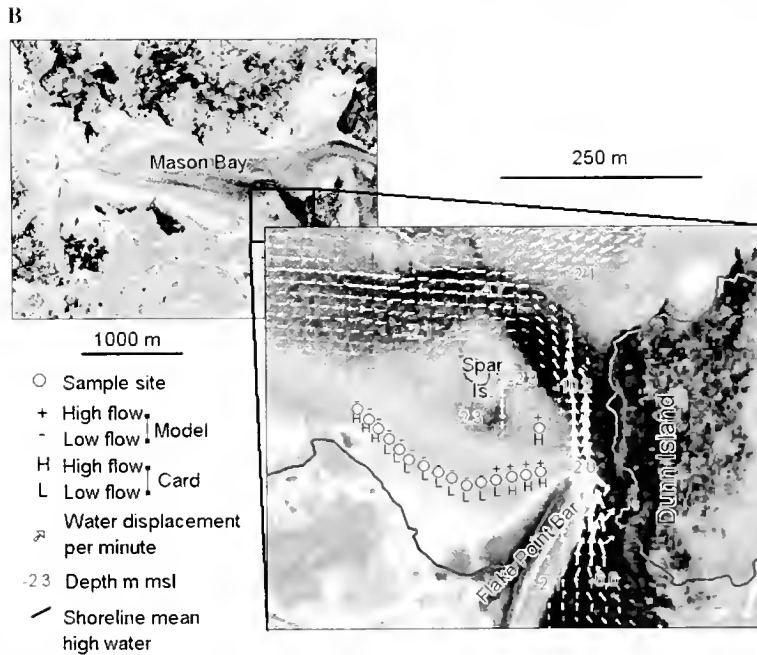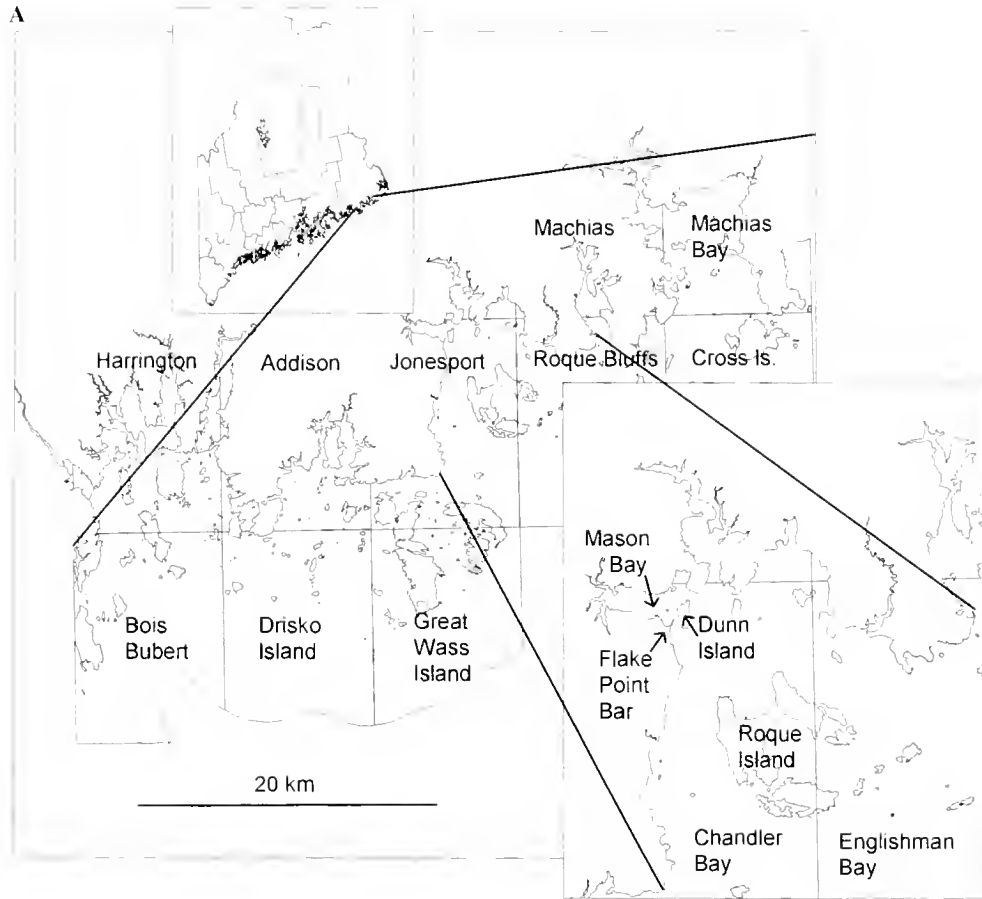
Figure 1. (A) Location of Mason Bay in Eastern Main near Jonesport, Maine with Englishman Bay and the Atlantic Ocean to the east connected by channels north and south of Dunn Island. Lines and labels show locations and extent of 7.5 min USGS quadrangles. (B) Aerial photos of Mason Bay. Right image is the rectangular area in SE image of the entire Bay. The array of sample locations (−2 m msl) are spaced 40 m apart except for the site nearest Spar Island. Vectors show water displacement/minute at maximum flood tide.

intervals for an average 2 m amplitude tide (Congleton et al. 1999). A vertical average of the current velocity for each time step was used because tidal amplitude and shallow water depths would inhibit stratification. Bottom friction was proportional to the square of the vertically averaged bulk flow. Vectors showing the current magnitude and direction estimated by the model for each grid cell were imported into the GIS. Layers of current vectors at different times in a tide cycle described flow throughout the bay.

For the statistical analysis of clam growth, the time series of velocities were averaged over the flood phase. The layer showing the sample site locations was placed over a layer of average current velocities to estimate velocities at each site. Because the sample locations were not centered on the grid used by the numerical model, the mean velocity of adjacent grid cells with the same approximate elevation were averaged.

FIELD MEASUREMENT OF CURRENTS—CLOD CARDS

Plaster of Paris hemispheres (clod cards) were used for measuring relative water motion at each of the fifteen sampling sites. In previous studies, rectangular clod cards were used (Doty 1971, Thompson & Glenn 1994). Clod cards used in this study (Fig. 2) were molded in hemispheric plastic capsules (32.36 cm³), creating a uniform surface area exposed to the current regardless of card orientation.

Commercial Plaster of Paris or gypsum was mixed two parts powder to one part water. The slurry was poured into the capsules and leveled off with a straightedge and left at room temperature for a week to insure thorough drying. After attachment to a 9×6.5 cm sheet of plastic with silicone epoxy, initial dry weights for each of the clod cards were measured and recorded.

For field deployment, the backing sheet of each clod card was attached to a brick with rubber bands. One clod card was placed at each of the 16 clam sample sites (Fig. 1B), and a total submersion time was estimated for the period that included air exposure at low tide. Because all clod cards were deployed on a spring tide in April (−0.7 m mllw), they were recovered after 4 days while the locations were still accessible at low tide. After recovery, cards were lightly rinsed to remove mud and were left to dry at room temperature for one week and weighed. The percentage loss and the change in weight were calculated.

The calibration of clod cards in quiescent water or under free convection conditions is necessary for the overall calculation of integrated field water speed. Four clod cards from the same lot as those used in the field trial were suspended 5 cm below the surface of a 22-l cylindrical container containing seawater (30–32 ppt salinity). The container was placed in a larger recirculating tank

maintained at 7°C, which corresponded to the average water temperature during the field trial.

Every 24 h, the water inside the container was replaced with fresh salt water and the dissolved Plaster of Paris on the bottom of the container discarded. After the calibration period of four days, each card was dried at room temperature for a week and then weighed. The average of the initial weights and average of the final weights for the four calibration cards were used in the water velocity calculations.

The scalar arithmetic mean velocity of the water in the field (V) was estimated for the 16 sites following the methods of Thompson and Glenn (1994):

$$V = 4.31 \ (W_{initial}/A_{initial})^{0.25} \ (S_{field}^{1.25}/S_{calibration}) \quad (1)$$

where $W_{initial}$ is the initial clod card weight of the field deployed card; $A_{initial}$ is the initial exposed surface area; S_{field} and $S_{calibration}$ are calculated as

$$[1 - (W_{final}/W_{initial})^{1/3}]/\theta \quad (2)$$

where W_{final} and $W_{initial}$ are the final and initial weights of the field and calibration tests, and θ is time submerged in the field and calibration tests.

Theta for the field trial θ was total time between deployment and recovery even though the clod cards in the field experienced air exposure during low tides. During periods of air exposure, field clod cards remained wet and continued to dissolve. On average, the cards were subjected to aerial exposure for approximately 1 h during each low tide.

STATISTICAL ANALYSIS

Tidal velocities for each site were categorized as high or low using estimates from the clod cards and numerical model. Because the mean (±SD) velocity estimated using the clod cards (4.96 ± 0.88 cm/s) was higher than the mean estimated by the numerical model (3.85 ± 1.34 cm/s), high flow sites were identified as having flows greater than 5 cm/s using clod cards and 4 cm/s the numerical model. Mean flow at the seven high flow sites identified using clod cards averaged 5.87 ± 0.63 cm/s and at the five high flow sites identified using the numerical model averaged 5.85 ± 0.47 cm/s. Mean flow at the nine low flow sites identified by clod cards averaged 4.35 ± 0.37 cm/s and at the 11 low flow sites identified by the numerical model averaged 3.02 ± 0.33 cm/s. High and low flow means categorized using either clod cards or the numerical model were statistically different ($P < 0.001$) using pooled variance t-tests.

Variability in shell growth increment during the preceding growth season was analyzed by analysis of variance (ANOVA)
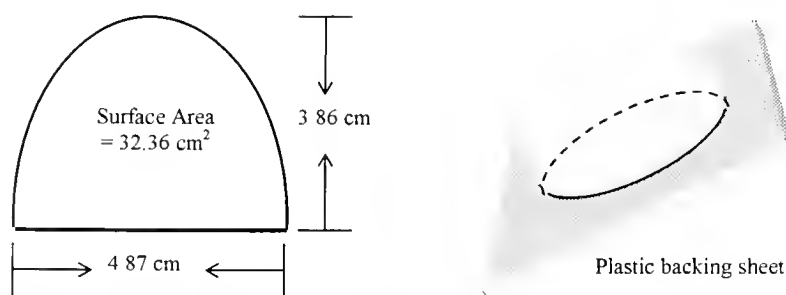
Figure 2. Gypsum clod cards constructed from a plastic mold cemented to a 9×6.5 cm backing sheet of plastic.

using the GLM procedure in SYSTAT v. 10. Shell length at the beginning of the growing season (distance between the last most anterior and posterior margins of the last growth check), years of sampling (1996 and 2000), and water velocity category of either high or low as indicated by either clod card weight loss or the numerical model were the independent factors. The initial model included main effects and all interactions.

$$Y_1 = B_0 + B_1X_1 + B_2X_2 + B_3X_3 + B_4X_1X_2 + B_5X_1X_3 + B_6X_2X_3 + B_7X_1X_2X_3 + e$$

Where Y_1 is the annual growth increment; B_0 is the intercept; B_1X_1 are the coefficient and categorical variable for year; B_2X_2 are coefficient and value for initial size and B_3X_3 are the coefficient and categorical current estimate (H,L) either from clod cards or the model; $B_5X_1X_3$, $B_6X_2X_3$ and $B_7X_1X_2X_3$ are coefficients for two and three way interactions; and e is the random error term.

Terms that were statistically insignificant ($P > 0.05$) were deleted from the model using the backward elimination procedure (Draper & Smith 1966).

To ensure independence of residual errors in predicting growth increment of spatially proximate observations, the Durbin-Watson Test Statistic was used to test for the existence of autocorrelated errors. Because 50 clams were collected at each location, residual error terms remaining after fitting the GLM model might not be independent if there is a site effect independent of local currents. First-order autocorrelation (lag = 1) results in the error term consisting of a fraction of the previous error term plus a new random disturbance term (Neter et al. 1996). Error terms are uncorrelated only at the time the autocorrelation term (ρ) is statistically equal to zero.

RESULTS

Composition of the sediments at the 16 sites ranged from 47–55% sand, 29–41% silt, 12–16% clay and 1.15 to 1.27% carbon. Shell lengths at the start of the two years ranged from 6.9 mm to 55.7 mm, with average (±SD) of 23.1 ± 10.8 mm. After excluding the juveniles or individuals without a growth check mark, sample size was 729 with an average (±SD) growth increment of 9.4 ± 4.0 mm with clams sampled once.

Trends in estimated water velocities from the numerical model and clod cards were similar. Velocities estimated with clod cards were highest at the site nearest Spar Island and at the sites near Flake Point Bar. Velocities decreased at the sites near the center and increased at the western end of the cove (Fig. 1B). Estimates from the numerical model displayed a similar trend generally decreasing moving westward from Flake Bar, but without the increase at the most western locations.

The correlation coefficient between the 16 estimates of current velocity from the numerical model and Eq. 1 was 0.74 ($P < 0.05$). Velocity averages over the flood tide at the sixteen sites ranged from 2.2 cm/sec to 7.14 cm/sec as estimated by the numerical model and ranged from 3.8 cm/sec to 7.52 cm/sec as estimated by Eq. 1. The estimation of current velocities by a numerical computer model and Eq. 1 were similar although the estimates from the numerical model were lower. The velocities estimated by the clod cards were during a spring tide, which would be expected to be higher than the velocities predicted by the numerical model during an average tide. Clod card measurements, however, were near the bottom where velocities are decreased by bottom shear.

The maximum water speed on the flood tide was also estimated

for the sixteen sites. Maximum velocities predicted by the numerical model ranged from 4.0 cm/sec for some of the western and central sites to 21.4 cm/sec at the site closest to Flake Point Bar.

All linear models used growth increment as the dependent variable, year (1996 vs. 2000) and flow (high vs. low as categorized by either clod cards or the model) as categorical variables and included initial size as a continuous variable. The Durban-Watson Statistic indicated that the GLM models had statistically significant first order autocorrelations. An inspection of autocorrelation plots of correlation versus lag indicated significant but diminishing positive autocorrelations up to lag 10 (Fig. 3). Autocorrelation significance ($P < 0.05$) was determined from the 95% confidence interval for the sampling distribution of the autocorrelation of lag k or r_k, which is normal with $\mu_{rk} = 0$ and $\sigma_{rk} = 1/n^{1/2}$ with a sample size of n (Lin et al. 1995).

A difference transformation replaced values for the dependent variable (growth increment) with the difference between it and the preceding value. Differencing is a popular and effective method of removing trend from spatial (location effect) and time series (temporal effect) data. Autocorrelation plots following the transformation had no trend because as lag increased there was a random distribution of positive and negative autocorrelations (Fig. 3). To ensure validity of significance tests using the transformed data, a linear regression with a hierarchical layout with clams (or trial) nested or stacked within site was used. The trial or clam within site effects was insignificant for hierarchical models tested ($P = 0.87$). Consequently, independence of error terms could be assumed and significance tests based on the difference-transformed data would be valid.

The ANOVA tables for the difference transformed growth increment as the dependent variable and high-low current category estimated by clod cards or the numerical model are in Tables 1 and 2. Both models explained 57–58% of the variability in growth increment. Estimates from both models indicated clams grew slower the first year of sampling (1996) and that larger clams grew less with –0.26 mm and –0.28 mm decrease in the growth increment for each mm increase in initial size depending on whether
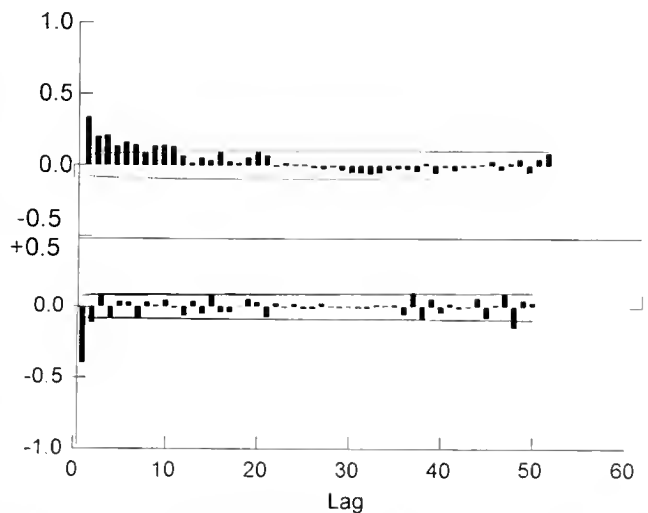
Figure 3. Autocorrelations between residual linear model errors with lags from 1 to 50 for predicting shell increment (top) and difference transformed measurements of shell increment (bottom). Lines above and below zero baselines are 95% confidence intervals for autocorrelation = 0.0.

TABLE 1.

ANOVA of growth increment with a difference transformation resulting from fitting a complete model reduced until only statistically significant effects remain. Current categories were average current <5 cm/s or average current ≥5 cm/s as estimated from clod cards using Eq. 1. R^2 of 58%.

Source	Sum-of-Squares	df	Mean-Square	F-Ratio	P
Year	392.378	1	392.378	65.049	0.000
Initial size	5138.024	1	5138.024	851.793	0.000
Clod card current	143.122	1	143.122	23.727	0.000
Current * size	33.430	1	33.430	5.542	0.019
Current * year	77.765	1	77.765	12.892	0.000
Error	4355.109	722	6.032		

currents were described with the numerical model or clod cards (Fig. 4). Larger average currents also stimulated growth although the effect on growth increment was less than that of year or initial size (Table 3). The adjusted least squares mean (±SE) for the growth increment at the sites identified by clod cards as low flow was 9.6 ± 0.25 and at the high flow sites was 11.9 ± 0.32 (Table 3). The least squares means (±SE) for growth increment at sites identified by the numerical model as low flow was 9.51 ± 0.274 cm/s and at the high flow sites was 11.70 ± 0.33 cm/s.

There was a significant interaction between year and current (Tables 1 and 2). Increased growth for high flow was expected during the second year because the highest flow site was only sampled in the second year. There were also significant two-way (clod card analysis) and three-way (numerical model analysis) interactions involving the effect of initial size indicating an inconsistent stimulatory effect of current on growth for animals of different size. However, interaction terms involving initial size made the smallest contribution to the model Sum of Squares or R^2.

DISCUSSION

A previous study (Congleton et al. 1999) also reported general agreement between water velocities estimated by the numerical model and measured by a portable current meter. The correlation between flows estimated by the numerical model and Eq. 1 in this study were lower than reported in Congleton et al. 1999. The 16 sites in this study, however, were a subset of the 25 sites in the previous study and had a smaller range of current velocities.

Numerous factors affect the accuracy of using clod dissolution in measuring currents. Mean current velocities estimated with the clod cards were higher than the velocities estimated using the model (Table 3). As previously noted, cards were deployed during a Spring tide when currents were stronger than an average tide that is simulated by the model. High estimates of currents using clod

cards compared with other techniques have been previously reported with dissolution rates in field experiments 16–18% high (Porter et al. 2000) compared with measured flows. Although flow estimates using cards in this study were higher than estimates using the model, there should also be some negative bias in the clod card estimated flows because θ in Eq. 1 included the time when the cards were air exposed at low tide while the θ used for calibration was total emersion time. Clod card accuracy could be increased by calibration in known steady flows rather than using a diffusion index factor as in this study (Porter et al. 2000).

Flows were anticipated to be greatest at the most easterly and most westerly sample locations because the flood tide entered the cove on either side of Spar Island. This anticipated pattern was seen in the flow rates estimated by the clod cards, but not the numerical model. The failure of the numerical model to predict increased currents west of Spar Island may be caused by the averaging of flow rates of the surrounding grid cells, because sample sites were not centered on the grid. Also, velocity estimates were an average for a cell with an area of 1305 m^2. A model with greater spatial resolution would show flow patterns in greater detail.

With a significant correlation between the current velocities estimated by the clod cards and numerical model, the similarity in the statistical analysis for the two sets of current measurements was not unforeseen. As expected, initial size had a significant effect on the growth increment of *M. arenaria*, resulting in slower growth in larger individuals (Fig. 4).

In an earlier study (Beal et al. 2001) placed clams at the same intertidal locations in Mason Bay and measured increment in shell length between time of removal from the hatchery and seeding on the flats in April and removal from the flats at monthly intervals until December. Mean shell length increased from 14.1 mm to 21.9 mm resulting in a 7.8 mm increase between June and August to December. Growth increment for the entire growing season was

TABLE 2.

ANOVA of growth increment with a difference transformation resulting from fitting a complete model reduced until only statistically significant effects remain. Current categories were average current <4 cm/s or average current ≥5 cm/s as estimated from the numerical model. R^2 of 57%.

Source	Sum-of-Squares	df	Mean-Square	F-Ratio	P
Year	304.810	1	304.810	49.360	0.000
Initial size	3524.739	1	3524.739	570.781	0.000
Model current	155.673	1	155.673	25.209	0.000
Current * year	142.192	1	142.192	23.026	0.000
Current * year * size	62.972	1	62.97	10.197	0.001
Error	4458.564	722	6.175		

Annual Shell Increment
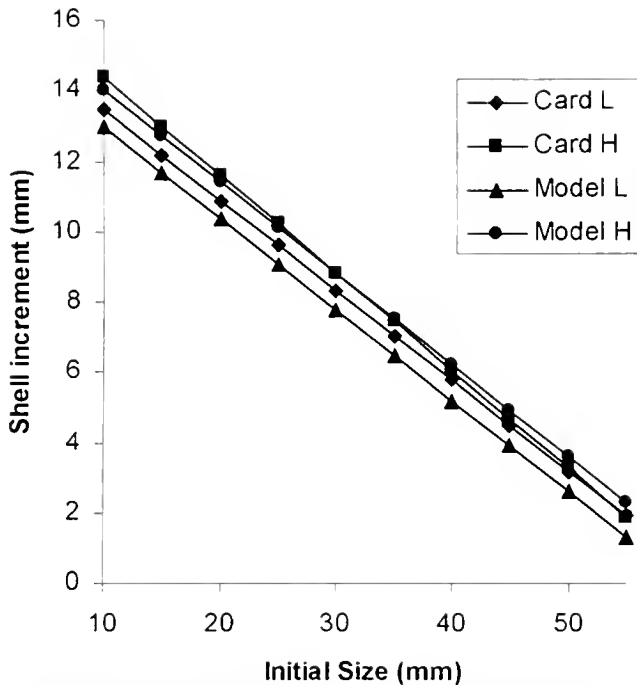
Figure 4. Increment in shell length for the year 2000 for clams in low and high flow sites as categorized using clod cards. (Card L, Card H) and the numerical model (Model L, Model H).

slightly less than 12 mm. Although juvenile clams without an initial growth check were excluded from the sample in this study, the growth increments predicted for 10 mm clams in Figure 4 is similar to the value reported by Beal et al. (2001). Brousseau (1979) predicted an asymptotic size of 108.12 mm and individuals in age class 5 reaching a harvestable size on Georgetown Island, Maine. Growth increments from both studies in Mason Bay would also result in a market size of 51 mm being reached in approximately 5 y. Results from this study also indicate market size would be reached earlier by clams at sites with average flows >5 cm/s than flows <5 cm/s.

Walne (1972) concluded that water current is a significant factor affecting filtration rates of bivalves, leading to higher growth

TABLE 3.

Adjusted least squares means for annual shell growth increment in low and high flows as estimated by clod cards and a numerical flow model. ANOVA and significance tests are in Tables 1 and 2.

Flow Estimate	Mean Flow (cm/s)	Growth Increment (mm) Least Square Mean	SE
Clod card			
Low flow	4.357 ± 0.370	9.565	.247
High flow	5.860 ± 0.618	11.899	.323
Numerical model			
Low flow	2.994 ± 0.428	9.505	.274
High flow	5.838 ± 0.457	11.699	.327

rates. The relationship, however, varies with species of bivalve. As velocities increase, an increased supply of particles corresponds to increased consumption rates in mussels (Fréchette et al. 1989). Higher currents would also cause sediment resuspension. Both frequency of sediment resuspension and sediment food value were found to be adequate to provide a nutritional benefit to scallops on George's Bank (Grant et al. 1997). However, filtration and growth rates were observed to be inhibited at higher flow levels. Mussels reduce filtration rates on average by 4.8% at velocities >25 cm/sec (Wildish & Miyares 1990). At a specified algal concentration, Cahalan et al. (1989) found that growth rates of bay scallops peaked at an intermediate flow velocity of 6.5 cm/sec. Sea scallop feeding is inhibited at currents >10 cm/sec (Wildish & Saulnier 1992, Wildish et al. 1987), and growth may even cease at 12 cm/sec (Kirby-Smith 1972).

Species differences in the stimulatory effect of water currents on growth were explained by an "inhalant pumping speed" hypothesis that predicts maximum growth at ambient flow the same as the inhalant pumping speed of the species. Siphonate taxa generally have greater inhalant pumping speeds. Hard clams (Grizzle et al. 1992) and mussels (Grizzle et al. 1994), however, increased growth rates over a wider range of currents.

Although year and initial size had more effect on clam growth in Mason Bay than did water velocity (Tables 1, 2), clams at high flow sites did have a larger growth increment than the low flow sites (Table 3). The results from this study show increasing shell increments of *Mya arenaria* of 23–24% at higher average current velocities. It is possible that the site closest to Flake Point Bar with a maximum estimated free stream flow 21.4 cm/sec could have had feeding inhibition at maximum flood tide. However, preliminary data (Turner 1991) found no decrease in average pumping velocity of *Mercenaria mercenaria* in flows between 20 to 30 cm. Additional studies need to be completed to identify the current velocity at which physiologic inhibition of feeding occurs in clams and other siphonate bivalves and also to determine the effect of a wider range of tidal flows on feeding and growth.

The R^2 values for the linear models accounted for 57–58% of the variability in the annual growth increment with differences in initial size responsible for most of this variability in growth. The range of water velocities across the study sites was not large. Some of the unexplained variability may have been partially caused by error in counting external growth lines particularly for older individuals as was reported for *Geukensia demissa* (Brousseau 1981).

Error in predicting current velocities would also decrease R^2 for the statistical models. Clod cards were wet and dissolving, but air-exposed during part of the tidal cycle resulting in overestimation of θ in Eq. 2 and a possible underestimation of current speed in Eq. 1. Field deployed clod cards could be eroded by waves and currents. Shallow water waves result in a local "to and fro" water motion on the bottom increasing gypsum erosion resulting in overestimation of tidal currents using clod cards.

Different calibration techniques for clod cards could increase accuracy of their use. Calibration of gypsum dissolution in flumes with known flows was superior to still water calibration (Porter et al. 2000) as used in this study. Porter et al. 2000 also found that the gypsum dissolution method should not be used to compare flows in different flow environments or to measure flows in an environment different from the calibration environment. These considerations limit the usefulness of clod cards in tidal environments because the flow environment changes during a tidal cycle. However, gypsum dissolution experiments should be interpreted as

measuring mass transfer relationships rather than flow speed. Biologic response variables such as shell growth in this study may be directly influenced by mass transfer of nutrients and indirectly affected by flow.

Another limitation to the predictive capability measured in this study is the bivalves in the present were not maintained in a controlled environment. Numerous factors could cause stress and affect growth. In a mariculture operation, trampling, predation, and reburial after digging could be eliminated. Under these conditions, the impact of water movement on variation in growth may be greater.

Differences in clam density could also affect growth. Clam density was not controlled in the present study. Beal et al. 2001 varied seed clam densities between 330 m^{-2} and 1320 m^{-2} at the same location in Mason Bay without significantly affecting the growth increment in shell length (Beal et al. 2001). Low clam densities at all study sites were apparent during field sampling from the digging effort required to collect the clams. Density was also found not to have a significant effect on final shell length of *Mercenaria mercenaria* grown in bags (Fernandez et al. 1999).

Application to Mariculture Siting

The relationship between bivalve growth and the clod card erosion should be useful in evaluating mariculture sites. Although the contribution of current magnitude to the R^2 of the linear model of growth was small relative to year and initial size, the increase in growth predicted for clams of uniform size that are seeded at the same time (or year) would be increased by 22–24% in high flows sites relative to low flow sites.

Relative water flow can be estimated by measuring percentage weight loss of cards deployed at different sites. The use of Eq. 1 for calculating an estimated velocity requires laboratory measurement of clod card loss in quiescent water, but determining the percent weight loss of cards should be sufficient for estimating relative flow rates at locations with the same air exposure and water temperature.

The number of cells required in a grid with sufficient resolution to estimate local tidal currents is a possible limitation on using a numerical model. Grid scale is an important aspect of tide modeling in the Gulf of Maine (Suesy et al. 1993). For use in mariculture siting, grid cells should be of the same size or smaller than the location where the clams are to be seeded. Ramming and Kowalik (1980) considered using a grid with irregular steps with the smallest grid distance in the region of primary interest with larger grid cells away from the region of high resolution. The solution for the irregular grid, however, is much more complicated compared with an equidistant grid with spurious effects decreasing the accuracy expected from grid refinement. Despite these limitations, Kowalik and Murty (1993) gave a number of examples of models using a combination of coarse and fine grids in their consideration of the problem of using nested and multiple grids to describe tidal flats.

A frequently used approach is to use the solution from a model using a coarse grid as input for the boundary conditions for a fine mesh grid for the area where higher resolution is required. The development of multiple models at different scales would be facilitated by using an object-oriented approach. The object-oriented feature of inheritance allows a general description of model components in a base class to be inherited by a child or derived class with the specific components to be added for a specific implementation. An object-oriented, two-dimensional landscape model with biologic components has been previously developed (Congleton et al. 1997).

For time series descriptions of current magnitude and direction over large areas, obtaining estimates from a numerical model would be the most practical. The incorporation of current estimates from a numerical model in a GIS, as described by Congleton et al. (1999), would make the information readily retrievable for use in aquaculture siting and other applications.

ACKNOWLEDGMENTS

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MATURITY AND GROWTH OF THE PACIFIC GEODUCK CLAM, *PANOPEA ABRUPTA*, IN SOUTHERN BRITISH COLUMBIA, CANADA

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ABSTRACT Measurements were made to determine size and age at maturity and growth of the Pacific geoduck clam, *Panopea abrupta*, from two areas in southern British Columbia, Canada. Growth rates were slower for *P. abrupta* from Gabriola Island than those from Yellow Bank. Histological examination of gonads indicated that at sizes <90 mm SL considerably more males matured than females, but at sizes ≥90 mm SL the sex ratio was similar for males and females. Size at 50% maturity was similar for *P. abrupta* from both areas (58.3 and 60.5 mm SL, respectively), but age at 50% maturity was slower for geoduck from Gabriola Island (3 y) than those from Yellow Bank (2 y). Although one hermaphrodite was recorded, *P. abrupta* was considered basically gonochoristic (dioecious).

KEY WORDS: Pacific geoduck, *Panopea abrupta*, maturity, sex ratio, hermaphrodite, reproduction

INTRODUCTION

The Pacific geoduck clam, *Panopea abrupta* (Conrad, 1849) (Pelecypoda: Hiatellidae), is distributed along coastal areas from southern California to Alaska and west to southern Japan (Bernard 1983, Coan et al. 2000). Geoduck are found buried up to 1 m deep within soft substrates (e.g., mud and sand) from the low intertidal to at least 100 m (Jamison et al. 1984, Goodwin & Pease 1989). There are commercial fisheries for geoduck in Alaska, British Columbia, and Washington State (Campbell et al. 1998, Bradbury & Tagart 2000, Hand & Bureau 2000). Geoduck are long-lived, reaching ages up to 168 y (Bureau et al. 2002). Adult geoduck have separate sexes and broadcast spawn annually, usually during summer (Andersen 1971, Goodwin 1976, Sloan & Robinson 1984). Planktonic larvae settle on substrates within 47 days, and juveniles burrow into the substrate (Goodwin et al. 1979, Goodwin & Pease 1989). Geoduck juveniles and adults feed by filtering food particles (e.g., phytoplankton) from seawater (Goodwin & Pease 1989). Geoduck growth is variable but most rapid in the first 10 y; thereafter, although growth in shell length is greatly reduced, shell thickness and meat weight continue to increase at a slow rate (Bureau et al. 2002).

Andersen (1971) found 50% maturity occurred at about 75 mm SL in geoduck sampled in the Hood Canal, Washington State, but little is known about the rate of sexual maturity for *P. abrupta*, especially in British Columbia. (Sloan & Robinson 1984). The purpose of this paper is to present information on the sexual maturity and growth rates of *P. abrupta* from two areas in southern British Columbia.

MATERIALS AND METHODS

Samples from as wide a range as possible of *P. abrupta* were obtained from Yellow Bank, near Tofino on the west coast of Vancouver Island, (Lat. 49°14.18′, Long. 125°55.48′) during 28 May, 1991 and Gabriola Island, near Nanaimo in Georgia Strait, (Lat. 49°07.6′, Long. 123°45.05′) during 22 to 23 May, 1991, at depths between 5–15 m for both areas. The clams were transported to the laboratory in coolers (2°C) and kept in running sea water (ambient temperature) until processed within 48 h of capture.

For each geoduck, shell length was measured as the straight-line distance between the anterior and posterior margins of the shell to the nearest mm with vernier calipers. The age of each geoduck was estimated using the acetate peel method of Shaul and Goodwin (1982). Each right valve was sectioned through the hinge plate, the cut surface polished, etched with a 1% hydrochloric acid solution for 1.5 min, washed with distilled water, dried, and an acetate peel made by applying an acetate sheet on the hinge surface with acetone. Growth rings imprinted on the acetate peel were counted on a digitizing table after ×40 magnification using a Neo-Promar projector. Although most individuals had their SL and age measured, there were some that had only the SL or only the age measured; these latter individuals were included in the analysis where appropriate. Reproductive condition of each geoduck was determined by removing a sample from the central portion of the gonad and preserving the tissue in Davidson's Solution (Shaw & Battle 1957). Histological slides were prepared with sections of the gonad stained with hematoxylin-eosin. Histological sections of the gonads were classified into six stages according to Andersen (1971). Stage 0 was immature (no differentiation in gonadal tissue; loose vesicular connective tissue in gonad). The other stages were for mature geoduck (connective tissue well developed, primary cells evident on follicle walls or eggs or sperm development evident) and classified as: (1) early active; (2) late active; (3) ripe; (4) partially spent; and (5) spent.

Average von Bertalanfy growth curves were fitted to all data points of size at age using the equation:

$$L_t = L_\alpha (1 - e^{-k(t-t_0)})$$

where t is age in years, L_t is shell length (mm) at age t, L_α is theoretical maximum size, k is a constant, determining rate of increase or decrease in length increments, t_0 is the hypothetical age at which the organism would have been at zero length. The parameters L_α, k, and t_0 were estimated using a non-linear Gauss–Newton least squares method (SYSTAT 2000).

The proportion of mature geoduck (P) at shell length or age (X) was estimated using the equation:

$$P_X = X/(X + e^{(A-BX)})$$

where A and B are parameters estimated using a non-linear Gauss–Newton least squares method (SYSTAT 2000). Data for both sexes were combined for each of the growth and maturity curve analyses since sex could not be distinguished in the immature sizes.
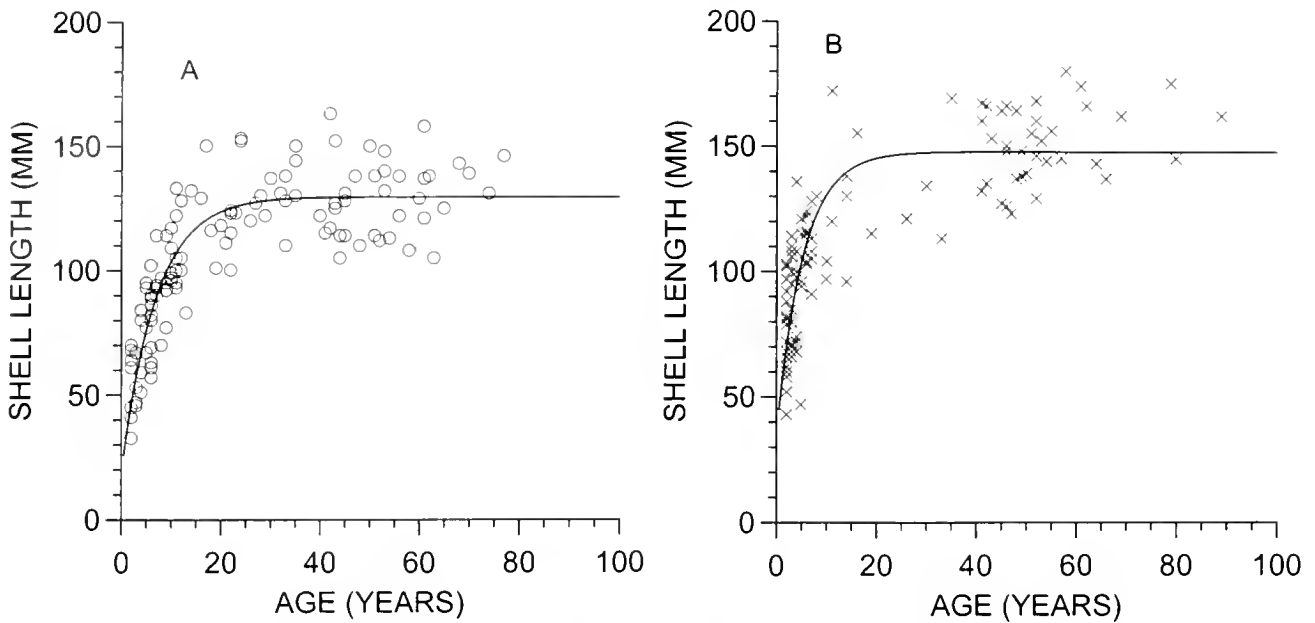
Figure 1. Growth curves for *P. abrupta* collected from (A) Gabriola Island, and (B) Yellow Bank. Curves calculated from the von Bertalanfy growth parameters (Table 1).

RESULTS

Growth

The oldest *P. abrupta* collected was 77 y (146 mm SL) from Gabriola Island, and 117 y (154 mm SL) from Yellow Bank. The smallest and largest geoduck, respectively, was 10 mm SL (age unknown, probably 1 y) and 163 mm SL (42 y) from Gabriola Island, and 43 mm SL (2 y) and 180 mm SL (58 y) from Yellow Bank. Growth was fastest in the first 10 y followed by slow growth thereafter for geoduck from both areas (Fig. 1). There was considerable variability of size within each age group. Growth rates of *P. abrupta* from Gabriola Island were slower than those from Yellow Bank (Fig. 1, Table 1).

Gonadal Condition

Immature gonads comprised 10.85% and 12.10% of the total geoduck gonads sampled from Gabriola Island (*n* = 129) and Yellow Bank (*n* = 124, includes three individuals without SL measurements), respectively (Fig. 2). The largest immature geoduck was 80 mm SL (5 y) and 72 mm SL (4 y) from Gabriola Island and Yellow Bank, respectively. There were insufficient data to determine spawning periods because seasonal monthly samples were not collected. However, most mature gonads were in the ripe or partially spent condition for geoduck collected from both areas (Fig. 2). There were no gonads that were spent (gonadal

TABLE 1.

Von Bertalanfy growth parameters for *P. abrupta* from Gabriola Island and Yellow Bank during May 1991. Values in brackets are approximate 95% confidence intervals.

Area	$L\infty$	K	t_0	N
Gabriola Island	129.6 (±4.1)	0.146 (±0.020)	−1.02 (±0.95)	120
Yellow Bank	147.7 (±5.8)	0.189 (±0.055)	−1.42 (±1.17)	108

condition 5). This suggested that geoduck spawning had begun at both areas during mid to late May 1991.

Sex Ratio

For geoduck <90 mm SL, in both areas combined, 41.18% were immature, and 54.41% were males (Table 2). The sex ratio for mature geoduck <90 mm SL was predominantly (92.5%) male
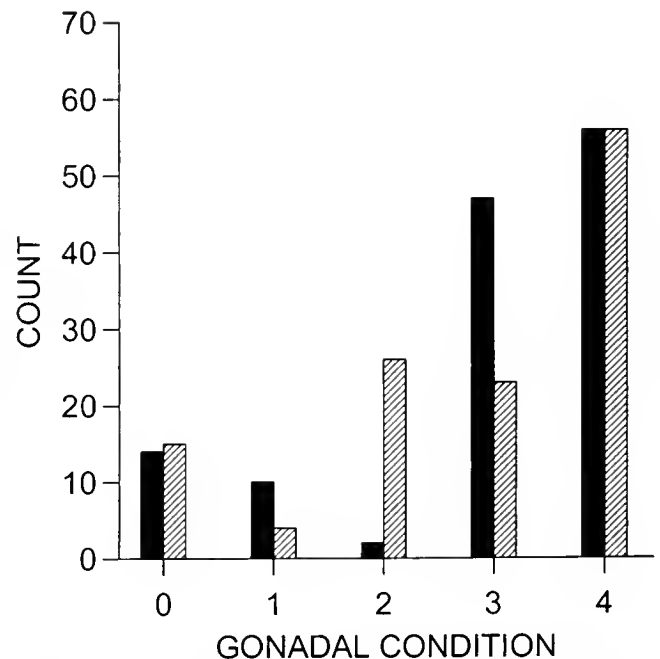
Figure 2. Frequency of gonadal condition stages found in gonads of all *P. abrupta* collected from Gabriola (black bars) and Yellow Bank (hatched bars). Gonads classified as 0 = immature, and mature stages that are 1 = early active; 2 = late active; 3 = ripe; and 4 = partially spent.

TABLE 2.

Percent of total gonads differentiated into mature males and females
and immature *P. abrupta* from Gabriola Island and Yellow Bank
during May 1991. One 91 mm SL hermaphrodite was found. N =
total number. Includes only individuals with SL measurements.

Area	Percent of Total				
	Male	Female	Immature	Hermaphrodite	N
<90 mm SL					
Gabriola Island	56.76	5.40	37.84		37
Yellow Bank	51.61	3.23	45.16		31
Total	54.41	4.41	41.18		68
≥90 mm SL					
Gabriola Island	57.61	42.39			92
Yellow Bank	45.56	53.33		1.11	90
Total	51.65	47.80		0.55	182

with few (7.5%) females for both areas combined. In contrast,
geoduck ≥90 mm SL had generally a more equal sex ratio, al-
though males were slightly more abundant than females in the
Gabriola Island sample, whereas there were slightly more females
than males in the Yellow Bank sample (Table 2).

Hermaphroditism

Although most of the histological material of mature *P. abrupta*
gonads allowed differentiation between females (follicles with oo-
cytes) and males (follicles with spermatozoa) (Fig. 3) there was
one individual that was a hermaphrodite, with a gonad showing
both male and female characteristics (Fig. 4). This gonad had some
follicles containing only either female or male gametocytes per
follicle, and other follicles, which contained spermatozoa and oo-
cytes in the same follicle. The geoduck was 91 mm SL (age was
not determined).

Maturity

Mean size at 50% maturity was similar for geoduck from
Gabriola Island, 58.3 mm SL (55.2–59.4 mm SL, lower and upper
95% confidence intervals, CI), and Yellow Bank, 60.5 mm SL
(51.1–64.0 mm SL, 95% CI) (Fig. 5, Table 3). Mean age at 50%
maturity was about 1 y slower for geoduck from Gabriola Island,
3.09 y (2.68–3.25 y, 95% CI), than at Yellow Bank, 2.04 y (1.72–
2.16 y, 95% CI) for Yellow Bank geoduck (Fig. 6, Table 3). The
smallest mature male was 45 mm SL (2 y) and 60 mm SL (2 y),
the smallest mature female was 59 mm SL (4 y) and 88 mm SL
(2 y), and the largest immature geoduck was 80 mm SL (5 y) and
72 mm SL (4 y), respectively, in the samples from Gabriola Island
and Yellow Bank.
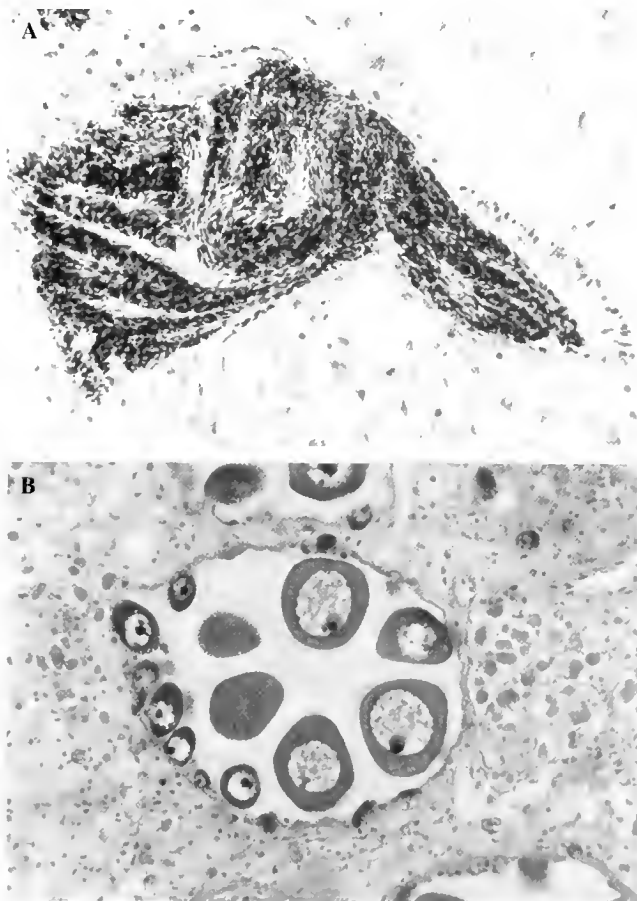
Figure 3. Photomicrographs of *P. abrupta* gonadal tissue cross-
sections of (A) Male (×400 magnification) showing spermatozoa-filled
follicle surrounded by connective tissue, (B) Female (×400) showing
oocyte-filled follicle surrounded by connective tissue.
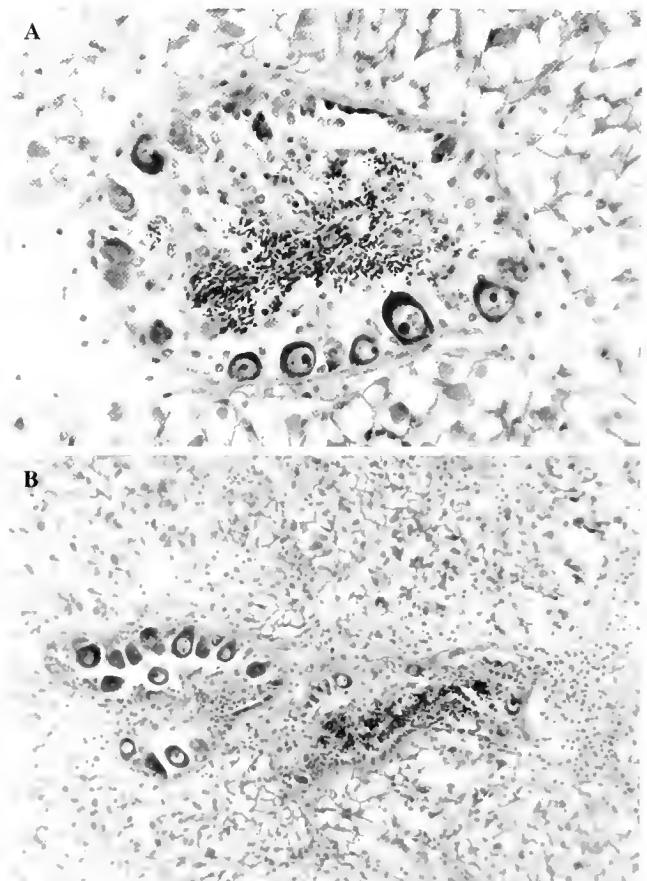
Figure 4. Photomicrographs of hermaphrodite *P. abrupta* gonadal tis-
sue cross-sections of (A) (×250 magnification), and (B) (×160) showing
single follicles containing oocytes and spermatozoa.
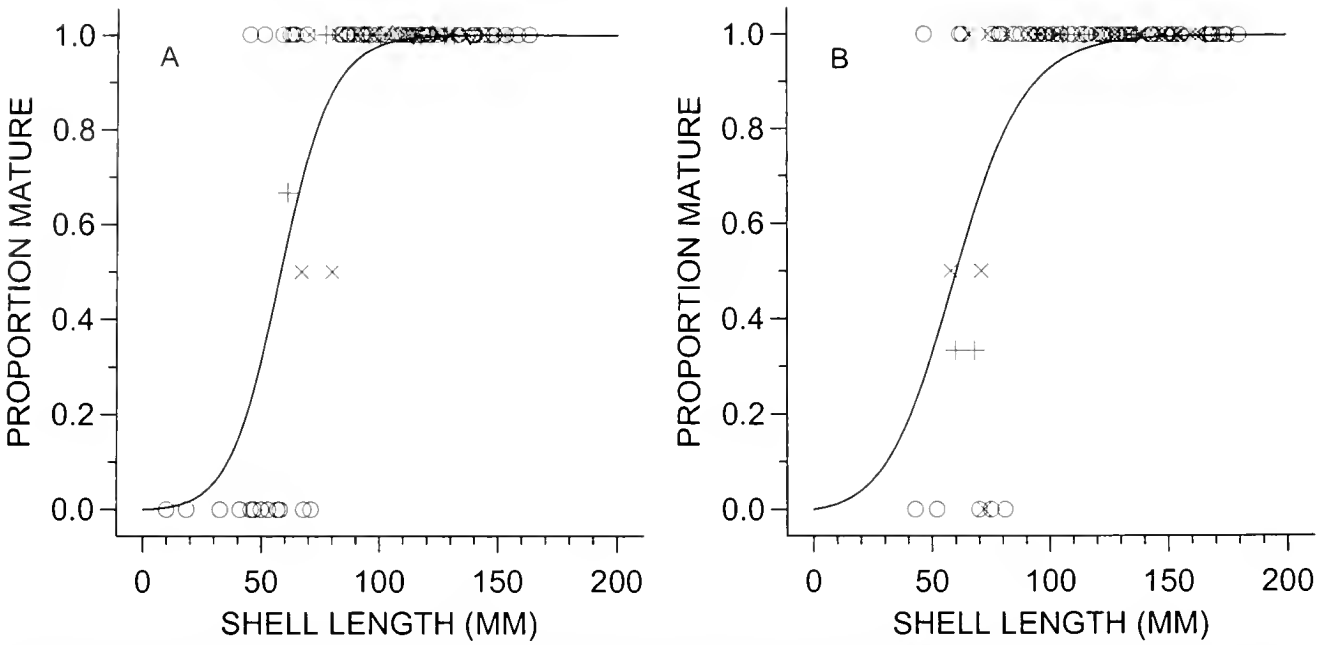
Figure 5. Size at maturity curves for *P. abrupta* collected from (A) Gabriola Island, and (B) Yellow Bank. Symbols indicate number of individuals per shell length: "O" = 1; "X" = 2; "+" = 3. See text for equation for the predictive curve and Table 3 for parameter values.

DISCUSSION

Our findings indicated that growth rates were faster for geoduck from Yellow Bank than those from Gabriola. Results were similar to those of Burger et al. (1998) and Bureau et al. (2002) who found that geoduck from Georgia Strait were generally smaller than those from the west coast of Vancouver Island. Reasons for the differences in *P. abrupta* growth rates between areas could be attributed to a variety of environmental and biological factors associated with different habitats (e.g., substrate type, temperature, exposure to water surge activity, pollution, food availability, and geoduck density or genetic characteristics) (Breen & Shields 1983, Harbo et al. 1983, Goodwin & Shaul 1984, Goodwin & Pease 1991, Noakes & Campbell 1992, Hoffman et al. 2000, Bureau et al. 2002).

Our examination of gonadal condition suggested that the spawning period for geoduck from both study areas was just beginning in mid to late May 1991. Results agree with other gonadal studies of geoduck, which found the main spawning period was during June and July (Andersen 1971, Goodwin 1976, Sloan & Robinson 1984).

The male:female sex ratio of mature *P. abrupta* found in this study (52:48) was similar to that reported by Goodwin (1976) (53:47) and Sloan and Robinson (1984) (57:43). The high percentage of males in the small sizes (young ages) in this study was

TABLE 3.

Parameter estimates for equation indicating relationships between proportion that are mature with shell length (SL in mm) or age (years) of *P. abrupta* from Gabriola Island and Yellow Bank during May 1991. See text for equation formula. Values in brackets are approximate 95% confidence intervals.

Area	Variable X	Parameter Estimates		N
		A	B	
Gabriola Island	SL	8.512 (±2.741)	0.076 (±0.044)	79
Yellow Bank	SL	7.224 (±2.314)	0.052 (±0.033)	80
Gabriola Island	Age	2.956 (±1.551)	0.591 (±0.435)	15
Yellow Bank	Age	2.397 (±1.540)	0.828 (±0.644)	14
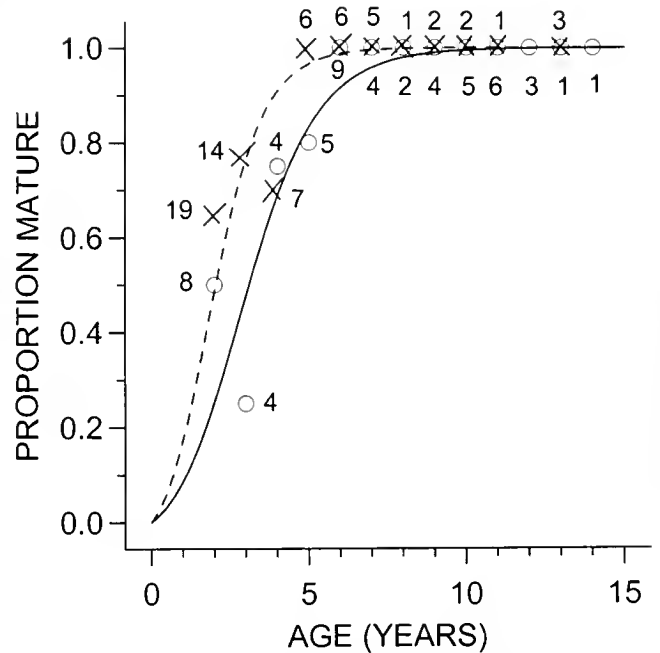
Figure 6. Age at maturity curves for *P. abrupta* collected from Gabriola Island ("O" solid curve), and Yellow Bank ("X" and dashed curve). Number by each symbol indicates number of individuals per age group. See text for equation for the predictive curve and Table 3 for parameter values.

similar to Andersen's (1971) findings of 94.4% males among geoduck with <100 mm SL.

Our findings indicated the first recording of a *P. abrupta* hermaphrodite. Most bivalve species are dioecious (sexes are separate) although hermaphroditism does occur in some species of this group (Coe 1943, Coan et al. 2000). Factors causing hermaphroditism in *P. abrupta* are unknown. Whether the "simultaneous" hermaphroditism (Coe 1943, Eversole 1989) in this geoduck was fully functional in producing viable eggs and sperm is unknown. However, sexuality of different sizes (or ages) in *P. abrupta* has not been studied extensively. We estimated that only ~1,200 individual gonads have been histologically examined to date from mature *P. abrupta* sampled in Washington State and British Columbia (Andersen 1971, Goodwin 1976, Sloan & Robinson 1984, this study). Andersen (1971) and Goodwin (1976) suggested that *P. abrupta* might be gonochoristic where sex is determined by development with males maturing at a smaller size (earlier age) than females. Although we suspect that hermaphroditism is rare in *P. abrupta*, the probability that some level of protandry, sex reversal, or "simultaneous" hermaphroditism in *P. abrupta* (especially for sizes <100 mm SL) may occur and should be investigated further.

Sexual maturity was variable between *P. abrupta* individuals and sexes. Males started to mature at an earlier age than female geoduck in Yellow Bank than Gabriola Island. Although size at 50% maturity was similar for *P. abrupta* from both areas (58.3 and 60.5 mm SL, respectively) age at 50% maturity was slower for geoduck from Gabriola Island (3 y) than Yellow Bank (2 y). Andersen (1971) found sexual maturity of geoduck to be variable, the smallest sexually mature geoduck to be 45 mm SL, and 50% size at maturity to be 75 mm SL (which Andersen estimated to be an age of 3 y). Our study is the first to show that although size at maturity may be similar for geoduck from two different areas, differences in growth rates may influence the age at which geoduck matures sexually. These findings are similar to some studies of other bivalve species, which suggest that onset of maturity may depend more on size than age (e.g., Nakaoka 1994). However, size and age at sexual maturity can also vary between populations in the same bivalve species (Ponurovsky & Yakovlev 1992, Sato 1994). Variation in environmental (e.g., temperature, current patterns, substrate type, and depth) and biological (e.g., genetics, food supply, growth and mortality rates, predation, and parasitism) factors may affect maturity rates within bivalve populations at different locations (Thompson et al. 1980, Ponurovsky & Yakovlev 1992, Nakaoka 1994, Sato 1994, Taskinen & Saarinen 1999).

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THE EFFECTIVENESS OF N-HALAMINE DISINFECTANT COMPOUNDS ON *PERKINSUS MARINUS*, A PARASITE OF THE EASTERN OYSTER *CRASSOSTREA VIRGINICA*

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ABSTRACT The pathogenic protozoan *Perkinsus marinus* (Mackin, Owen and Collier) is the cause of extensive mortalities in Eastern oyster, *Crassostrea virginica*, populations along the Gulf and East Coasts of the United States. A series of experiments was undertaken to determine the effect of N-halamine disinfectants on this protozoan parasite. The organic N-halamine disinfectants, 1,3-dichloro-2,2,5,5-tetramethyl-4-imidazolidinone (DC) and 1-chloro-2,2,5,5-tetramethyl-4-imidazolidinone (MC), apparently damage the permeability of the parasites outer membrane and alter the osmoregulatory functions of the cell. Damaged parasites were unable to reproduce at concentrations as low as 14.9 mg/L DC at 8 h exposure, or for the chemical MC at 24.9 mg/L at 12 h exposure. The chemical compounds appear to lyse the larger meronts first, followed by lysis of the daughter spores. These studies strongly suggest that the chemical compounds DC and MC can be used to disinfect seawater allowing the production of specific pathogen-free stock in oyster hatcheries, and having the potential to prevent the spread of these parasites from contaminated oysters to uninfected oysters.

KEY WORDS: oyster, *Perkinsus marinus*, disease, disinfection, N-halamine

INTRODUCTION

The Eastern oyster, *Crassostrea virginica* (Gmelin 1791) naturally occurs in North America from the Gulf of St. Lawrence in Canada to the Gulf of Mexico. It is common in estuaries in coastal areas of reduced salinity, and is an important commercial species. Once considered the most abundant source of oysters in the world, eutrophication, overharvesting and the parasites *Haplosporidium nelsoni* and *P. marinus* have caused the Chesapeake Bay oyster population to be reduced to a critically low level (Andrews 1988, Haskin & Andrews 1988, Hargis & Haven 1988). The parasites inhibit growth, reduce fecundity, and lower the oyster's condition and glycogen content (Menzel & Hopkins 1955, Newell 1985, Barber et al. 1988, Crosby & Roberts 1990). Oyster populations that have incurred high infection prevalence and intensities typically have low mortalities during their first year, but suffer higher mortalities in the following years (Paynter & Burreson 1991). The parasite does not have the same drastic effects on the oyster population in the Gulf of Mexico as it does in the Chesapeake Bay. An oyster requires three or more years to reach marketable size in the cooler waters of the Atlantic; however, only two years are required in the warmer waters of the Gulf of Mexico. In the Gulf of Mexico, this parasite infects over 80% of Eastern oysters with annual mortalities typically 50% of the adult oyster population. Transmission of the parasite occurs through the water by release of infective stages from the feces of living oysters, the tissues of dead oysters (Ray 1952, Mackin & Hopkins 1962), and by the gastropod ectoparasitic snail, *Boonea impressa* (White et al. 1987).

Perkinsus marinus has several life stages in the host oyster (Mackin & Boswell 1956, Perkins 1969). These include immature thalli, mature unicellular thalli (trophozoites), and presporangia. When released into seawater, presporangia develop a resistant cell wall, and then enlarge to become hypnospores. Under aerobic conditions, hypnospores differentiate into sporangia and produce motile zoospores (aplanospores in Mackin & Boswell 1956) within the hypnospores cell wall. One sporangium of *P. marinus* is capable of releasing approximately 354,700 zoospores (Chu & Greene 1989). Zoospores are released from hypnospores and undergo free-living stages in seawater.

Eradication of these pathogens in the wild is not possible because of the widespread nature of the diseases and the lack of knowledge regarding other species that might carry the disease (Elston 1990). Resistance to *H. nelsoni*, but not to *P. marinus* (Barber & Mann 1991) has been achieved through selective breeding of *C. virginica* (Ford & Haskin 1987, Ford et al. 1990, Burreson 1991).

Developing and maintaining hatcheries to produce larval oysters for grow out for commercial production or to repopulate depleted areas is one approach to alleviate the lack of natural reproduction. This method, however, requires the incoming seawater to be specific pathogen free. The traditional methods of using ozone and ultrafiltration are expensive for continuous production. Chlorine is an inexpensive alternative for water disinfection; however, its chemistry changes when combined with seawater.

Observations of oyster larvae exposed to chlorine-treated seawater indicate a lethal concentration for 50% of the test organisms (LC 50) for *C. virginica* larvae of 0.005 mg/L free chlorine (Cl+), regardless of whether static or intermittent addition of chlorine was used (Roberts et al. 1975, Bellanca & Bailey 1977, Roberts & Gleeson 1978). Concentrations as low as 0.05 mg/L of bromate, bromoform and chloroform caused some *C. virginica* 48 h larval mortality (Stewart et al. 1979). Galtsoff (1946) noted a 46% decrease in pumping action at a dose of 0.2 mg/L chlorine. He and other workers concluded, however, that chlorine was an effective means for disinfecting shells of contaminated oysters and that the oxidant would not interfere with depuration if chlorine levels were kept at a minimum. Later studies agreed with this finding but cautioned that oysters reduce pumping when chlorine concentrations exceed 0.01 mg/L. At chlorine concentrations above 1.0 mg/L, pumping cannot be maintained; thus, the use of chlorine as an effective means of depuration is limited by the tolerance of the species. The ability of adult shellfish to respond to low concentrations of total residual oxidant and to cease pumping may be

beneficial because it allows the animal to survive chlorine-produced oxidant (CPO) concentrations as high as 10 mg/L for 30 days (Galtsoff 1964). The corresponding decrease or cessation, however, of shell growth and feeding is disadvantageous. The most severe restrictions to chlorine use arise from the formation of chemical compounds from adding this to seawater. Halogenated organic compounds are formed that display complex chemistry. The products of chlorination of seawater are complex and not fully understood (Carpenter & Macalady 1975, Davis & Middaugh 1977, Wong & Davidson 1977, Carpenter et al. 1980). In seawater and brackish water, chlorine replaces some of the bromine in hypobromous acid releasing the bromine cation that is considered the disinfecting compound. Full strength seawater has a bromide ion concentration of 65 mg/L, and chlorine reacts with it to produce hypobromous acid and hypobromite ion. Bromamines and chloramines may be formed in the presence of ammonium ion. For normal seawater of pH 8, the initial products of chlorination are a mixture of hypobromous acid and hypobromite ion that are unstable with respect to decomposition and disproportionation (Macalady et al. 1977).

The N-halamine compounds used in this study were 1,3-dichloro-2,2,5,5-tetramethyl-4-imidazolidinone (DC; dichloro) and 1-chloro-2,2,5,5-tetramethyl-4-imidazolidinone (MC; monochloro). Both compounds were synthesized at Auburn University in the laboratory of S. D. Worley, Department of Chemistry. The compound MC can be produced in the laboratory and as a result of the hydrolysis of the compound DC. The compounds will be marketed by Vanson/HaloSource Corporation, Seattle WA[1]. These compounds are more stable in water and dry storage than free chlorine and other commercial products, such as the hydantoins and isocyanurates (Tsao et al. 1991). The N-halamine compounds do not produce trihalomethanes or react with bromide in seawater and should be more stable and more effective than free chlorine. The compound DC is the faster acting compound and the amide N-Cl moiety is more labile than the amine N-Cl group, providing a small amount of free chlorine. The hydrolysis decomposition product MC, having only the more stable amine N-Cl moiety, acts more slowly as a disinfectant.

In this series of experiments, the parasites were exposed to the chemical compounds in sterile artificial seawater (SASW) to determine the effectivity of the compounds. A related compound, 3-chloro-4,4-dimethyl-2-oxazolidinone, has been shown to kill *Giardia lamblia* more effectively than free chlorine (Kong et al. 1988), and it was speculated that DC or MC would penetrate oyster tissues and the thick parasite walls at a reduced level of chlorine.

A previous study using *Anadara trapezia* (blood cockles) and *Haliotis laevigata* (greenlip abalone) showed that free prezoosporangia of *Perkinsus* sp. (removed from oyster tissues) died within 30 min in chlorine solutions of 40 mg/L (Goggin et al. 1990); however, within tissues the parasites presumably are more protected and survived at least 2 h. Their study was concerned primarily with disinfecting meats of abalone. The objective of this study is to determine if the parasite *P. marinus* could be eliminated in the water column. The possibility of controlling *P. marinus* in an oyster hatchery by treating incoming water, or as an interim control preventing the spread of the parasite between oysters, could mean economic gains associated with increased health and growth characteristics.

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[1] Use of trade or manufacture's name does not imply endorsement.

METHODS

A series of three experiments were conducted to evaluate the effectiveness of these compounds on *P. marinus*.

Perkinsus marinus cultures were obtained from the American Type Culture Collection (ATCC), and cultured according to La Peyre and Faisal (1995). In experiment one, an aliquot was removed from culture, vortexed briefly to break up cell clumps, and then centrifuged at 500g for 5 min. These cells were rinsed twice with 15 ppt sterile artificial seawater (SASW), then resuspended in SASW at a concentration of approximately 5×10^5 cells mL^{-1}. The chemicals DC and MC, which were synthesized according to the method of Tsao et al. (1991), were prepared in three concentrations: 0.3, 14.9 and 29.8 mg/L and 0.5, 24.9 and 49.8 mg/L, respectively. These concentrations are based on molar equivalents of chlorine. Four replications of each chemical at each concentration were prepared in sterile, 50 mL, polypropylene centrifuge tubes. Approximately 5000 parasites were added to tubes containing 50 mL of each chemical concentration. The same amount of SASW with and without parasites served as the positive and negative controls. Contact time consisted of eight time intervals: 0.5, 1, 2, 4, 8, 12, 18, 24, and 48 h. At the appropriate time, the samples were mixed and 1 mL removed from each tube. Sodium thiosulfate (0.02 N) was added to neutralize the chlorine (i.e., to quench disinfecting action) and the cells were observed microscopically at ×100 with and without staining with Lugols Iodine.

A second experiment was initiated to determine the percent mortality at various concentrations and time intervals using a vital dye, trypan blue, which distinguishes between living and dead cells. This viability test evaluates the breakdown of membrane integrity determined by the uptake of the dye to which the cell is normally impermeable. Cell and chemical preparation was the same as previously described. Contact time consisted of three time intervals: 1, 2, and 8 h. At the appropriate time, the samples were mixed and 1 mL removed from each tube. The cells were washed with Hanks Balanced Salts Solution (HBSS) (Sigma, St. Louis, MO) and resuspended in 0.3 mL HBSS to which 0.5 mL trypan blue was added. The cell suspension was mixed and allowed to stand at room temperature for 5–15 min. Living and dead cells were counted and enumerated using a hemacytometer at ×100. Dead cells stained a dark blue, but living cells were able to exclude the dye. Cells with an intermediate blue color stain were considered dead.

A third experiment was performed to determine the viability of the cells after exposure to the two chemicals, targeting the cells that lightly stained indicating damage to the membrane. It was important to know whether these damaged cells would be able to recover and initiate a new infection.

Cells were removed from culture, centrifuged to pellet the parasites then resuspended in SASW. Four concentrations of DC (7.4, 14.9, 29.8, 44.6 mg/L) and 4 concentrations of MC (12.9, 24.9, 49.8, 76.6 mg/L) were prepared in sterile, polypropylene centrifuge tubes, and then 2 mL were transferred to individual wells of tissue culture plates. Three replications of each chemical concentration were prepared. Approximately 20 µL of the *P. marinus* (4.5×10^4 parasites mL^{-1}) cell suspension were added to each disinfectant chemical. The same amount of SASW with and without parasites was added to the positive and negative controls. Contact time consisted of four time intervals: 1, 2, 8, and 12 h. At the appropriate time, the chlorine in the samples was neutralized with 20 µL of 0.02 N sodium thiosulfate and the cells resuspended

TABLE 1.

Experiment 2: the effect of DC and MC concentration and exposure time on mortality of *P. marinus*.

mg/L	% Staining		
	1 hour	2 hours	8 hours
DC 0.3	0.3	1.3	12.9
DC 14.9	11.4	77.4	88.2
DC 29.8	80.0	80.8	99.8
MC 0.5	0	0.2	0.2
MC 24.9	3.1	16.3	16.0
MC 49.8	19.2	22.9	25.0

TABLE 2.

Experiment 3: the effect of DC and MC concentration and exposure time on mortality and replication of *P. marinus*.

mg/L	% Staining			
	1 hour	2 hours	8 hours	12 hours
DC 7.4	4.1	11.0	6.9	13.4
DC 14.9	12.7	34.8	83.0[a]	33.0[a]
DC 29.8	10.4	29.8	36.0[a]	98.6[a]
DC 44.6	98.1[a]	99.6[a]	100[a]	100[a]
MC 12.4	0	3.4	6.2	7.1
MC 24.9	10.9	14.3	20.7	70.0[a]
MC 49.8	17.2	14.6	82.0[a]	87.2[a]
MC 76.6	0	0	98.6[a]	90.3[a]

[a] Indicates cultures in which all parasites died without producing viable offspring when observed 48 hours after chemical treatment.

in 3 mL of culture media. A portion of the cell suspension was removed and evaluated with typan blue staining as previously described. The remainder of the samples were incubated in the dark at 25°C and evaluated at 24 and 48 h.

RESULTS

In the first experiment, no visible effects on *P. marinus* were observed for DC or MC treatments at any tested concentration up to 4 h. At 8 h exposure to either DC or MC, all parasite cells appeared to decrease in size, and at 18 h all cells were completely lysed at all concentrations. The negative controls appeared free of debris and bacterial contamination during the test. The positive controls appeared unchanged and did not exhibit any decrease in size, nor did they lyse.

The second experiment attempted to refine the earlier one by determining viability at various contact times. The viability of the cells exposed to DC has been reduced by 80% at 1 h at a concentration of 29.8 mg/L (Table 1). At a concentration of 49.8 mg/L MC at 1 h, a reduction of only 19.2% was observed. At the end of 8 h, 99.8% mortality was observed at 29.8 mg/L DC, as compared with 25% with 49.8% MC.

In the study addressing the viability and the ability of the parasite to recover from exposure to the DC and MC compounds showed a trend towards more rapid deactivation of the parasites by DC as compared with MC, at similar concentrations (Table 2). Cells in the positive control treatment exhibited normal growth and development.

DISCUSSION

Results of this study demonstrated that the compounds MC and DC eliminated the pathogen *P. marinus* in 15 ppt seawater under laboratory conditions. It is important to kill all parasites because a single sporangium of *P. marinus* is capable of releasing approximately 354,700 zoospores (Chu & Greene 1989).

Mortalities of 100% of *P. marinus* can be achieved using the faster acting chemical DC at concentrations of 14.9 mg/ for 8 or 12 h, 29.8 mg/L for 8–12 h or 44.6 mg/L for a minimum of 1 hour.

The slower acting chemical MC can achieve 100% mortality at concentrations of 24.9 mg/L for 12 h, 49.8 mg/L for 8 or 12 h, or 76.6 mg/L for 8–12 h. Additional testing would be desirable to determine lower concentration effectivity against this pathogen.

Both DC and MC are effective against the oyster parasite *P. marinus in vitro* at concentrations less than the estimated LD_{96} of the oyster larvae exposed to these same chemicals (Delaney et al. 2002). Histologic and physiologic information would be required on the long term effects of chemical exposure to oyster larvae; however, either compound has the potential to be used in oyster hatcheries to prevent infections of *P. marinus* from occurring, or to prevent the spread of the disease through the water column if the contact time is sufficient. Electron microscopy would provide additional insight on the mechanism of damage to the parasite's cell walls at different stages in the life cycle of the parasite.

N-halamines DC and MC at concentrations of total chlorine within the larval and adult oysters range of tolerance, are effective for the control of a protozoan pathogen, *P. marinus*, of Eastern oysters. These compounds have the potential to be used in oyster hatcheries and in recirculating based systems to produce specific pathogen free oysters. The use of these compounds as a substitute for free chlorine or chloramines would mitigate deleterious physiologic effects currently observed on oyster recruitment and survival in estuaries receiving chlorinated discharges.

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HATCHERY REARING OF THE BLACK SCALLOP, *CHLAMYS VARIA* (L.)

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ABSTRACT This work describes methods used for conditioning, spawning, and growing larvae of *Chlamys varia* in hatcheries and the results obtained. Conditioning in winter results in fast ripening. Oocytes are easily obtained by injecting serotonin. Different antibiotics were tested and the results compared. Different systems for setting were compared. *C. varia* prefers flat surfaces rather than monofilament as settlement substrate.

KEY WORDS: *Chlamys varia*, hatchery, conditioning, spawning, larval culture, settlement, antibiotics

INTRODUCTION

Worldwide production of pectinids has increased spectacularly in recent years, rising from 200,000 t in 1970 to 1.7 million t in 1996. The rise is largely the result of an increase in production of these shellfish by aquaculture, which accounts for 90% of the total production (Bourne 2000).

In Spain, as in the rest of Atlantic Europe, *Pecten maximus* is the most commercially valuable of the pectinid species exploited; however, experiments have recently been performed to assess the possibility of cultivating smaller pectinids, such as *Aequipecten opercularis* (Román et al. 1999) and *Chlamys varia* (Acosta & Alvarez 1990, Acosta et al. 1990, Román 1991).

Chlamys varia is found in the eastern Atlantic, ranging from southern Norway to Senegal and also in the Mediterranean (Ansell et al. 1991, Brand 1991). It displays rhythmic consecutive hermaphroditism; most younger/smaller specimens are males that undergo a gradual sex change so that most older animals are females (Lubet 1956, Lucas 1965, Reddiah 1962, Burnell 1983).

This species is relatively scarce in Spain. It is therefore rarely sold commercially, and there is very little information available about its biology and ecology. However, the potential for culturing the species in Galicia is presently being considered. Methods of obtaining gametes have been determined (Román & Fernández 1990), and spat have been cultivated in suspension from rafts (Acosta et al. 1990); Parada et al. (1993) provide information on the reproduction of *C. varia* cultivated in suspension. In Galicia the use of collectors to capture spat in natural environments has proven unsuccessful (Román et al. 1987, Ramonell et al. 1990) and therefore spat production must be conducted in hatcheries. Hatchery cultivation of this species has been described by Burnell (1983), Le Pennec and Dis-Menguss (1985, 1987), Acosta and Alvarez (1990), and Román (1991).

The aims of the present study were to investigate (1) the larval behavior of *Chlamys varia* under the standard conditions established at the Centro Oceanográfico de A Coruña (COAC), for the culture of *P. maximus* larvae, as summarized below; (2) the effect of different antibiotics on larval growth and survival; and (3) the behavior of the larvae at settlement, with the aim of optimizing the culture methods to increase the yield of spat.

At the COAC, culture of *P. maximus* larvae has been conducted intermittently since 1976, with some modifications to the tech-niques described by Román and Pérez (1979) and Román (1986, 1991).

The use of antibiotics in larval cultures is controversial. In general in Europe pectinid larvae cannot be consistently cultivated without chloramphenicol (González & Román 1983, Samain et al. 1992, Torkildsen et al. 2000), the use of which is presently prohibited by the EU. Other antibiotics must therefore be used commercially.

During settlement of pectinid larvae, mesh bottomed cylinders, or collectors made of different materials are often used. Pearce and Bourget (1996) have reviewed the use of different materials for the settlement of competent spat of various pectinid species, although no reference is made to hatchery rearing of *C. varia*. Only Rodhouse and Burnell (1979) mention settlement preferences of *C. varia* on undersurfaces or on shaded areas in sections, of PVC slats, in laboratory experiments.

MATERIALS AND METHODS

Conditioning

Adult *C. varia* of between 30 and 50 mm in height were transported from the sea to the COAC and conditioned from the end of December 2000 until March 2001. The trial started when scallops were totally spent. Scallops were placed in tanks ($180 \times 50 \times 30$ cm), through which sea water flowed at a rate of 6 L min^{-1} at ambient temperature (12–14°C). An average number of 21.8×10^9 cells day^{-1} of *Skeletonema costatum*, 13.7×10^9 cells day^{-1} of Tahitian *Isochrysis aff. galbana* and 14.0×10^9 cells day^{-1} of *Pavlova lutheri* were added to the circulating sea water using a dosing pump for density of 183 cells/μL. Males and females were kept separately after their sex was established by microscopic examination of gonad samples taken by needle puncture.

Stimulation

When females were observed to have well-developed gonads (swollen appearance and color range between white, cream or yellow), they were injected intramuscularly with 0.2 mL of 0.2 mM serotonin (Román & Fernández 1990). Once spawning began, 8 to 10 males were injected, and the sperm suspension from various specimens was mixed. The oocytes were sieved (100-μm mesh) to remove large particles and feces. The number of oocytes shed by each female was counted using a 1-mL Gallemkamp counting cell (Sedgewik-rafter S50). Sperm suspension was added to the containers in which the oocytes were held, so that there were approximately five sperm per oocyte (Gruffydd & Beaumont 1970).

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Incubation

Incubations were performed in 150-L conical-bottomed fiberglass tanks containing 0.45-μm filtered sea water at 16–18°C with slight aeration, for 3 days. Food was added on the second day (25 cells μL⁻¹ of a 1:1 mixture of Tahitian *I.* aff. *galbana* and *P. lutheri*) and on the third day the tanks were emptied and the larvae collected in 60-μm mesh sieves. Larvae of normal appearance were counted and the hatch yield was calculated. Three ranges of incubation density (<6; 6–10; >10 eggs/mL) was tested.

Larval Culture

Larvae were cultured in 150-L tanks containing 0.45-μm filtered sea water at ambient temperature (16–18°C) at an initial density between 0.5 and 8 larvae mL⁻¹; 8 mg L⁻¹ of chloramphenicol was added, and a mixed diet of 50 cells μL⁻¹ of Tahitian *I.* aff. *galbana*, and *P. lutheri* (1:1) was provided. The water was changed three times a week and the mesh size of the sieve used to retain the larvae was increased depending on the size of the larvae; each time the water was changed a sample of larvae retained was measured. Larvae reached a final density of less than 1 larva mL⁻¹ at the time of settlement. When competent pediveliger larvae appeared, the culture was 140-μm mesh sieved. If the number of pediveligers with eye spots was greater than 50%, they were placed in settlement systems.

Effect of Different Antibiotics

Larvae were cultivated at three different treatments: chloramphenicol (8 mg L⁻¹), penicillin plus streptomycin (30 mg L⁻¹ + 50 mg L⁻¹), and erythromycin (8 mg L⁻¹) and no antibiotic as control from hatching until settlement. The number of settled larvae was counted for each treatment. All treatments were carried out in duplicate.

Larval Settlement Systems

Three trials were performed with *C. varia* using two settlement systems, i.e., the traditional and the modified system. These two settlement systems were compared in the first experiment. The traditional system, consisting of a PVC cylinder that was 43 cm in diameter and 40 cm in height with a 140-μm mesh base through which water was circulated in an upwelling system, was placed in a 150-L tank. The method developed at the COAC (modified system) using artificial seaweed as a settlement substrate was prepared in another tank of the same size. A total of 172,500 pediveliger larvae were added to each tank. Water was changed by displacement. Food was added daily according to larval culture and *S. costatum* was included in the diet.

The effect of the substrate and the density of pediveligers on settlement was investigated in a second trial. The traditional system was used, but with a settlement substrate also provided. Nine 140-μm mesh-bottomed cylinders 25 cm in diameter and 19 cm in height (1983 cm² internal surface area) were placed in 200-L capacity tanks (180 × 50 × 30 cm). Three larval densities (10,000, 20,000, and 30,000 larvae/mL) and two settlement substrates (nylon monofilament, artificial seaweed, no substrate control) were used.

In the third trial, different settlement substrates were tested. For this, collectors comprising of artificial seaweed, nylon monofila-

ment filling and scallop shells were placed in a 400-L tank along with 312,125 pediveliger larvae. The numbers of spat on each substrate and on the tank walls were determined after approximately 45 days.

RESULTS

Conditioning

After 6 or 7 wk on the conditioning system, scallops were observed to have swollen gonads, from which viable gametes were obtained after stimulation of spawning.

Stimulation

Scallops were artificially stimulated by serotonin injection, in January, February, and March, and gametes were obtained on each occasion. A total of 58.3% of the females and 80.0% of the males responded to serotonin stimulation. The time needed to obtain sperm and oocytes ranged between 7 and 43 min and 9 and 52 min, respectively. An average number of 0.6×10^6 (range: 0.05×10^6–$2.4 - 10^6$, $n = 16$) oocytes were obtained from each female; the mean diameter of the oocytes was 68.8 μm ± 1.9 (SD).

Incubation

Incubation yields for three eggs density ranks were 25.5% (0–5 eggs/mL, $n = 11$); 34.1% (5–10 eggs/mL, $n = 5$); and 31.8% (>10% eggs/mL, $n = 6$). Statistical differences were not found between them (analysis of variance, $P > 0.05$). Mean size of larvae D obtained was 110.28 μm ± 2.61.

Standard Culture

Larval development (until 50% of the larvae developed eye spots) lasted an average of 19.3 days ± 2.0 ($n = 16$); 8 days after the spawning (larvae size = 134 μm ± 1 a purple spot, which is characteristic of this species, appeared on the dorsal posterior region of the larvae. Although larvae with eye spots may appear after 13 days, the proportion did not reach 20% until day 17 (larvae size = 194.1 μm ± 13.1). At the end of the culture period, the average yield of pediveliger larvae was 31.2 ± 17% (larvae size = 211.8
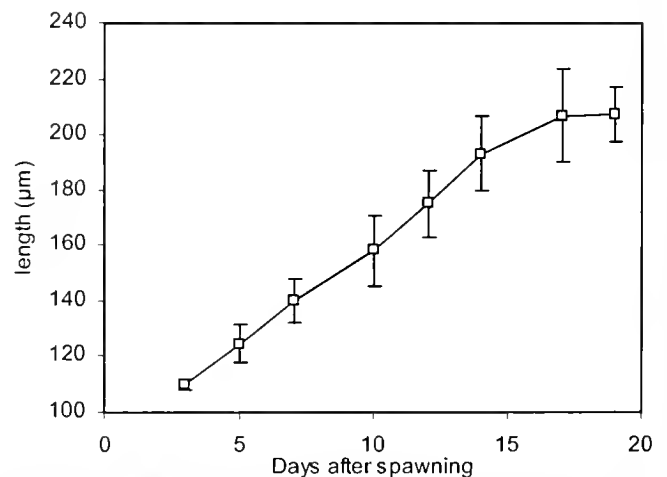
Figure 1. Larval growth of *Chlamys varia* (mean ± SD of 16 larval cultures).

TABLE 1.

Effect of different antibiotics on larval yields.

	Percent Pediveliger	Percent Settlement
Cloramphenicol	75.2 ± 5.1	10.1 ± 4.9
Penicillin + streptomycin	71.7 ± 9.3	10.8 ± 1.4
Erythromycin	83.6 ± 4.5	10.0 ± 3.3
Control	78.0 ± 8.3	1.7 ± 1.0

μm ± 9.9). The rate of growth from hatching until the final day of culture was 5.3 μm day^{-1} (Fig. 1).

Effect of Different Antibiotics

The percentage survival of the larvae, at the time of recording 50% with eye spots, exceeded 70% in all treatments, including the control in which no antibiotics were used (Table 1). However, during settlement, 1.7% larvae settled compared >10% for the antibiotic treatments.

Settlement

First Trial

Similar spats settlement was recorded in the tanks in which artificial seaweed and mesh bottomed PVC cylinders were used (30.1% and 30.6%, respectively) and 51,907 and 52,700 spat were obtained, respectively. More spat settled on the sides of the cylinder than on the mesh bottom. In the tank containing artificial seaweed, most spat settled on the walls of the tank. Although the spat on each substrate were not counted, there was a marked preference for vertical walls in both cases.

Second Trial

Effect of substrate and density of pediveligers on settlement. The number of spat settled in each cylinder was determined, the numbers that settled on the walls and the substrates provided were counted separately. The results are shown in Table 2. Most of settlement took place on the walls.

Third Trial

Settlement in 400 L capacity tank with various substrates. The results are showed at Table 3. A total of 19.7% spat

settled were recorded. The higher settlement was on tank walls (12.7%) with preference on bottom (Table 3).

DISCUSSION

Cultivation of *C. varia* larvae was performed using the techniques developed over several years at the COAC for cultivating *P. maximus* (Román, unpublished data). However, *C. varia* behaves differently from *P. maximus*. The most important differences were associated with settlement and effect of antibiotics. At the COAC, *P. maximus* larvae have not been successfully cultivated without antibiotics (González & Román 1983, Ruiz 1996), and to date, artificial seaweed has been found to be the best settlement substrate for this species (Román, personal communication). In contrast, *C. varia* can be cultivated to pediveliger successfully without antibiotics and artificial seaweed was not a particularly good settlement substrate for this species, the larvae preferring to settle on the tank walls.

Part of the standard cultivation method of *C. varia* involves discarding batches in which the oocytes are not spherical or in which there is a low hatching rate (<10%). Not all times of the year are suitable for obtaining good quality larvae and hatcheries do not have unlimited space, therefore when larvae are available the best possible production rates must be obtained. Early removal of batches of poor quality larvae allows culture of other batches obtained from different spawns. With this method, time and money are saved and better average yields are obtained, as cultures that would probably die are eliminated.

Conditioning of *C. varia* during the winter months allows viable gametes to be obtained from January onwards, thereby bringing forward the natural spawning times, which usually take place in spring and early summer (Parada et al. 1993). Unlike other pectinid species that have been cultivated at the COAC (*P. maximus, P. jacobaeus,* and *Aequipecten opercularis*) *C. varia* matures quickly during the conditioning period (4–5 wk) and gametes are obtained using serotonin, allowing the timing of the larval cultures to be planned. Furthermore, there is no risk of self-fertilization and polyspermy is easily avoided.

The result of the response of *C. varia* to stimulation by serotonin was similar to those described by Román and Fernández (1990) although complete emptying of the gonads was not always observed in this study.

The average number of oocytes per female obtained in the present study (0.6×10^6, maximum 2.4×10^6) was less than those previously reported: 1.54×10^6 (Román & Fernández 1990), 4.5×10^6 (Le Pennec & Diss-Mengus 1985) and 5×10^6 (Burnell 1983).

TABLE 2.

Effect of larval density and settlement substrates on yield of spat of *C. varia* (Trial 2).

		Number of Pediveligers		
Settlement Substrate Provided	Percent of Settlement	10,000	20,000	30,000
Control (mesh bottomed cylinder only)	Cylinder (%)	35.1	52.3	48.3
Mesh bottomed cylinder + monofilament	Cylinder (%)	32.2	41.4	19.1
	Monofilament (%)	1.5	9.2	4.1
	Total (%)	33.7	50.6	23.2
Mesh bottomed cylinder + artificial seaweed	Cylinder (%)	9.1	37.7	13.8
	Artificial seaweed	9.9	20.8	8.4
	Total (%)	19.0	58.5	22.2

TABLE 3.

Effect of settlement substrates on yield of spat of *C. varia* (Trial 3).

Collector Substrate	No. of Spat	Percent Settlement
Tank walls	39500	12.7
Standard net filling	10455	3.3
Scallop shell	9722	3.1
Artificial seaweed	1885	0.6
Total		19.7

However, these authors used larger adult stock than in the present study (30–50 mm; Román and Fernández used specimens of between 50–75 mm and Burnell, specimens >50 mm).

The mean diameter of the oocytes was similar (average range, 68–72 μm) to those found by Burnell (1983; 65–70 μm) but larger than those found by Le Pennec and Diss-Mengus (1985; 50–60 μm).

The density of eggs incubated did not appear to affect the yield of larvae. This is consistent with the results of Román and Fernández (1990), who found no significant effect of density (using between 1 and 50 eggs mL^{-1}) on the yields. O'Connor and Heasman (1995) obtained yields of up to 40% with cultures of *C. asperrima* using a density of 100 eggs mL^{-1} and 48% with a density of 1 egg mL^{-1}. Le Pennec and Diss-Mengus (1987) obtained hatching yields of 77.7% after a period of incubation of 2 days (density 2.3 eggs mL^{-1}), after which D larvae of 90 μm were collected (using sieves of mesh size 43 μm). Román and Fernández (1990) also incubated the eggs for 48 h and obtained a yields of 17.9%. O'Connor and Heasman (1995) reported that 54% of *C. asperrima* eggs hatched, and veliger larvae were obtained, following 2 days incubation. With the culture technique developed at the COAC, larvae were incubated for 3 days, then 60 μm mesh sieves were used to remove small or abnormal larvae. Although the yield of D larvae (29.2%) was lower than that reported by the authors mentioned above, better results were subsequently obtained because dead or abnormal larvae, which usually appear at the end of the incubation period, have already been removed.

The duration of the larval period of *C. varia* has been reported as 22 days at 18°C (Burnell 1983), 19 days at 16–18°C (present study and Acosta & Álvarez 1990), and 15 days at 17°C (Le Pennec & Diss-Mengus 1985).

The characteristic purple spot that occurs in this species, has been reported to appear at different ages and in different sizes of larvae: on day 4, in larvae of 120 μm (Le Pennec & Diss-Menguss, 1985); on days 10–12, in larvae of 130–140 μm (Burnell, 1983); and on day 8, in larvae of 134 μm, (present study).

Larvae with eye spots appeared from day 13 onwards. In the present study, 20% of the larvae had eye spots on day 17 (average size of larvae, 194.1 μm). Acosta and Álvarez (1990) detected the pigmentation on day 14 (161.7 μm), whereas Burnell (1983) detected it in 20-day-old larvae (200 μm).

Similar growth rates have been reported: 5.3 μm day^{-1} (present study), 4.8 μm day^{-1} (Acosta & Álvarez 1990), and 5.3 μm day^{-1} (Burnell, 1983), all of which are much lower than that reported by Le Pennec and Diss-Mengus (1987; 10 μm day^{-1}).

The larval culture yield obtained (31.2% pediveliger larvae, average size 211.8 μm) was lower than those obtained by Le Pennec and Diss-Mengus (1985, 1987; of between 65.5% and 70%, of larvae of 210 μm). Using the same conditions, Burnell (1985) did not obtain more than 4% survival of larvae of size 215 μm.

Despite the fact that few studies have been made of this species, there is considerable variation in the results obtained by different authors. This may be because of genetic differences or more probably, to different culture conditions, such as the quality of the gametes or the diet. De la Roche (pers. com.) cultivated *C.varia* larvae obtained from adults originating from Málaga and from Galicia simultaneously and did not observe any differences in the diameter of the oocytes, the age and size at which the pigmented mark appeared, size at the time of appearance of the eye spot or growth rate. Of the studies compared, the best results (in terms of growth rate and yields), were obtained by Le Pennec and Diss-Mengus (1985, 1987), possibly because of the diet provided, which included diatoms, and to better conditioning conditions.

It appears that antibiotics are necessary for successful cultivation of pectinid larvae but not all give good results. Chloramphenicol appears to give the most consistent results. Uriarte et al. (2001) reported higher growth and survival rates in *Argopecten purpuratus* using chloramphenicol at doses of 2 and 8 mg L^{-1} than without the antibiotic. Mendes et al. (2001) obtained survival rates of 20–25% in cultures of *Nodipecten nodosus* using cloramphenicol, in contrast with almost total mortality on using florphenicol. Ruiz (1996) reported high mortality in *Pecten maximus* larvae cultured with erythromycin and high rates of survival with tetracycline and trimethoprim plus sulphamethoxazole. González and Román (1983) reported no yield of *Pecten maximus* larvae cultured without antibiotics, in contrast to cultures in which chloramphenical was used at a concentration of 2.5 mg L^{-1}. Samain et al. (1992) found much higher survival and growth rates employing antibiotics and Torkildsen et al. (2000) obtained larval yields of 30% when chloramphenicol was added to the cultures.

The percentage of settlement was variable in the different cultures [30% (trial 1), between 19 and 58% (trial 2), and 20% (trial 3); approximately 10% in the cultures conducted with different antibiotics]. This variability may have been due to intrinsic factors, but there were also variations within the same culture batches, depending on the quality of the substrates provided (extrinsic factors). It is clear that *C. varia* prefers to settle on the tank walls than on nylon monofilament. O'Connor and Heasman (1994) found that *Chlamys asperrima* also preferred the tank bottom and walls to the collectors provided for settlement. *C. varia* showed a preference for the more sheltered, poorly lit areas of the collectors (Rodhouse & Burnell 1979). However, in experiment 3 of the present study, we found a very low settlement rate on the scallop shells, despite the fact that they were hung with the concave part of the shells facing downwards, an arrangement which should have provided the most sheltered conditions in the tank.

Although improvements in conditioning (quality of gametes), larval diet and the substrate and settlement conditions must be made, hatchery culture of *C. varia* larvae is possible, and commercially viable numbers of spat can be obtained, which would allow development of an industry dedicated to the production of this species.

ACKNOWLEDGMENTS

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EFFECT OF DEPLOYMENT DATE AND ENVIRONMENTAL CONDITIONS ON GROWTH RATE AND RETRIEVAL OF HATCHERY-REARED SEA SCALLOPS, *PLACOPECTEN MAGELLANICUS* (GMELIN, 1791), AT A SEA-BASED NURSERY

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ABSTRACT The effect of date of deployment on growth and subsequent retrieval of hatchery-reared scallop spat from a land-based hatchery to a sea-based nursery was studied to provide information for management of juvenile-size scallops, ranging from 1.4–7.0 mm in shell height. The objective of this study was to determine the optimal time period for spat deployment to a sea-based nursery to yield commercially acceptable growth rates and retrieval (scallops remaining after mortality and loss through nets). Spat of the same size class and stocking density were deployed over five consecutive 16–23 day intervals beginning in August 1997. Environmental factors were monitored weekly. Scallops were sampled after each deployment period for determination of shell height and retrieval. Scallops were then re-deployed and sampled before (November) and after (June) the winter season. Results demonstrated that there were significant differences in scallop growth and retrieval among the five consecutive deployments. Only scallops that had been deployed in August were greater than 7 mm by November and could be sorted and transferred to larger mesh equipment for ongrowing prior to winter. The findings of this study demonstrated that early deployment (August) to sea-based nursery yielded high growth rates and retrieval. Deployment later than early September required over-wintering in nursery culture before transfer to ongrowing. Significant correlations were found between both growth rates and retrieval and some of the environmental parameters (e.g., temperature, chlorophyll-a, particulate organic matter). Acclimation to the new farm conditions may be necessary for nursery-sized scallops to adjust physiologically without a major lag in growth following transfer from the hatchery to the sea.

KEY WORDS: growth, nursery culture, *Placopecten magellanicus*, scallop spat, sea star

INTRODUCTION

The aim of a nursery stage in bivalve aquaculture is to foster the development of young postmetamorphic settled animals to an optimal size for ongrowing and handling. For scallops, the nursery stage starts with the transitional period between a planktonic larval phase in a well-maintained hatchery setting and a benthic postlarval phase where the settled spat are deployed to a sea-based nursery or to a semicontrolled land-based growout environment. Sea-based nursery culture can be improved by determining the variation of environmental factors at the nursery site and by manipulating the timing of the deployment of spat to nursery culture to coincide with optimal conditions.

Determining the timing of deployment at the sea-based nursery is necessary to optimize growth rates of hatchery-reared *Patinopecten yessoensis* (Bourne & Hodgson 1991). Spat deployed during optimal food density and temperatures have higher growth rates and survival.

The window of opportunity of deployment to the sea-based nursery can be assessed by determining growth rates and retrieval as functions of measurable natural factors, such as water quality, food availability, and the presence of potential predators over time. When adequate nursery conditions are provided, growth rates and survival are maximal, and the time scallops spend in the nursery stage exposed to other risk factors decreases.

Growth rates of scallops vary seasonally as a result of fluctuations in food supply and temperature (Kirby-Smith & Barber 1974, Vahl 1980, Grecian et al. 2000). Growth rates of cultured *P. magellanicus* are highest in the summer and lowest in the winter

(Dadswell & Parsons 1991, 1992, Côté et al. 1993, Kleinman et al. 1996, Parsons et al. 2002) and show no increase during the autumn bloom compared with summer (Emerson et al. 1994). Sea scallops in some areas of Atlantic Canada are able to naturally produce two cohorts annually of which the summer (June to July) cohort grows faster than the autumn (September to October) cohort over the entire culture period (Dadswell & Parsons 1992). Dadswell and Parsons (1992) proposed that the higher growth rates of the first cohort were caused by the initial exposure of spat to the summer food conditions in the water column and a longer, more favorable period of warmer water. Thus, in bivalve hatcheries and nurseries, the early production of scallop spat is important for deployment to nursery culture early in the summer, as is the practice for oysters. This may result in the growth of scallop spat to a size of 7 mm or greater by the autumn, at which time spat would be large enough to transfer to intermediate culture gear as well as for sale to commercial growers. This growing period is much shorter than waiting until the following summer, which is the current protocol in the sea scallop industry (Dadswell & Parsons 1991, Couturier et al. 1995).

Salinity, temperature, and predation impact survival of scallops. Salinity concentrations below 13 psu and 18 psu cause mass mortality in scallops in short-term and long-term exposures, respectively (Bergman et al. 1996, Frenette & Parsons 2001). As well, sea star predation on scallops can be significant in wild or bottom seeded scallops (Dickie & Medcof 1963, Scheibling et al. 1991, Barbeau & Scheibling 1994a). Sea star predation on scallops is limited in suspended nursery culture gear, unless the nursery gear is deployed prior to the settlement and growth of sea stars (Dadswell & Parsons 1992, Parsons 1994). Survival of post larval scallops, *Pecten maximus*, transferred from hatchery to nursery was dependent on the immersion time during transfer, temperature differential and spat acclimation to the thermal regimen of the

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sea-based nursery (Christophersen 2000, Christophersen & Magnesen 2001).

Timing of deployment of nursery-sized spat at the sea-based nursery is critical for optimizing growth rates and survival. The objective of this study was to determine the window of opportunity for deployment of hatchery-reared sea scallops at a sea-based nursery that enhances growth rates and retrieval and provides availability of spat for intermediate grow-out. Based on previous research on sea scallops, the hypotheses for this study are: (1) growth will be highest in scallops deployed earliest in the summer (August) when temperature and food availability are highest and (2) retrieval of scallops will decline with the onset of sea star settlement.

MATERIALS AND METHODS

Study Site

Scallops were deployed on a scallop farm, Shell Fresh Farms Ltd., based in Poole's Cove, Newfoundland, Canada. The main study site was located in North Bay, head of Fortune Bay, NL at the Ladder Garden lease (47°42'N, 55°26'W).

Experimental Design and Sampling Protocol

This experiment was designed to determine the optimal period for the deployment of nursery-size, post larval scallops at a sea-based nursery. Scallops were deployed over consecutive treatment intervals from the time they were first available from the hatchery and were large enough to be handled (>1.4 mm shell height) until no new cohorts of spat were available in the autumn. The spat were reared at 15°C from several spawnings undertaken at the Belleoram Sea Scallop Hatchery, Belleoram, Newfoundland (47°32'N, 55°25'W). Spat were sorted by screening and those between 1.4 and 2.0 mm in shell height were used in the study. A sample of spat was obtained for initial shell height measurements ($n = 30$) for each deployment.

Scallops were counted and deployed on five occasions at 500 spat/collector in 1.2-mm-mesh collector bags on August 4, August 22, September 7, September 26, and October 19, 1997. Two collector bags, each filled with 1 m of Netron™ (34 g), were held in individual plastic bread trays (69 cm × 57 cm × 15 cm) at a 5 m depth (Grecian et al. 2000). The number of replicate bags varied from two to four depending on scallop spat availability. The initial "short-term" interval duration between successive deployment and retrieval dates ranged from 16 to 23 days and depended on site accessibility. Each short-term deployment interval ended when the next set of collector bags was deployed and the final short-term deployment interval ended on November 8, 1997.

Scallop retrieval (defined as number remaining after mortality and any potential loss through the mesh of the nets) was assessed by counting scallops remaining at the end of each interval and scallops were measured for shell height ($n = 30$). All scallop treatments were then redeployed and again counted and measured for shell height before and after the winter season on November 8, 1997 and June 24, 1998, respectively. During the experiment, all scallop treatments were handled in a similar manner.

Water samples were pumped from a 5-m depth for phytoplankton identification, density and determination of total particulate matter (TPM), particulate inorganic matter (PIM), particulate organic matter (POM), and chlorophyll-a concentration. Temperature and salinity were measured through the water column to a depth of 10-m using a YSI Model No. 30 S-C-T meter. Sea star settlement was also determined (see below). Each parameter was sampled approximately weekly during the short-term intervals (August to November).

Immediately after water samples were collected, the phytoplankton samples were fixed with Lugol's Iodine and 1% formaldehyde. These samples then sat undisturbed for at least two weeks to allow the seston particles to settle. The top 90% of water was siphoned off and its volume was measured. The remaining volume, which contained all settled algal particles, was also measured. This concentrated volume was mixed thoroughly and 10 mL were transferred to a 10-mL Ütermohl settling chamber for overnight settlement. The sample was analyzed visually for total number of cells and species composition using a Zeiss Axiovert 35 microscope under phase contrast at 400× magnification.

The total plankton assemblage was categorized into 8 major groups (McKenzie, 1997). Seven of these were on the basis of size while the final group comprised "unidentified species." The size categories included microzooplankton including tintinnids and ciliates (>20 μm in diameter), autotrophic and heterotrophic dinoflagellates (12 to 60 μm), prymnesiophytes comprising small (2 to 12 μm in diameter) spherical nanoflagellates, auto-nanoflagellates comprising spherical flagellates from 2 to 20 μm in diameter, cryptophytes comprising small (8 to 18 μm in length) tear-drop shaped biflagellates, centric diatoms (12 to 30 μm in diameter, connected in long chains), and pelagic pennate diatoms (30 μm in length, single cells). Phytoplankton were identified according to Rott (1981).

For TPM and chlorophyll-a samples, 15 L of seawater were pumped from a depth of 5 m and pre-screened at 300 μm into separate 20-L buckets and taken to the hatchery. Water samples (4 L) for TPM were filtered onto Whatman GF/C 45-mm diameter glass microfiber filters, which had been previously combusted in a muffle furnace at 500°C for 4 h to remove organic matter and were then weighed. The filters were then stored frozen at −20°C and ultimately oven-dried at 80°C for 24 h, weighed for TPM, transferred to a muffle furnace for 4 h at 500°C, and reweighed to determine PIM. From these weights, ash-free dry weight or POM was calculated according to the formula TPM = POM + PIM.

An additional 4 L of seawater was filtered onto Whatman GF/C filters for chlorophyll-a and pheopigment determination. Filters were frozen (−20°C) for later processing according to the fluorometric methods of Strickland and Parsons (1968) and Parrish et al. (1995).

Sea star settlement was monitored weekly from July 15 to November 8, 1997, by deploying strings of eight empty pearl nets (34-cm × 34-cm square base pyramidal-shaped nets, 6-mm mesh) weekly at the farm with retrieval after approximately two weeks. Individual pearl nets were washed and all material greater than 250 μm was collected on a mesh screen and preserved in 40% methanol. Samples were analyzed using a dissecting microscope for determination of numbers of sea stars present.

Data Analysis

Data were analyzed using the SPSS statistical package (Version 8.0). All percent data were arcsine-square-root transformed before statistical analysis (Sokal & Rohlf, 1995). Differences in growth rates and retrieval were analyzed using an analysis of variance (ANOVA) and the post hoc Tukey's b test was used to test for differences among treatments. Equality of means was analyzed

using an Independent sample t-test. Pearson correlation analyses were also performed on growth and retrieval data with the environmental parameters. The level of significance was set at $\alpha = 0.05$.

RESULTS

Growth Rates

Initial shell height among the replicates was not significantly different for all dates ($P > 0.01$) except September 7 (One-way ANOVA; $F = 9.735$, df $= 2, 87$, $P < 0.001$). This was because the scallops in one of the replicates were from a slow-growing batch of larvae and they were not randomly assigned among the replicates for that date, hence this replicate was not used for further analysis or in figures. The initial mean size ranged from 1.41 to 1.62 mm shell height (Fig. 1).

The mean shell heights of spat at the end of each short-term deployment interval were significantly different from the initial mean shell heights (t-tests, $P < 0.05$, Fig. 1). As well, mean shell heights at the end of each short-term interval were significantly different among the different deployment dates and decreased from 3.54 mm to 1.51 mm shell height (one-way ANOVA; $F = 556.621$, df $= 4, 445$, $P < 0.001$).

Growth rates declined over the short-term intervals (Fig. 2). Significant differences were found among growth rates for the different intervals (one-way ANOVA; $F = 95.162$; df $= 4, 11$, $P < 0.001$). Highest growth rates occurred during the first deployment interval at 118 μm d^{-1} (SE ± 1.3), whereas the lowest growth rates occurred during the last interval at 3.3 μm d^{-1} (SE ± 0.7). The mean growth rate of all spat deployed between August 4 to November 8, 1997 was 43.2 μm d^{-1} (SE ± 0.8).

Growth rates of scallops from the earliest deployment were higher in the autumn and over winter than those from the subsequent deployments (Fig. 3). For scallops deployed on August 4 and 22, growth rates were high until November 8. For the same scallops, growth rates from November to June 24, 1998, declined to a level similar to that of scallops deployed from September 7, 1997. Scallops deployed on September 26 and October 19, had lower overall growth rates to November [11.4 μm d^{-1} (SE ± 1.1) and 3.3 μm d^{-1} (SE ± 0.7), respectively] and to June [21.5 μm d^{-1} (SE ± 1.4) and 7.2 μm d^{-1} (SE ± 1.3), respectively].

Figure 2. Mean growth rates and retrieval of scallops over consecutive deployment intervals at Shell Fresh Farms Ltd., Poole's Cove, NL. The initial date of an interval is the final date of the previous interval. Common letter denotes no significant difference in growth rates or retrieval among intervals (Tukey's b test). Vertical bars are ±SE.

Retrieval

Retrieval of spat at the end of each deployment interval (number remaining after mortality and loss) declined over time (Fig. 2) and was significantly different among the different short-term deployment intervals (one-way ANOVA; $F = 47.129$, df $= 4, 11$, $P < 0.001$). Highest retrieval was obtained from spat deployed during the first interval (97%), whereas lowest retrieval was for spat deployed on September 26 (53%). Retrieval of spat from their initial deployment to November 8 was not significantly different than their retrieval after the short-term intervals (Paired t-test; t $= 0.013$, df $= 14$, $P = 0.990$; Fig. 3).

Environmental Characteristics

Water temperature declined over the deployment periods (August–November, Fig. 4A). Mean temperatures for the five consecutive deployment intervals were 14.7, 13.6, 11.3, 11.2, and 7.9 °C, respectively. Spat were not acclimated from 15°C in the hatchery to ambient seawater temperatures before sea-based deployment. Salinity increased over the study period (Fig. 4A). Mean salinity was 28.3 psu whereas the range was from 26.5 to 31.5 psu.

Chlorophyll-a concentrations (one-way ANOVA; $F = 0.544$, df $= 14, 24$, $P = 0.881$), pheopigment concentrations (one-way

Figure 1. Mean shell height of scallops deployed over five consecutive 2-week intervals in 1997 and on November 8, 1997, and June 24, 1998, at Shell Fresh Farms Ltd., Poole's Cove, NL. The initial date of an interval was the final date of the previous short-term interval. Common letter denotes no significant difference among mean shell heights for each sample period (Tukey's b test). Vertical bars are ±SE.

Figure 3. Mean growth rates and retrieval of scallops deployed at a sea-based nursery at Shell Fresh Farms Ltd., Poole's Cove, NL, on five dates in 1997 and sampled on November 8, 1997, and June 24, 1998. Common letter denotes no significant difference in growth rates or retrieval among intervals (Tukey's b test). Vertical bars are ±SE.
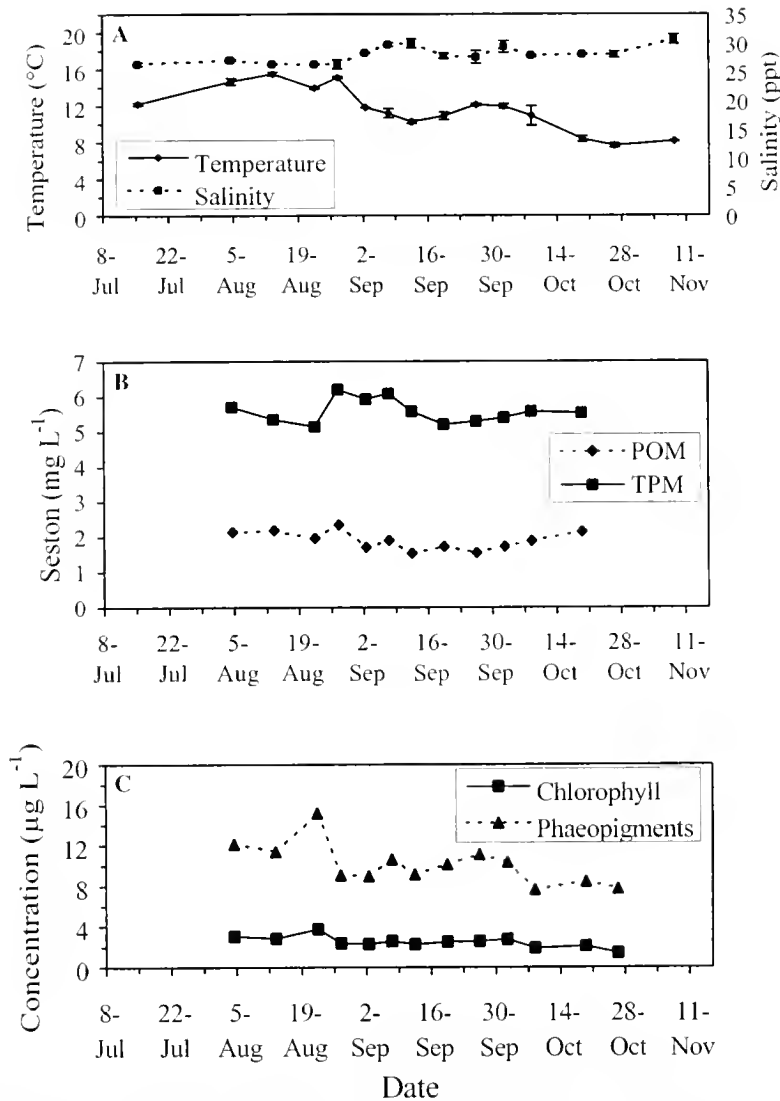
Figure 4. Water quality at Ladder Garden site, Shell Fresh Farms Ltd., Poole's Cove, NL, from July 15 to November 8, 1997. A) Temperature and salinity (±SE; $n = 3$), B) seston, C) chlorophyll and pheopigments at 5 m. (TPM, total particulate matter; POM, particulate organic matter).

ANOVA; $F = 0.500$, df $= 14, 24$, $P = 0.910$), and POM (one-way ANOVA; $F = 0.715$, df $= 14, 21$, $P = 0.737$) were not significantly different over the duration of the study.

TPM remained constant at Ladder Garden (Fig. 4B) with weekly mean TPM being 5.6 mg L^{-1}. POM was also constant at Ladder Garden with a mean of 1.9 mg L^{-1}. Chlorophyll-a and pheopigments averaged 2.4 and 10.1 mg L^{-1}, respectively (Fig. 4C).

There was a significant difference in total phytoplankton density among the weekly samples (one-way ANOVA; $F = 7.084$, df $= 13, 28$, $P < 0.001$; Fig. 5). The total phytoplankton density peaked around the middle of August, followed by a decline. The decline was also evident when the mean total phytoplankton density was calculated for each interval (Fig. 6). The autotrophic nanoflagellates, pelagic pennate diatoms, and dinoflagellates were the numerically dominant groups present (Fig. 7A and B). The species that contributed to the peak abundance were *Navicula* sp., *Chlamydomonas* sp., *Ochromonas* sp., *Micromonas* sp. (Fig. 8A and B). Percent abundance of phytoplankton size groups indicated that species <5 μm had the greatest contribution to phytoplankton biovolume (Fig. 9).

Sea star settlement at the Ladder Garden site peaked between September 19 and October 23 (Fig. 10). There were significant differences in sea star settlement over the different sampling dates (ANOVA; $F = 99.674$, df $= 13, 336$, $P < 0.001$). Maximum
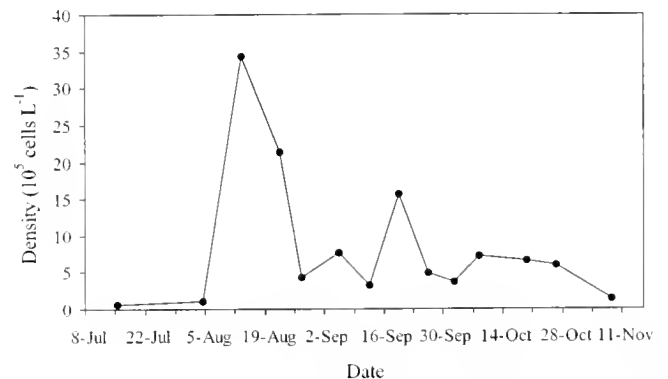
Figure 5. Total phytoplankton density at Ladder Garden site of Shell Fresh Farm, Poole's Cove, NL, from July 15 to November 8, 1997.
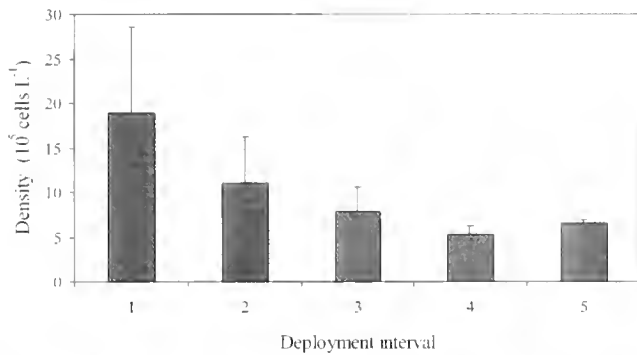
Figure 6. Mean density of total phytoplankton over five intervals of scallop deployment on a sea-based nursery at Shell Fresh Farm, Poole's Cove, NL. Intervals began on August 4 and ended on November 8, 1997. Vertical bars are ±SE.

settlement was 310 sea stars per collector per day and mean sea star settlement was 79 sea stars per collector per day.

Most environmental factors were highly correlated with growth rates and retrieval (Tables 1 and 2). TPM and dinoflagellates were not correlated with growth rates and TPM and PIM were not correlated with retrievals.

DISCUSSION

Effects of Deployment Date on Growth Rates and Retrieval

The date of transfer or deployment of scallop spat from hatchery to nursery was a useful predictor of growth and retrieval. The higher growth rates and retrievals in the earlier deployments were related to several parameters in this study, where ambient tem-
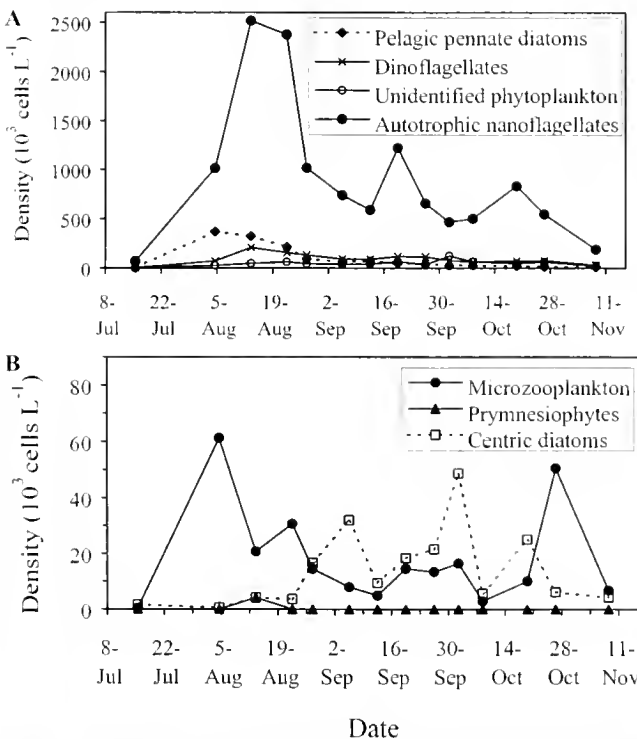
Figure 7. Mean density of "(A)" four dominant and "(B)" three less dominant groups of major plankton at Shell Fresh Farms Ltd., Poole's Cove, NL, from July 15 to November 8, 1997.
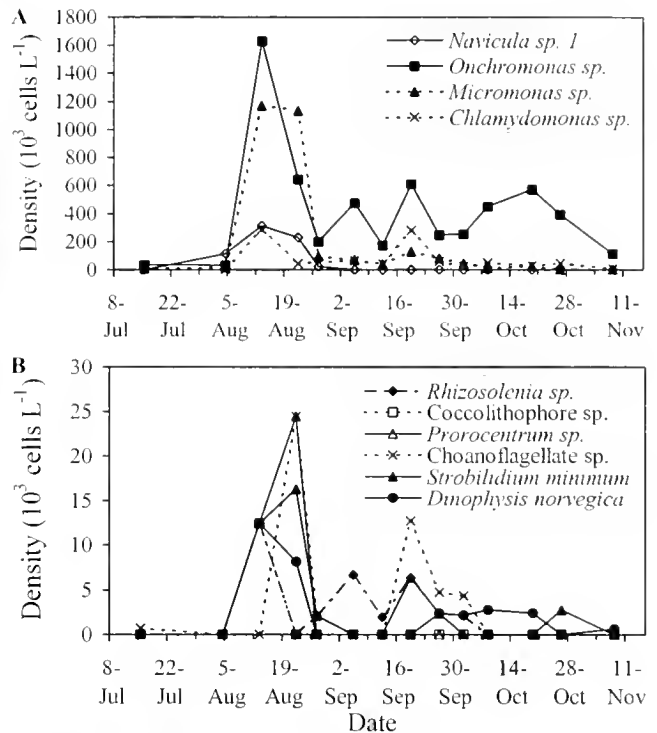
Figure 8. Mean density of "(A)" dominant and "(B)" less dominant plankton species that showed a declining trend over intervals of scallop deployment at a sea-based nursery at Shell Fresh Farms Ltd., Poole's Cove, NL, from July 15 to November 8, 1997.

perature and food availability and quality (species composition, organic content and lipid characteristics inferred from literature reports) were higher initially, then declined after early August. Predator (sea star) abundance peaked near the second deployment date before declining. Spat growth and retrieval from the initial deployment demonstrated that there is an optimum time or window of opportunity, which could be used to maximize nursery growth. After this period, scallops face increasing adversity in terms of declining temperature and food quantity and quality, and increasing predation and temperature shock (the difference between hatchery and ambient temperatures). In a similar study in Southern Norway, *Pecten maximus* spat transferred from hatchery to sea-based nursery from March to August showed increased growth and survival during the summer when water temperatures were >10°C and when temperature differences between the hatchery and nursery were minimal (Christophersen & Magnesen, 2001).

Temperature and food availability declined from August to November while sea star settlement began in mid-September. Variations in temperature and food availability were similar to those found in other areas of Atlantic Canada, including Conception Bay, NL, and Bedford Basin, NS (Mayzaud et al. 1989, Navarro & Thompson 1995). In earlier studies of sea scallop aquaculture, temperature and food availability were the main predictors of growth (Parsons & Dadswell 1992, Côté et al. 1993, Emerson et al. 1994, Kleinman et al. 1996). Likewise, sea stars are a significant predator of scallops (Barbeau & Scheibling 1994a, b). Changes in these parameters may best explain the variation in growth and retrieval of the scallops over the different deployment intervals.

A negative correlation of salinity with growth and retrieval of scallops in the deployment study was probably a coincidence as
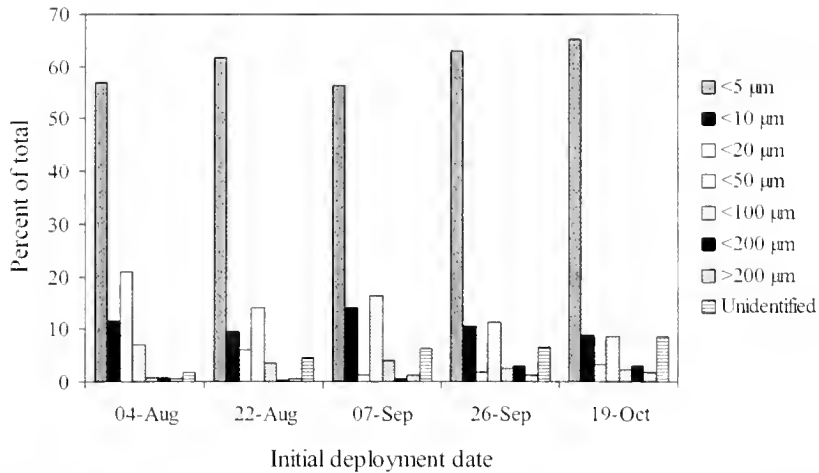
Figure 9. Particle size frequency distribution of plankton at Ladder Garden, Shell Fresh Farms Ltd., Poole's Cove, NL, over five consecutive deployment intervals of scallops at a sea-based nursery.

the salinity tolerance range for wild juvenile sea scallops is ≥25 psu (Frenette & Parsons 2001), which is lower than the salinity during the present study. The increase in salinity over the study period reflects the decreased runoff and the increased upwelling that occurs in the autumn in this area.

Decreases in metabolic processes due to declining temperature may explain why reduced growth rates were observed in scallops deployed on different dates in this study as has been found for *Pecten fumatus* (Cropp & Hortle 1992). Respiration rates in sea scallops decrease with declining temperature (Shumway et al. 1988), but clearance rates are correlated with ambient temperature in sea scallops (MacDonald & Thompson 1986) as well as in the eastern oyster, *Crassostrea virginica* and the bay scallop, *Argopecten irradians* (Rheault & Rice 1996). In the present context, reduced clearance rates would be expected to decrease food intake and result in reduced growth.

Declining retrieval over time was correlated with deployment temperature. This however, does not indicate that scallops died as a direct result of decreasing temperature. Scallops are able to live within a temperature range of −2°C to 22°C (Dickie 1958). Hence their survival should not have been influenced by decreasing temperatures *per se*. Christophersen and Magnesen (2001) found that

when *Pecten maximus* spat were deployed at water temperatures >10°C, spat had up to a 4-fold increase in survival compared with scallops deployed at temperatures <10°C. The sea scallops were likely influenced more by the temperature difference from the hatchery to the sea-based nursery environment than their physiologic condition or predation by sea stars.

Effects of Food Variation on Growth Rates and Retrieval

Scallops deployed when *Prorocentrum*, *Dinophysis* and *Navicula* spp. densities were elevated exhibited higher growth rates than scallops deployed when densities of these phytoplankton species had declined. All these mircoalgae have been found in gut analyses of adult scallops (Shumway et al. 1987). We found all three species in high abundance and the first two species are considered to add greatly to the energy uptake of scallops (Shumway et al. 1987). Cryptophyte densities also peaked during August when growth rates were highest. Cryptophytes are rich in the fatty acids, 22:6w3 and 20:5w3 (Volkman et al. 1989, Viso & Marty 1993) and are important for a good diet and membrane fluidity in bivalves (Enright et al. 1986, Napolitano et al. 1992). Cryptophytes are a preferred alga in mixed diets and are related to growth
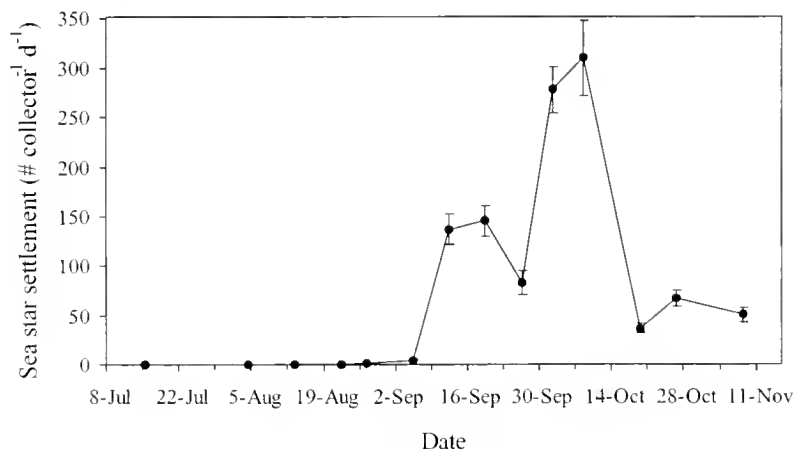
Figure 10. Mean sea star settlement at Ladder Garden lease of Shell Fresh Farms Ltd., Poole's Cove, NL, from July 15 to November 8, 1997 (*n* = 8). Vertical bars are ±SE.

TABLE 1.

Pearson's correlation coefficients of short-term growth rates and retrieval of nursery-size scallops with mean water quality parameters at a sea-based nursery at Shell Fresh Farms Ltd., Poole's Cove, NL, from August 4 to November 8, 1997.

	Temperature	Salinity	Chlorophyll-a	Phaeopigments	TPM	PIM	POM	% POM	Sea Star Settlement
Growth rate									
r value	0.840	−0.826	0.901	0.940	−0.043	−0.573	0.700	0.773	−0.796
Significance (two-tailed)	<0.001	<0.001	0.001	<0.001	0.439	0.013	0.002	0.001	<0.001
Retrieval									
r value	0.828	−0.698	0.849	0.870	0.233	−0.358	0.714	0.644	−0.890
Significance (two-tailed)	<0.001	0.002	<0.001	<0.001	0.201	0.095	0.001	0.005	<0.001

$n = 15$ for all parameters.

in sea scallops (Shumway et al. 1985, Parrish et al. 1995). It is expected that scallops exposed to a higher quality diet allowing adaptation to declining conditions would perform better than scallops exposed to a lower quality diet (see Shumway et al. 1997).

MacDonald and Thompson (1985) found that shell growth was higher under favorable conditions of food and temperature, and that this was site specific. Location of sea-based nursery sites should consider food quantity and quality. However, because there have been so few growth studies of juvenile bivalves with respect to natural phytoplankton composition, the actual quantity and quality of food required is not known (Newell et al. 1989, Parrish et al. 1995, Grant 1996). Phytoplankton is a major component of the diet of adults (Shumway et al. 1987, Cranford & Grant 1990); however, further research is necessary to determine the actual quantity and quality that allow juvenile scallops to perform optimally (Shumway et al. 1997).

Predation Effects on Retrieval

There was an increased negative correlation between retrieval of scallop spat and sea star settlement during the short-term intervals. Increasing sea star settlement coupled with declining sea scallop retrieval was expected (Dadswell & Parsons 1991, 1992, Barbeau & Scheibling 1994a, Parsons 1994). Successful predation may be due to the similar size of the settling sea stars and scallop spat as well as debilitation caused by significant temperature changes between hatchery and nursery environments (Dickie 1958, Barbeau & Scheibling 1994a). In the present study temperature

difference between hatchery and nursery progressively increased with deployment date from 0 to 7.1°C. Although sea star predation may be reduced with decreasing temperature (Barbeau & Scheibling 1994a), the temperature shock may have rendered spat more susceptible to sea star predation.

Dickie (1958) observed a lack of mobility of scallops for about a month when they were exposed to drops of 4–7°C in ambient temperature, which he speculated may be detrimental if predators are unaffected. Temperature debilitation may have coincided with highest mortality of scallops in the present deployment study, which was during the period of peak sea star settlement on the culture gear.

Importance of Acclimation on Growth Rates and Retrieval

The effect of increasing differences between hatchery and at sea nursery conditions on the performance of scallops raises concerns over handling protocols. Although acclimation to different conditions was not specifically examined in this study, a few general observations can be made regarding its importance. Sea-based nursery conditions were within the natural environmental ranges for scallops; however, scallops performed increasingly poorer with each consecutive deployment interval. Other studies have found that sudden changes in the environmental or rearing conditions can decrease survival and growth (Thompson 1984, Cranford & Grant 1990, Côté et al. 1993, Christophersen 2000, Christophersen & Magnesen 2001). Acclimation of *Pecten maximus* to lower ambient water temperature did confer a small increase in survival in

TABLE 2.

Pearson's correlation coefficients of short-term growth rates of nursery-size scallops with mean phytoplankton densities at a sea-based nursery at Shell Fresh Farms Ltd., Poole's Cove, NL, from August 4 to November 8, 1997.

	Total	Microzooplankton	Choanoflagellates	Dinoflagellates	Prymnesiophytes	Cryptophytes	Autotrophic nanoflagellates	Centric Diatoms	Unidentified	*Rhizosolenia* sp.
r value	0.994	0.558	0.772	−0.058	0.895	0.789	0.993	−0.635	0.991	0.893
Significance (two-tailed)	<0.001	0.031	0.001	0.837	<0.001	<0.001	<0.001	0.011	<0.001	<0.001

	Navicula sp.	*Chlamydomonas* sp.	*Ochromonas* sp.	*Micromonas* sp.	Coccolithophore	*Prorocentrum* sp.	Choanoflagellate sp.	*Strombilium minimum*	Pelagic Pennate Diatoms
r value	0.726	0.987	0.687	0.913	0.980	0.895	0.944	0.772	0.974
Significance (two-tailed)	0.002	<0.001	0.005	<0.001	<0.001	<0.001	<0.001	0.001	<0.001

$n = 15$ for all parameters.

juvenile scallops (Christophersen & Magnesen 2001). *Mytilus edulis* requires 14 days to acclimate oxygen consumption, filtration rates and assimilation efficiency (Widdows & Bayne 1971). Hall (1999) observed that in sea scallops 15–21 days were required for membrane fluidity to adjust to a temperature decrease from 15 to 5 C. The temperature and diet differentials between hatchery and nursery may have been too great or too abrupt for scallops to maintain optimal performance without the opportunity to acclimate, particularly later in the deployment season.

Implications for Hatchery, Nursery and Grow out

The findings of this study provide growers with a protocol for working with animals in a dynamic environment, under optimal and suboptimal conditions. Hatchery managers may be able to use our results to improve decisions on when to deploy sea scallops and nursery managers may now have the ability to optimize growth and retrieval of sea scallops reared in a sea-based nursery system and to better plan for transfer to grow out when scallop spat reach the desired target size.

CONCLUSIONS

Growth rates and retrieval of nursery-sized scallops were influenced by time of deployment at a sea-based nursery during a period that spanned early summer to late autumn. Highest growth rates and retrieval of nursery-sized scallops were observed during August and early September when the nursery site water column was characterized by high food densities, high temperature and low sea star settlement. However, scallops deployed in late September and October had low retrieval as well as low growth rates until the following spring or later.

The ability of nursery-sized scallops to grow and survive may be related to the differences between hatchery and sea-based nursery environments in terms of food quality and temperature differentials. There is a need to determine the nutritional requirements of nursery-sized scallops and to practice acclimation protocols.

ACKNOWLEDGMENTS

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DEVELOPMENT, EVALUATION, AND APPLICATION OF A MITOCHONDRIAL DNA GENETIC TAG FOR THE BAY SCALLOP, *ARGOPECTEN IRRADIANS*

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ABSTRACT As a component of an aquaculture-based, bay scallop stock-restoration program in west-central Florida nearshore waters, we developed a genetic tag for the bay scallop, *Argopecten irradians*. Using the polymerase chain reaction technique, we amplified segments of highly purified scallop mitochondrial DNA using 10-base-pair random primers and generated fragments that we investigated for use as genetic tags. We excised and cloned the amplicons obtained from six individuals to assess them for nucleotide variability. We chose one, highly polymorphic amplicon of 1049 base pairs and designed a set of sequence-specific polymerase chain reaction primers for it. We used these primers to sequence portions of the fragment, from both the 5′ and 3′ ends, respectively, effectively dividing the fragment into two distinct segments separated by 66 nucleotide base pairs. The two segments contained sufficient polymorphism such that 72% (segment 1) and 80% (segment 2) of the individuals were unique in a sample of 97 wild individuals; 97% of these individuals were unique when both segments were considered. Nucleotide sequences appeared to be faithfully transmitted from one parent to its presumed offspring; indications of heterozygosity and heteroplasmy were not observed for any individual throughout the study. Our analysis of this DNA fragment suggested that it is an mtDNA component, but we were unable to characterize the gene region that it encompasses. To test our genetic tag, we used these two segments to preliminarily assess the contribution of the stock restoration program to the bay scallop population at a single area targeted for stock restoration. Our analysis suggests that the stock restoration effort either did not contribute or contributed at a low level to the local population, but our postrestoration sample sizes may have been too small to detect very small contributions. Our work demonstrates the utility of using random primers to develop mtDNA genetic tags for species for which little is known about the nucleotide sequence or gene order of the mtDNA molecule and the potential for application of that tag as a preliminary evaluation tool in a stock restoration or stock enhancement program.

KEY WORDS: *Argopecten irradians*, bay scallop, DNA sequencing, Florida, genetic tag, mitochondrial DNA, stock enhancement, stock restoration, random primers

INTRODUCTION

Throughout the world, many commercially and recreationally valuable species of shellfish and finfish are declining as a result of overfishing, pollution, habitat degradation, and disease (e.g., Hargis 1999, Marelli et al. 1999, Hutchings 2000). Management of these fisheries through quotas and closures has not always been effective in preventing further decline or allowing natural recovery to take place. For this reason, aquaculture-based stock restoration and enhancement are now accepted methods for restoring depleted fishery stocks (Tettelbach and Wenczel 1993, Peterson et al. 1995, Southworth and Mann 1998, Svasand et al. 2000, Arnold 2001).

The bay scallop, *Argopecten irradians* (Lamarck, 1819), a species valuable to the people of Florida as both a commercial and recreational resource, was once plentiful in west-Florida nearshore waters and high-salinity embayments. By the early 1960s, population numbers and abundances had severely declined in many regions, due in part to dwindling seagrass beds and pollution during the 1950s (Haddad 1988, Blake et al. 1993, Arnold et al. 1998). Later, concerted efforts by state and federal governments and environmental groups led to water-quality improvement and restoration of habitats suitable for scallop propagation (Blake 1998). In the 1990s, the commercial fishery was closed, and area-specific restrictions or prohibitions on harvesting were implemented for the recreational fishery (Arnold et al. 1998). Despite these efforts and those of a small-scale bay–scallop stock-enhancement project ongoing throughout the 1990s (Blake 1998),

bay scallop population numbers and abundance continued to decline in west-central Florida waters. Therefore, in 1997, a multiyear, aquaculture-based, stock restoration program was initiated in west-central Florida nearshore waters to re-establish extirpated bay scallop populations and enhance depleted populations.

One way to assess the success of a stock restoration endeavor is to estimate the contribution of the original aquaculture broodstock to the local or regional population. For bay scallops, the most reliable method is a genetic tag, generated from nuclear or mitochondrial DNA (mtDNA). A genetic tag can be used to estimate the success of a stock restoration or enhancement program because an assessment of the contribution of hatchery-reared or hatchery-derived individuals to the recipient population can be made (Bert et al. 2002). The genetic tag must be sufficiently powerful to discriminate aquaculture-derived individuals from wild individuals, or at least the tag should be composed of genotypes of sufficient rarity to allow detection of the stock restoration contribution through changes in the percentages of these genotypes in the population. In either case, the contribution of the restoration effort must be sufficient to enable detection by sampling.

There are several methods of genetic tagging (Palsbøll et al. 1997, Palsbøll 1999, Bert et al. 2002), but one of the easiest is to find and use a highly variable portion of mitochondrial DNA (mtDNA). Nontranscribed regions of the mtDNA molecule serve as excellent genetic tags (Simon et al. 1994) because they typically mutate more rapidly than most other DNA segments (Meyer 1993). In addition, if the mode of inheritance is uniparental, tracking it in first-generation offspring is straightforward.

In invertebrates, the mtDNA molecule can vary greatly in both

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gene order and nucleotide sequence, even among closely related taxonomic groups (e.g., Boore & Brown 1994, Boore et al. 1995, Wilding et al. 1999). Thus, the universal mtDNA primers developed for vertebrates (Palumbi 1996) are not always successful in amplifying invertebrate mtDNA segments. Here, we report on the development of a compound mtDNA genetic tag for the bay scallop using an unidentified bay scallop mtDNA fragment initially amplified by 10-base-pair (bp) random primers obtained commercially. We evaluate its genetic diversity and applicability through a preliminary assessment of the contribution of our stock restoration program to the bay scallop population at one location (Homosassa Bay, Florida; Fig. 1). Last, we discuss the general utility of single-gene genetic tags.

MATERIALS AND METHODS

Development of the Mitochondrial DNA Genetic Tag

To search for an mtDNA fragment that could serve as a genetic tag, we first obtained highly purified mitochondrial DNA from the gonad tissues of sexually mature bay scallops from Homosassa, Florida ($n = 6$); mature bay scallops contain both male and female reproductive tissues. We used a modified homogenization buffer containing 100 μM sucrose and the standard extraction method of cesium-chloride density gradient ultracentrifugation (Lansman et al. 1981). The mtDNA band was collected in a 1-mL syringe by side puncture with a hypodermic needle and purified by dialysis. The purified mtDNA yielded a single fragment of approximately 16,000–20,000 bp when run through a 2%, ethidium-bromide-stained, low-EEO, agarose gel (Fisher Biotechnologies, Pittsburgh, PA). According to the methods described by Williams et al. (1990), we amplified portions of the highly purified mitochondrial DNA of these individuals using the twenty 10-bp random primers supplied in RAPD Kit A (Qiagen Operon Technologies, Inc., Alameda, CA). Five microliters of each polymerase chain reaction (PCR) product was run in a low-EEO agarose gel to view the amplifications. Multiple bands were obtained for most primers except for OPA-3 (AGTCAGCCAC) and OPA-18 (AGGTGAC-
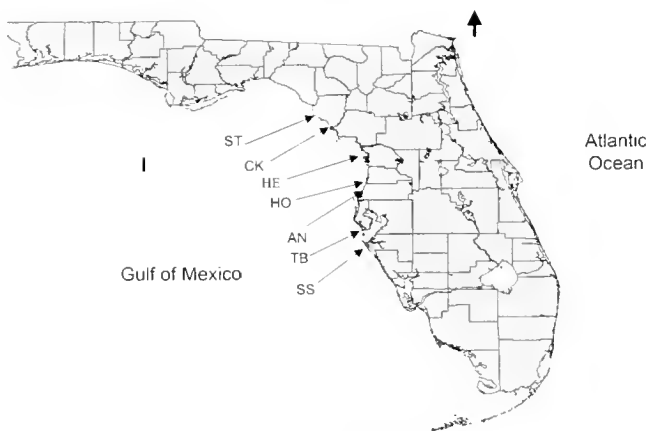
Figure 1. Collection locations for bay scallops (*Argopecten irradians*) in Florida to estimate the frequencies of original-broodstock haplotypes in the wild prior to the restoration effort (sample sizes are given in Table 1B). HO was the location of the stock restoration evaluation presented in this report. Abbreviations: ST = Steinhatchee; CK = Cedar Key; HE = Hernando; HO = Homosassa Bay; AN = Anclote Estuary; TB = Tampa Bay; SS = Sarasota Bay.

CGT). OPA-3 gave a single band of approximately 1,000 bp and OPA-18 gave two intense bands of approximately 1,000 and 1,600 bp.

The remaining 45 μL of the OPA-3 and OPA-18 PCR reactions were run in a gel of 2% low-melting-point agarose (NuSeive, FMC, Rockland, ME). According to the standard method of Sambrook et al. (1989), the fragments were excised and cloned in a plasmid vector (Bluescript PBC KS, Stratagene, La Jolla, CA) that was initially cleaved with *Eco*RV and tailed with 2-mM dTTP (Marchuk et al. 1990). After plating the transformed cells, the three fragments were amplified from two colonies of each of the six individuals using the T3 and T7 primers (Stratagene, La Jolla, CA), which annealed to the Bluescript vector on either side of the insert. The PCR products were electrophoresed in a 1.5%, low-EEO, agarose gel, excised, and purified using a Strata Prep DNA Gel Extraction Kit (Stratagene, La Jolla, CA). The purified DNA was resuspended in 50 μL of sterile distilled water.

Cycle sequencing was performed from both the 5′ and 3′ ends of each fragment using 0.5 μL of the purified DNA, two μl of Big Dye™ Terminator Cycle-Sequencing Ready-Reactions with AmpliTaq FS DNA polymerase (PE Biosystems, Foster City, CA) and 0.5 μL of 3.2-pM solutions of the T3 and T7 primers, in a total volume of 5 μL. The reaction product was then ethanol precipitated and resuspended in 20 μL of Template Suppression Reagent (PE Biosystems, Foster City, CA), according to the manufacturer's instructions.

The resuspended product was analyzed by using an ABI Prism™ 310 Genetic Analyzer (PE Biosystems, Foster City, CA). The sequences obtained were aligned and edited using the AutoAssembler™ DNA Sequence Assembly Software (PE Biosystems, Foster City, CA); further electropherogram editing was performed using Chromas v. 1.6 (Technelysium Pty, Ltd, Helesville, Queensland, Australia). The two OPA-18 fragments cloned and sequenced for the six individuals were composed of multiple sequences, most of which matched very poorly when aligned and were therefore considered nonhomologous. Some sequences aligned very well and were thus presumed to be homologous, but they were invariable in this fragment. However, sequences of the OPA-3 fragment for the six individuals were homologous and differed among all six individuals at one or more nucleotides. This 1,049-bp fragment was named SCAOPA-3 and was identified as a possible genetic tag. A representative sequence of the SCAOPA-3 fragment has been deposited in GenBank under accession number AF261938. Highly specific primers for the fragment were designed: SCA-1, composed of 5′–AGTCAGCCACCCACTAAA-TTAGATCTCA–3′ and SCA-2, 5′–AGTCAGCCACTGGTT-TATAGTGGAATAGTT–3′. The first 10 bp of these primers constituted the sequence of the 10-bp primer that was initially used to amplify the SCAOPA-3 fragment.

Using each custom-made primer, we sequenced the SCAOPA-3 fragment from the two ends toward the center, thereby effectively dividing it into two segments. The sequences for the first portion (termed segment 1) consisted of 471 bp beginning at position 33 and ending at position 503; the second segment (termed segment 2) consisted of 450 bp beginning at position 569 and ending at position 1018. These segments did not overlap and 66 bp between these segments were not included.

For the remaining genetic analyses, bay scallops were obtained alive and from each individual, a section of adductor muscle was excised, labeled, and stored at −80°C. For each individual, we purified total DNA from the adductor muscle using the modified

PureGene DNA Extraction protocol for small tissue samples, according to the manufacturer's instructions (Gentra Systems, Minneapolis, MN).

To identify the origin of the SCAOPA-3 fragment (i.e., mitochondrial or nuclear DNA), we used our broodstock bay scallops. The bay scallop stock-restoration project involved two generations of broodstock (an "original-broodstock" [parental] generation and a "restoration-broodstock" [F_1] generation). The offspring of the restoration-broodstock generation constituted the aquaculture-derived "brood" (F_2) generation that should supplement the wild population. We determined the nucleotide sequences of 26 restoration broodstock raised from eight original broodstock collected from the wild Homosassa Bay population in 1997 and 23 restoration broodstock raised from five original broodstock collected from the wild Homosassa Bay population in 1998. We examined these sequences for among-individual heteroplasmy and for within-individual heterozygosity. We also compared the sequence of SCAOPA-3 to published sequences and to those available in the computer database GeneBank.

Testing the Genetic Tag

To assess the natural level of polymorphism of the SCAOPA-3 fragment and the potential of each segment to serve as an independent component of a compound genetic tag, we sequenced from one direction each of the two segments for 97 individuals collected in 1997 and 1998, prior to the time of potential input from the stock restoration program. Twenty-three of these were from Homosassa, of which 13 were the original-broodstock scallops used in the Homosassa Bay stocking effort; the remainder of these were collected from Tampa Bay ($n = 50$) and the Anclote Estuary ($n = 24$) (Fig. 1). Scallops from these nearby sites were used because the Homosassa bay scallop population had collapsed and thus individuals from that location had to be used with discretion. To estimate the frequencies of the original-broodstock haplotypes in the wild population, we collected and analyzed 54 individuals from Homosassa and 271 individuals from six other west-Florida nearshore locations (Fig. 1). These individuals were collected prior to 1999, the first year that aquaculture-derived individuals could have contributed to the population.

To test the utility of our genetic tag, we examined the SCAOPA-3 sequences from bay scallop recruits collected from Homosassa Bay during appropriate years, determined as follows. In west Florida, bay scallops, which are hermaphrodites, commence spawning in October and generally cease by December (Barber and Blake 1983; Arnold et al. 1998). Therefore, from September through early October of each year, the original-broodstock scallops were collected from wild populations at locations targeted for restoration, brought into the laboratory, and spawned under controlled conditions. Their offspring (the restoration broodstock) were reared in containment through the winter and the following spring until they attained approximately 20–30 mm shell height. These scallops were then planted in cages in the vicinities of collection of the original broodstocks. There, they were to complete their growth through the summer and, hopefully, contribute to the spawning stock when they sexually matured in the fall. Their recruits (the brood generation), along with wild recruits also inhabiting the restoration locations, would be of sufficient size to be collected and tested for parentage in summer of the year after they were spawned by the restoration broodstocks and 2 y after collection and breeding of the original broodstocks.

Bay scallops can live for 2 y (Orensanz et al. 1991), but it is not known whether they contribute to the spawning stock in the second year of their lives. To insure that we accounted for this possibility, we collected bay scallop recruits from restoration sites and assayed them for the genetic tag for two years after the planting of the restoration broodstocks, if those broodstocks survived for 2 y. Thus, a single cycle of bay scallop stock restoration, including the genetic monitoring, was a 3- or 4-y process.

We searched for haplotypes that could be from the offspring of the restoration broodstocks that were planted in Homosassa Bay in 1998 and 1999; these were derived from original broodstocks collected in 1997 and 1998. We removed the 1998 restoration broodstock after the 1998 spawning season because most of those individuals died. However, we left the 1999 restoration broodstock in their cages through both the 1999 and 2000 spawning seasons. Therefore, we assayed bay scallop recruits collected from Homosassa Bay in 1999 for genotypes that matched the 1997 original-broodstock genotypes and assayed bay scallop recruits collected from the bay in both 2000 and 2001 for genotypes that matched the 1998 original-broodstock genotypes.

To obtain these post-restoration "assessment" collections of bay-scallop recruits, we randomly allocated 20 sampling stations within an area of Homosassa Bay defined by the 0.7 m and 2.0 m depth contours and by somewhat arbitrary latitudinal borders that were selected based upon our knowledge of the area. Using SCUBA, at each station we searched within 1 m on each side of a 300-m transect line and collected all scallops within that zone (600 m² per transect, 12,000 m² total). We also collected scallops using vessel-deployed rollerframe trawling gear. Those samples were obtained from deeper water sites (approximately 1.5-m to 3.5-m depth). The Global Positioning System locations (available upon request) of these collections were recorded. All assessment collections were potentially composed of an admixture of wild recruits and hatchery-derived recruits, the latter of which could have as parents either two restoration-broodstock individuals or one restoration-broodstock and one wild individual. (We recognize that, if mtDNA is maternally inherited in scallops, any recruit generated by the union of an egg from a wild individual and a sperm from a restoration-broodstock individual would not be identified as a possible aquaculture-derived bay scallop.)

Bert and Tringali (manuscript in preparation) describe the samples needed to perform a complete assessment of a stock restoration or enhancement effort. Following their suggestions, we analyzed the following individuals for their genetic-tag nucleotide sequences. After they completed spawning, we assayed the eight original-broodstock individuals used in fall 1997 and the five original-broodstock individuals used in fall 1998 for both segments 1 and 2 of our genetic tag. Because bay scallops are hermaphroditic, any or all of the original-broodstock individuals may have passed their mtDNA on to the restoration broodstocks. We did not assay the restoration broodstocks because many individuals died before we could collect them. We assayed the following numbers of bay scallop recruits: 199 collected in 1999, 253 collected in 2000, and 242 collected in 2001. To detect individuals with aquaculture-derived mtDNA haplotypes in these assessment collections, we first compared the SCAOPA-3 segment-2 haplotype of each recruit to that of each original-broodstock scallop from the appropriate year. We then sequenced for segment 1 any recruit that had a segment 2 haplotype that matched that of an appropriate-year, original-broodstock scallop. We used the seg-

ment 2 component of our genetic tag first because it was slightly more variable than segment 1 (see below).

Data Analyses

To examine the level of genetic diversity of our SCOPA-3 fragment, provide baseline data for estimating the contribution of our stock restoration effort to the Homosassa bay scallop population, and estimate the sensitivity of our genetic tag, we first calculated a number of standard measures of genetic diversity for each segment using the Arlequin statistical package (Schneider et al. 2000) on the 97 bay scallops collected from the three west-Florida locations. We then estimated the frequencies of original-broodstock haplotypes in the seven wild bay-scallop collections and used the AMOVA program in Arlequin to obtain a baseline estimate of the distribution of the original-broodstock haplotypes in those collections, which included the Homosassa Bay collection. We also used Arlequin to quantify the genetic diversity of the segment 2 haplotypes in each of the wild bay-scallop collections and in the collective wild population. In addition, we searched for original-broodstock haplotypes in the collections of wild bay scallops. We tested our ability to detect original-broodstock haplotypes in the wild population by calculating the minimum detectable frequency (MDF) of the original-broodstock haplotypes, using the basic binomial sampling equation

$$MDF = 1 - \exp\left[\frac{\ln(\alpha)}{n_\nu}\right] \qquad (1)$$

where $\alpha = 0.05$ and $n =$ number of individuals, and defined as the frequency below which the probability of detecting at least one individual bearing an original-broodstock haplotype would be <0.05. We assumed that our sampling and the distribution of the haplotypes in the wild population were random.

To estimate the contribution of our stock restoration effort to the Homosassa Bay scallop population, we first examined the appropriate assessment collections for the presence of original-broodstock haplotypes. Then we used Equation 1 to calculate the probability of detecting those haplotypes in those assessment collections. (Detailed mathematical and statistical approaches for the overall assessment of the restoration effort will be described in a later manuscript [Wilbur et al. in preparation]).

We further explored the limitations of our genetic tag by conducting probability assessments on simulated data based on haplotype frequencies observed in individuals from the 1999 and the 2000 + 2001 assessment collections. For the simulations, we randomly eliminated 5%, 10%, 15%, 20%, or 25% of the individuals in the collections and substituted at the designated frequency a single, randomly chosen haplotype from the 1997 or 1998 original broodstock, as appropriate for the assessment collection(s) undergoing the simulation analysis (Table 2). We then calculated haplotype diversity, nucleotide diversity, and the percentage of different haplotypes in the population for these simulated collections and compared these statistics to those calculated for the corresponding actual assessment collection without the hypothetical stock restoration contribution. If the stock-restoration program was successful, we would expect to see significant shifts in the frequencies of haplotypes possessed by the original broodstock in populations following restoration efforts. To determine the minimum post-restoration frequency differences that would be needed to detect contributions from restoration broodstock, we used the V-test (DeSalle et al. 1987) to compare the haplotype frequency distributions of our simulated assessment collections with the appropriate actual assessment collection. For the 5% increment within which we detected significance, we simulated assessment collections for each 1% increment of stock restoration contribution and tested each of those simulated collections for significant differences in haplotype distribution compared with our actual assessment collections.

RESULTS AND DISCUSSION

Evaluation of the SCAOPA-3 mtDNA Fragment

Our characterization the origin of the SCAOPA-3 fragment suggested that it is of mitochondrial DNA origin. Each of the SCAOPA-3 sequences from our 49 restoration-broodstock scallops strictly matched only one of the haplotypes in the appropriate pool of original-broodstock haplotypes. The DNA sequencing protocol that we used allowed for detection of heterozygous individuals if they were present; that is, heterozygous sequences characteristically appear as two peaks of approximately equal intensity at a given nucleotide site. However, none of these bay scallops were heterozygous, and we found no heterozygous individuals in any of our subsequent analyses. Therefore, we conclude that SCAOPA-3 is transmitted from parent to offspring as a haploid molecule and we presume that it is mitochondrial DNA. At present, we cannot say if SCAOPA-3 is inherited maternally; paternal mtDNA inheritance occurs in other bivalves (e.g., *Mytilius*; Liu et al. 1996, Zouros et al. 1994). Despite comparing its nucleotide and presumptive amino acid sequences to those reported for other organisms, including other mollusks (Hoffmann et al. 1992, Boore and Brown, 1994) and to unpublished sequences, (e.g., GeneBank accession numbers ABO55625, ABO65375), we were unable to characterize with certainty the gene region it encompasses. However, this will not affect the study, provided that SCAOPA-3 is faithfully transmitted as a haploid molecule from parent to offspring.

Sensitivity and Application of the SCAOPA-3 Fragment

The eight 1997 original-broodstock individuals had only seven different segment 2 haplotypes. However, the two individuals that were identical for segment 2 differed for segment 1. Thus, each of our broodstock individuals had a SCAOPA-3 haplotype that was unique in the aquaculture hatchery.

All differences among individuals in segment 1 and segment 2 of our mtDNA fragments were in the form of single bp substitutions. In Table 1A, we present estimates of genetic diversities for the two segments as determined by sequencing the individuals used to characterize the fragment. Separately, these segments distinguished high percentages of individuals; collectively, they distinguished nearly all of the individuals.

Results of the AMOVA analysis suggest that the bay scallops comprising the west-Florida pre-restoration collections were genetically homogeneous with respect to the SCAOPA-3 mtDNA fragment. In Table 1B, we summarize the segment 2 genetic diversities for these collections and for the west-Florida population. Both the percentage of individuals with different haplotypes and the proportion of haplotypes that were unique were very high in the individual samples and high in the combined data. Eighty-five individuals (26%) were defined by four haplotypes in the proportion 42:19:18:6; thus, the most common haplotype present in the population occurred in only 13% of the individuals. Correspond-

TABLE 1.

Estimates of bay scallop (*Argopecten irradians*) genetic diversities for the SCAOPA-3 mitochondrial DNA genetic tag in (A) 97 wild individuals from Tampa Bay, Homosassa, and Anclote, Florida, (segments 1 and 2 are defined in Materials and Methods) and (B) western Florida collections made prior to the stock restoration effort (segment 2 only).

Segment	No. bp	HN	HQ	RS	h	p	K
A							
1	451	72	91	0.19	0.97 ± 0.01	0.86 ± 0.48	3.9 ± 2.0
2	450	80	86	0.20	0.99 ± 0.00	1.35 ± 0.07	5.6 ± 2.7
Total	901	97	98	0.20	1.00 ± 0.00	1.10 ± 0.56	9.5 ± 4.5

Location	Year	N	HN	HQ	h	p	K
B.							
ST	1997, 1998	61	75	89	0.97 ± 0.01	1.22 ± 0.66	5.4 ± 2.6
CK	1997	20	75	87	0.94 ± 0.04	1.07 ± 0.61	4.7 ± 2.4
HO	1997, 1998	54	81	89	0.99 ± 0.01	1.38 ± 0.74	6.2 ± 3.0
HE	1997, 1998	32	81	85	0.98 ± 0.01	1.12 ± 0.63	4.8 ± 2.4
AN	1997, 1998	67	84	93	0.99 ± 0.01	1.30 ± 0.70	5.4 ± 2.7
TB	1997	65	80	90	0.98 ± 0.01	1.30 ± 0.70	5.6 ± 2.7
SS	1998	26	85	86	0.98 ± 0.02	1.80 ± 0.60	4.8 ± 2.4
Total		325	62	85	0.98 ± 0.00	1.26 ± 0.68	5.3 ± 2.7

Abbreviations: No. bp = number of base pairs; HN = percentage of individuals with different haplotypes; HQ = percentage of haplotypes unique to single individuals; RS = number of polymorphic sites per nucleotide site; h = haplotype diversity; p = nucleotide diversity in %; K = number of pairwise nucleotide differences; N = number of individuals; ST = Steinhatchee; CK = Cedar Key; HO = Homosassa; HE = Hernando; AN = Anclote; TB = Tampa Bay; SS = Sarasota Bay.
h, p, and K are mean values ± standard deviations.

ingly, all standard measures of genetic diversity were comparatively high (e.g., haplotype diversity ranged 0.94–0.99).

Twenty-four wild-individual haplotypes matched five 1997 original-broodstock haplotypes for segment 2. However, none of the individuals that matched original-broodstock segment-2 haplotypes also matched the same broodstock individual for segment 1. No wild individuals collected in 1998 matched any of the original-broodstock segment-2 haplotypes. If our assumptions associated with Equation 1 were valid, we could expect to obtain a match between a wild-individual haplotype and an original-broodstock haplotype if the broodstock haplotype was present in our wild-population sample at a frequency of approximately 1% or greater (MDF_{95} = 0.00917). Thus, the estimated prerestoration frequency of each of the 1997 and 1998 broodstock haplotypes in the wild population probably was less than 1%.

Ten of the assessment scallops collected in 1999 matched three of the 1997, segment-2, original-broodstock haplotypes. Eight of those were identical to the single original-broodstock scallop with the haplotype that was the second most common in the wild population. However, the haplotypes of all of those individuals differed from that original-broodstock individual's segment 1 haplotype. No segment-2 haplotypes from assessment bay scallops collected in 2000, and only one segment-2 haplotype from an assessment bay scallop collected in 2001, matched any 1998, original-broodstock, segment-2 haplotype. That individual did not match for segment 1 the original-broodstock individual that it matched for segment 2. Thus, our collective sample size of 694 individuals gave no indication that the bay scallop restoration project contributed to the local Homosassa bay scallop population during 1999–2001.

The MDF_{95} for detection of an original 1997 or 1998 broodstock haplotype in the appropriate assessment collection(s) was, respectively 0.015 (1999 assessment collection) or 0.0060 (2000 + 2001 assessment collections). Original-broodstock haplotypes that were present in the putative admixed Homosassa population at

frequencies near or below the MDF_{95}s were at statistical risk of not being detected. However, these frequencies were so low that stock restoration contributions at or below these levels may essentially be inconsequential.

Although haplotype diversity and nucleotide diversity in our hypothetical assessment of stock restoration contribution were proportionally reduced with increasing stock restoration contribution, they were not as sensitive to the input of stock restoration contribution as was the percentage of different haplotypes (Table 2). Nevertheless, our simulations indicate that a stock restoration contribution of at least 15% in the 1999 assessment collection and 10% in the 2000–2001 combined assessment collection would be needed to generate a significant difference between those assessment collections with versus without stock restoration contributions.

Genetic Tags and Molluscan Stock Restoration

The general strategy in a stock restoration program is to collect animals from the targeted restoration site, produce large quantities of aquaculture-reared or, in the case of our bay scallop program, aquaculture-derived (one generation removed) individuals, and use them to supplement or replenish the population at the same site. Determining the success of such an effort depends on the ability to detect the contribution (in numbers or percentages) of hatchery-reared or hatchery-derived offspring in the post-restoration recruits. In supplemented populations, the frequency of aquaculture-generated individuals can range from undetectable to a complete swamping of the admixed population. A single-gene genetic tag such as ours can indicate whether restoration effort has resulted in essentially undetectable input, substantial input, or a complete swamping of the local population. However, the capacity of this tag to estimate the contribution of the stock restoration effort between the extremes of essentially no input and very high input is

TABLE 2.

Hypothetical analysis of stock restoration contribution in the assessment collections from Homosassa with levels of contribution varying from 0% (original assessment collection) to 25% (see Materials and Methods for method of simulating stock restoration contributions). (A) 1999 assessment collection (N = 199 individuals). (B) 2000 + 2001 combined assessment collections (N = 495 individuals).

SRC (%)	N1	N2	HN1	HN2
A.				
0	199	0	0.72	0.72
5	189	10	0.73	0.70
10	179	20	0.73	0.66
15	169	30	0.72	0.62
20	159	40	0.75	0.60
25	149	50	0.77	0.58
B.				
0	495	0	0.69	0.69
5	470	35	0.69	0.66
10	445	69	0.69	0.62
15	421	104	0.70	0.73
20	396	139	0.72	0.57
25	370	174	0.73	0.55

Abbreviations: SRC = hypothetical stock restoration contribution; N1 = number of individuals taken from the specified year assessment collection; N2 = hypothetical number of individuals contributed from the stock restoration program (within a single percentage, all of which were taken from a single, randomly chosen broodstock individual); HN1 = percentage of individuals with different haplotypes without stock restoration contribution (calculated based on N1 only); HN2 = percentage of individuals with different haplotypes with stock restoration contribution (calculated on N1 + N2).

related to the degree of statistical uniqueness, as measured by statistical probability, of the tag in each application. To precisely define an intermediate-level contribution from a stock restoration effort, the assessment collection must consist of a very high number of individuals; the genetic tag must be complex (e.g., composed of our compound mtDNA genetic tag plus several microsatellite loci), or, if it is a single-gene tag, extremely variable; or the method for determining the contribution must differ from ours.

Because we found no original-broodstock haplotypes in either the wild population or the assessment collections, we can combine all of these collections to estimate the uniqueness of our original-broodstock haplotypes and calculate the *MDF* above which we might expect to encounter one of these haplotypes. We can estimate with 95% probability that we would have detected at least one original-broodstock haplotype in this combined sample (1,019 individuals) if the frequency of any of these haplotypes was 0.003 or greater. Clearly, frequencies below this *MDF* would represent inconsequential contributions from a stock restoration effort. Thus, our single-gene genetic tag should be useful for assessing the success of our entire bay scallop restoration effort.

In many cases, a single-locus, preliminary genetic tag such as ours could be useful in assessing the contribution of stock restoration efforts. Multi-locus genetic tags can be laborious, time-consuming, and expensive to develop, test, and apply. Furthermore, in our case, the potential for reproductive mixing between restoration broodstock and wild scallops limits the ability for nuclear DNA-based assignment of individuals to either the brood generation or to the wild population. Our genetic tag can be used to preliminarily evaluate the success of a bay scallop stock enhancement or restoration effort and thereby to evaluate whether it is worth the expense and effort to develop a more definitive genetic tag. Then, if it appears that the stock restoration effort may have contributed a potentially significant fraction of the recruits to an area, a high-resolution, multi-gene tag can be developed. However, under certain conditions, the type of genetic tag presented here may be sufficient for an entire study.

The advantages of using a single-gene genetic tag composed of more than one hypervariable segment and in which the segments can be used sequentially are increased resolution and reduced effort. In our genetic tag, both segment 1 and segment 2 had ample and nearly equivalent variation. By sequencing first for segment 2, the expense and time required were reduced significantly because only the individuals that had segment 2 haplotypes identical to those of the original-broodstock haplotypes also needed to be sequenced for Segment 1.

The utility of a single-gene genetic tag such as that presented here is enhanced if the broodstock used possesses essentially unique haplotypes or genotypes. However, there are limitations to this type of approach. A large number of wild individuals or a high percentage of the wild population must be assayed to establish the frequencies of the genetic-tag haplotypes in the pre-restoration population, and individuals with "unique" haplotypes should be used as broodstock. Threatened or depleted populations can be further endangered if they are flooded with aquaculture-derived individuals that collectively possess only a few naturally rare genotypes or haplotypes, if those individuals interbreed extensively and successfully with the remnant wild population. Nevertheless, for some applications, the procedure that we described here provides researchers with a method for finding an mtDNA genetic tag in organisms for which little is known about their mtDNA. This type of genetic tag can be used to screen individuals and derive parentage or group associations for stock restoration efforts, conservation biology, or other suitable applications.

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GAMETOGENESIS IN A SYMPATRIC POPULATION OF BLUE MUSSELS, *MYTILUS EDULIS* AND *MYTILUS TROSSULUS*, FROM COBSCOOK BAY (USA)

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ABSTRACT To test the hypothesis that a temporal variation in species-specific spawning times is the mechanism limiting hybridization and maintaining genetic integrity in a *Mytilus edulis* (L.) and *M. trossulus* (Gould) hybrid zone in eastern Maine, mussels from a low intertidal site in Cobscook Bay were histologically examined at monthly to semi-monthly intervals throughout the year 2000. Analysis of gamete volume fraction and oocyte area measurements detected no difference in the timing of gametogenesis and spawning between *M. edulis* and *M. trossulus*. Differences in mature oocyte area measurements, however, indicated that *M. edulis* spawned larger eggs than *M. trossulus*. At this location, low frequency of hybridization and maintenance of genetic identity for these two species is unlikely the result of temporally distinct spawning times.

KEY WORDS: Mytilus, gametogenesis, hybridization, mussels

INTRODUCTION

The *Mytilus* species complex is composed of three closely related blue mussel species, *M. edulis*, *M. trossulus*, and *M. galloprovincialis*. In the northern hemisphere, *M. edulis* occurs principally in the eastern and western Atlantic; *M. trossulus* is found in the Baltic Sea, the northwestern Atlantic Ocean, and the northern Pacific Ocean; and *M. galloprovincialis* occurs in the Mediterranean Sea, the Atlantic coast of southern Europe, northern Africa, and the Pacific coast of North America (Gosling 1984, 1992, Koehn 1991, McDonald et al. 1991, Suchanek et al. 1997). An early survey of *Mytilus* spp. on the east coast of North America indicated the presence of only a single species, *M. edulis* (Koehn et al. 1976), but in a later study, Koehn et al. (1984) identified two genetically distinct taxa inhabiting Atlantic Canada. These two genetically distinct groups (*M. edulis* and *M. trossulus*) form a zone of sympatry from northern Newfoundland south to the eastern coast of Maine (Varvio et al. 1988, McDonald et al. 1991, Bates and Innes 1995, Comesaña et al. 1999, Rawson et al. 2001).

Hybridization is commonly reported wherever members of the *Mytilus* complex are sympatric (Gosling 1992). In the Baltic Sea, *M. edulis* and *M. trossulus* hybridize so readily that they are considered semi-species (Väinölä & Hvilsom 1991). *M. edulis* and *M. galloprovincialis* hybridize extensively in a zone of sympatry that extends from the coast of Spain through the British Isles. The frequency of hybrid genotypes varies significantly among locations but can reach values as high as 80% in some populations (Hilbish et al. 1994, Comesaña & Sanjuan 1997, Sanjuan et al. 1997). In contrast, the frequency of hybrid genotypes formed by interspecific matings between *M. edulis* and *M. trossulus* in the northwest Atlantic is much lower, ranging from 12 to 26% (Koehn et al. 1984, Varvio et al. 1988, Bates & Innes 1995, Mallet & Carver 1995, Saavedra et al. 1996, Comesaña et al. 1999, Rawson et al. 2001). Although variation among sampling locations and the use of different methodologies (e.g., morphologic analysis, allozyme electrophoresis, mitochondrial, and nuclear DNA-based markers) may be partly responsible for the variation in the frequency of hybrids observed, these studies suggest that hybridization is less prevalent among blue mussels on the Atlantic coast of North America than in the Baltic or European hybrid zones.

Mate choice, habitat specialization and differential environmental tolerance, spawning asynchrony, and gamete incompatibility are processes that can initiate and maintain reproductive isolation between closely related species in sympatric populations (Palumbi 1994). In free-spawning marine invertebrates, mate choice, *per se*, is unlikely to play an important role in limiting hybridization. Increasing evidence, however, suggests that gamete interactions can affect reproductive isolation. For example, rapid, divergent evolution in sperm proteins (bindin and lysin) limits interspecific hybridization in sea urchins and abalone (Swanson & Vacquier 1998, Palumbi 1999), respectively. The existence of similar mechanisms in bivalves has not been confirmed.

Additionally, any habitat-specific selection that creates patchy species distributions may also limit hybridization because fertilization is more likely among close neighbors. Gardner (1996) has suggested that blue mussel hybrid zones occur in regions of environmental discontinuity so that the general patterns of species distribution are determined by differential adaptation. Several studies have observed that the distribution of blue mussel species is correlated with changes in environmental parameters, both in the contact zone between *M. edulis* and *M. galloprovincialis* in western Europe (Hilbish et al. 1994, Gardner 1996, Gilg & Hilbish 2000, Hilbish et al. 2002) and between *M. trossulus* and *M. galloprovincialis* on the Pacific coast of North America (Sarver & Foltz 1993). In the northwest Atlantic, research has focused on differences in salinity and wave exposure in structuring the species composition of blue mussel populations. There has been little evidence to directly link any of these factors with either the distribution, or the relatively low frequency, of hybrids within the region where *M. edulis* and *M. trossulus* are sympatric.

Reproductive isolation and maintenance of genetic identity may also be dependent on temporal variation in spawning events. In sympatric populations of *M. galloprovincialis* and *M. edulis* in southwestern Europe, low hybridization is observed when spawning periods are out of phase, whereas sites with a greater degree of synchrony have a higher degree of hybridization (Gardner 1992, Seed 1992). The objective of the present study was to determine whether the relatively low rate of hybridization occurring between *M. edulis* and *M. trossulus* in eastern Maine could be attributed to temporal variation in spawning.

MATERIALS AND METHODS

Adult mussels (35 to 50 mm in shell length) were collected by hand from a sympatric, low intertidal population in East Bay (lati-

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tude 44°56'30"N; longitude 67°07'50"W; Cobscook Bay, Maine) throughout 2000 (Table 1). Samples of 120 mussels were obtained monthly from January through April, October through December, and semi-monthly between 4 May and 14 September. Mussels were transported on ice to the University of Maine, and a piece of mantle tissue approximately 0.5 cm^2 was removed and preserved in 95% ethanol for DNA extraction. The remainder of the mussel was preserved in Dietrich's fixative (Gray 1954) for subsequent histologic preparation. All preservation was completed within 24 h of collection.

DNA was extracted from gonadal tissue following the protocol of Rawson et al. (2001). Three polymerase chain reaction-based nuclear markers, polyphenolic adhesive protein (Glu-5'), internal transcribed spacer, *Mytilus* anonymous locus-1, and one mitochondrial marker (mt16s-F; Rawson et al. 2001), were used to identify mussels with *M. edulis* and *M. trossulus* genotypes from each sampling period. Initially, the Glu-5' marker was run on all samples and used to identify 30 (n = 40 on 17 and 30 August) individuals homozygous for both *M. edulis* and *M. trossulus* Glu-5' alleles. These 60–80 mussels were subsequently genotyped at the remaining three markers. Individuals not scored as multilocus homozygotes for *M. edulis* or *M. trossulus* alleles at all markers (i.e., hybrids) were eliminated from further analysis. The combined results of all four markers were used to pick 20 individuals (n = 30 on 17 and 30 August) of each species for assaying reproductive condition.

Preserved individuals were transversely sectioned (2- to 3-mm thick) anterior of the byssal gland, dehydrated in an ascending alcohol series, cleared with Xylenes, and embedded in Paraplast (Howard & Smith 1983). Cross sections (5 μm) of each block were cut on a rotary microtome, placed on glass slides, stained with Shandon instant hematoxylin and eosin Y, and permanently mounted. Slides were examined using a compound microscope (Nikon LABPHOT-2) equipped with a video camera (Dage CCD 72). Images were digitized with a frame grabber (Flash Point 128,

Integral Technologies Inc.) and measurements made using image analysis software (Image Pro Plus; Media Cybernetics).

Reproductive state was measured by two separate methods. First, the gamete volume fraction (GVF) of all individuals was calculated as the area of reproductive tissue present in one microscopic field divided by the entire area (Bayne et al. 1978). Thus, estimates of GVF indicate the proportion of mantle that is comprised of reproductive tissue. The mean of five random fields (300×) was calculated for each individual and used in subsequent statistical analysis. In addition to the GVF, mean oocyte area was estimated for each female from 50 measurements (1200×) of the cross-sectional area of oocytes with a clearly visible nucleolus (Garrido & Barber 2001).

GVF data were analyzed using a three-way ANOVA for sample date, species, and gender. Oocyte data were evaluated with a two-way ANOVA across sample date and species. Both data sets were evaluated at α = 0.05 using simultaneous Bonferroni pairwise comparisons of sample level means. Statistical analyses were performed using Minitab 13.0, which automatically adjusts the Bonferroni α level to compensate for the total number of possible pairwise comparisons. Because all possible combinations of pairwise comparisons were not of interest, the α level was manually readjusted to account for the appropriate number of comparisons used in the analysis.

RESULTS

Gametogenesis in *M. edulis* (mean length 44.8 mm ± 3.7) and *M. trossulus* (mean length 44.3 mm ± 3.5) was highly synchronous at the East Bay site throughout 2000. Species-specific mean gamete volume fractions (estimated for both male and female mussels) were relatively low in February and increased steadily in both species from February to June. The peak mean GVF of 0.89 in *M. edulis* was identical to the 0.89 estimated for *M. trossulus* mussels sampled on 4 June. GVF remained high in both species throughout

TABLE 1.

Mytilus edulis, Mytilus trossulus: relative number of males, females, and undifferentiated mussels sampled in East Bay, 2000.

| | *Mytilus edulis* | | | *Mytilus trossulus* | | | |
	Males	Females	Undifferentiated	Males	Females	Undifferentiated	Totals
19 Jan	7	9	4	5	5	1	31
20 Feb	9	6	5	8	11	1	40
21 Mar	11	7	2	11	8	1	40
17 Apr	7	11	2	9	10	1	40
4 May	8	10	2	8	12		40
18 May	12	8	–	11	9		40
4 Jun	8	12		8	11		39
18 Jun	11	9		13	7		40
30 Jun	8	11		9	11		39
17 Jul	12	8		12	8		40
1 Aug	10	10		9	11		40
17 Aug	19	10	1	17	11		58
30 Aug	15	14		13	16		58
14 Sep	7	10	3	13	4	3	40
15 Oct	9	11		7	5	8	40
17 Nov	10	9	1	7	7	4	38
9 Dec	6	12	1	6	8	6	39
Totals	169	167	21	166	154	25	702

Undifferentiated individuals were not used in statistical analysis.

June and July and then declined precipitously between 17 July and 1 August samples among mussels of both species. Following this initial dramatic decline, a less pronounced decrease in GVF was observed up to the 15 October sampling date, after which GVF estimates were constant and nearly equal to those observed in February (Fig. 1).

Analysis of gender-specific patterns of GVF variation indicated that while gamete development in the females of both species was comparable to that of males, it lagged behind that of the males. For example, mean GVF estimates for females were consistently lower than those observed in males from February to April but by June these differences had disappeared. In addition, spawning in females resulted in a greater loss in GVF relative to males. Overall, males had an average yearly GVF approximately 10% higher than females for both *Mytilus edulis* and *M. trossulus*. Bonferroni pairwise comparisons ($\alpha = 0.05$) indicated a significant difference in GVF between males and females on 30 August (Fig. 2A and B).

Consistent with the graphic analysis, a three-way ANOVA revealed that significant differences in GVF occurred between date and gender but not between species. Significant interactions occurred between date and species and between date and gender resulting from the seasonality of gamete development. Gametogenic cycles (as defined by GVF) were the same for both species and there were no significant interactions between species and gender or date*species*gender (Table 2). With respect to the sharp decrease in GVF, Bonferroni analysis indicated that significant decreases in GVF at both the species and gender levels corresponded with the initial spawning period between 17 July and 1 August. Though differences occurred between sexes because of the high postspawn variation, spawning times were still highly synchronous.

Similar results were obtained using mean oocyte areas to assess gametogenic cycles. Mean oocyte areas increased sharply for both species from 21 March through 4 June. After 4 June, oocyte areas gradually increased until maxima were observed on 17 July (*Mytilus edulis* 678.6 μm^2 and *M. trossulus* 530.1 μm^2). A sharp decrease in mean oocyte areas occurred between 17 July and 1 August. After 1 August, there were increases in oocyte area until 30 August for *M. edulis* and 14 September for *M. trossulus*, followed by a less pronounced and protracted period of decline until 9 December (Fig. 3).

The two-way ANOVA for oocyte areas indicated a significant

Figure 2. (A) *Mytilus edulis*. Mean (±1 SD) gamete volume fraction for male vs. female mussels from East Bay, Maine, USA. (B) *Mytilus trossulus*. Mean (±1 SD) gamete volume fraction for male vs. female mussels from East Bay, Maine.

interaction between date and species (Table 3). The difference between species was caused by variation in mean oocyte size rather than a variation in the timing of gametogenic events; average yearly oocyte area was 338.2 μm^2 for *M. edulis* and 308.2 μm^2 for *M. trossulus*. Significant declines in species-specific oocyte area were observed between 17 July and 1 August, corresponding with a period of spawning indicated by GVF analysis. Additional

Figure 1. *Mytilus edulis, Mytilus trossulus*. Mean (±1 SD) gamete volume fraction for mussels from East Bay, Maine, USA.

TABLE 2.

Gamete volume fraction of *Mytilus edulis* and *Mytilus trossulus*: results of a three-way ANOVA testing the effects of date, species, and gender on the gametogenic cycle.

Source	df	Mean Square	F Value
Date	16	4.4869	142.53***
Species	1	0.0003	0.01
Gender	1	1.8650	59.24***
Date × species	16	0.1061	3.37**
Date × gender	16	0.0827	2.63**
Species × gender	1	0.0407	1.29
Date × species × gender	16	0.0293	0.93
Error	587		

** *P* < 0.01. ***P* < 0.001.
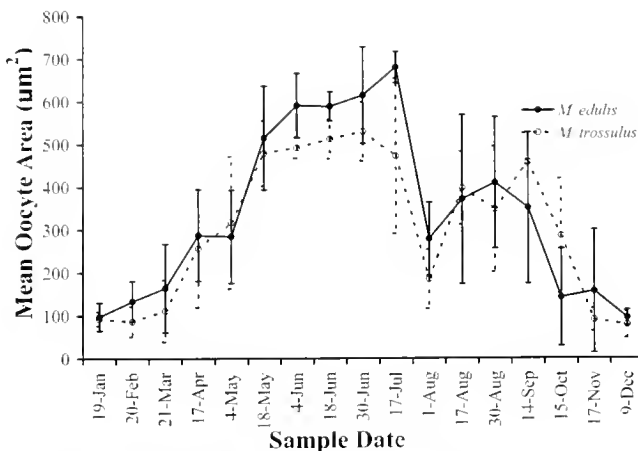
Figure 3. *Mytilus edulis, Mytilus trossulus.* Mean (±1 SD) oocyte area for mussels from East Bay, Maine.

significant decreases between dates were slightly out of phase, with the mean oocyte area of *M. edulis* decreasing from 14 September to 15 October and that of *M. trossulus* from 15 October to 17 November. A significant difference in oocyte area (t = 7.24, P < 0.001) was observed just prior to spawning on 17 July indicating that *M. edulis* spawned larger eggs than did *M. trossulus*. Mean shell length of females sampled on this date was not significantly different (t = 1.29, P = 0.220).

DISCUSSION AND CONCLUSIONS

The reproductive cycle of mussels in this population was highly seasonal which is typical of many benthic marine invertebrates in northern temperate zones. In this study, gonadal development was minimal through the winter as indicated by low GVF and small oocyte diameters. Increased gametogenic activity in spring corresponded to increasing water temperature and presumably food availability. A significant decrease in GVF and oocyte diameters, indicative of a major spawning event, took place in late July and involved a large proportion of the population. Interestingly, GVF for females increased slightly in samples collected after this initial spawning event. Such an increase could be caused by redevelopment of the gonad in preparation for a second spawning. However, we observed little histologic evidence of redevelopment in individual mussels that had already spawned. The predominant histologic feature at this time was empty follicles containing a few refractory oocytes. Thus, the few individuals that did not spawn or had only partially spawned after the peak-spawning event in late July were responsible for the observed increase in GVF.

TABLE 3.

Mean oocyte area of *Mytilus edulis* and *Mytilus trossulus:* results of a two-way ANOVA testing the effects of date and species on the gametogenic cycle.

Source	df	Mean Square	F Value
Date	16	1.6398	53.67***
Species	1	0.1236	4.04*
Date × species	16	0.0665	2.18**
Error	285	0.0306	

* P < 0.05, **P < 0.01, ***P < 0.001.

More importantly, the reproductive cycles of *Mytilus edulis* and *M. trossulus* sampled from this population were highly synchronous. For the year 2000 at the East Bay site, the results of this study indicate that interspecific fertilization between *M. edulis* and *M. trossulus* is possible based on spawning times. Similar findings have been reported elsewhere. Freeman et al. (1992) and Mallet and Carver (1995) observed synchronous reproductive patterns in populations of *M. edulis* and *M. trossulus* from Lunenburg Bay, Nova Scotia. Additionally, Toro et al. (2002) found that the initiation of spawning was coincident between these species and their hybrids in Trinity Bay, Newfoundland; although *M. trossulus* displayed a more protracted period of spawning at this location the variation alone was not sufficient to explain the limited numbers of hybrids observed. Thus, four studies covering a wide geographic region from Maine to Newfoundland have observed similar results all suggesting that hybridization is not limited solely by species-specific differences in spawning times.

It is possible that genetic identity is maintained between *M. edulis* and *M. trossulus* by a factor other than different spawning periods. Gamete recognition proteins have been shown to drastically reduce the hybridization potential between closely related taxa of marine invertebrates. Interestingly, molecular phylogenies suggest that *M. trossulus* is the most divergent of the blue mussel taxa (Rawson & Hilbish 1995). It has been recently shown that *M. edulis* and *M. trossulus* have also diverged significantly with respect to amino acid sequence at a sperm lysin locus (C. Riginos, pers comm). Divergence in gamete recognition proteins such as sperm lysin could act to limit hybridization between *M. trossulus* and other blue mussel taxa. Though no evidence of functional differentiation has been documented as yet, preliminary data indicates that cross-fertilization of *M. edulis* and *M. trossulus* is limited except at very high sperm concentrations (Rawson unpublished). Thus, future efforts should focus on more detailed observations of the spawning behavior of these two species as well as the potential for functional variation in gamete recognition proteins.

The present study found that *M. trossulus* had smaller mean oocyte size at maturity and presumably spawned smaller eggs than *M. edulis*. Given that *M. trossulus* has a higher reproductive output (Toro et al. 2002), it follows that similarly sized *M. trossulus* produced more (but smaller) eggs than *M. edulis*, which might provide a selective advantage for the more fecund *M. trossulus*. Similarly, *M. galloprovincialis* has a higher fecundity per unit length than *M. edulis* at Croyde in S.W. England, but genotypic ratios between these two species have not changed over time because of large numbers of small *M. edulis* (Gardner & Skibinski 1990). Smaller oocytes may also represent a response to environmental stress. Cobscook Bay in eastern Maine is near the southern distributional limit of *M. trossulus* (Rawson et al. 2001) and as such, may be a less than optimal environment for this species. However, *M. trossulus* from Newfoundland also produces smaller eggs, has a smaller size at first maturity than *M. edulis* (Toro et al. 2002), as well as a population structure containing a higher frequency of small *M. trossulus* individuals (Comesaña et al. 1999). Given that a difference in oocyte size has been observed in both Maine and Newfoundland it is more likely that this difference is the result of a difference in life history strategy rather than a response to environmental stress. Additional data are needed on extrinsic factors such as population structure, size at first maturity, reproductive output, and size dependent mortality to draw conclu-

sions concerning the intrinsic factors shaping the life history evolution of *M. edulis* and *M. trossulus*.

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MODELING OF FILTER-FEEDING BEHAVIOR IN THE BROWN MUSSEL, *PERNA PERNA* (L.), EXPOSED TO NATURAL VARIATIONS OF SESTON AVAILABILITY IN SANTA CATARINA, BRAZIL

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ABSTRACT   The aim of this study is to quantify and model the filter-feeding behavior of the mussel *Perna perna* feeding on natural seston. Models were generated that described each step of the feeding process and produced a predictive model of rates of food uptake by *P. perna* in culture areas from Southern Brazil. Feeding experiments using the biodeposition approach were conducted with mussels ranging in shell height from 3.94 to 9.22 cm of three sites, including turbid and clear water environments. Organic content of the seston (OCS, fraction) decreased as total particulate matter (TPM, mg L^{-1}) increased. The maximum filtration rate (FR, mg L^{-1}) measured for an individual mussel was 156.7 mg h^{-1} and was recorded when TPM was 33.9 mg L^{-1} and OCS was 0.18. Rejection rate of particles had a strong positive relationship with TPM, and an inverse relationship with OCS. Maximum rejection rate recorded was 124.1 mg h^{-1} and was measured under the same seston conditions as maximum filtration rate. Net organic selection efficiency by mussels (NOSE, fraction) was related to the amount of particulate organic matter (POM, mg L^{-1}) and particulate inorganic matter (PIM, mg L^{-1}) available in the water. NOSE was positive below PIM values of 2 mg L^{-1}, but had negative values when POM was above 3 mg L^{-1} and PIM between 2 and 15 mg L^{-1}, and positive values when POM was below 3 mg L^{-1} and PIM above 15 mg L^{-1}. Maximum NOSE was 1.71, when PIM was 1.02 mg L^{-1} and POM was 0.67 mg L^{-1}. Organic content of ingested matter (OCI, fraction) had a positive relationship with NOSE and TPM. Maximum OCI was 1.24 and was measured when TPM was 33.9 mg L^{-1}, OCS was 0.18, FR was 151.30 mg h^{-1}, and NOSE was 1.30. The net absorption efficiency of ingested organics (NAEIO) increased with increasing OCI in a hyperbolic relationship. The net organic absorption rate (NOAR, mg h^{-1}) increased with both FR and OCI. The coupling of the equations that described filter-feeding processes for *P. perna* in the STELLA software environment produced a robust model with relatively low complexity and specificity. The model can predict the *P. perna* feeding behavior in turbid or clear water and can be used with different species if the correct coefficients are used. The coupling of this feeding model with future models of energy budget, population dynamics, seston hydrodynamics, and primary production will be valuable for the evaluation of shellfish carrying capacity.

KEY WORDS: mussel physiology, model, *Perna perna*, STELLA

INTRODUCTION

Assessing carrying capacity, the environmental capacity for shellfish culture is generally approached using ecophysiological modeling (e.g., Brylinsky & Sephton 1991, Newell & Campbell 1998, Schölten & Smaal 1998). The inclusion of processes relative to rates of selectivity, rejection, and absorption by molluscan filter feeders is of primary importance for both ecosystem and local scales models (Smaal et al. 1998). Sessile suspension-feeders obtain energy by selectively feeding on seston, which includes a variable mixture of algae, detritus, and silt. Not only does the seston have a small fraction with nutritional value (Smaal & Haas 1997), but also the composition changes on time scales of minute to months (Grant 1993). The available organic content of the seston ranges from 5 to 80% (Bayne & Hawkins 1990). Such nutritional variability in the seston forces sessile organisms like mussels to maximize their energy intake and ultimately their net energy balance, by varying rates of feeding and digestion in response to seston concentration and organic content (Bayne et al. 1993).

The literature describing bivalve rates of filter feeding and digestion is extensive (see reviews by Bayne & Newell 1983, Griffiths & Griffiths 1987, Bayne 1993). However, recent findings suggest that previous studies have limited application because they used artificial diets, and it is unclear to what extent using artificial diets provides a realistic representation of "*in situ*" feeding behav-

ior (Bayne & Hawkins 1990). Normal feeding processes and behavior are better measured in experiments where the animals are allowed to feed on natural seston (Hawkins et al. 1996a, Wong & Cheung 2001, Gardner 2002).

Most research on the ecophysiological processes in shellfish has focused on temperate species (e.g., *Mytilus edulis*), and there has been limited work on tropical species and their environments (Hawkins et al. 1998a, Wong & Cheung 2001). Although bivalves use the same general selective mechanisms for food acquisition (Hawkins et al. 1998b), there are both intra- and inter-specific differences in feeding rates (Navarro et al. 1991). Describing the physiologic responses characteristic of each species is needed, rather than extrapolating data from other species (Gardner & Thompson 2001, James et al. 2001). There are likely to be a number of significant differences in tropical environments. Our understanding of the feeding physiology of *Perna perna* (Linnaeus, 1758) (Berry & Schleyer 1983, Bayne et al. 1984, van Erkon Schurink & Griffiths 1992) is limited to laboratory experiments using microalgae monocultures or a mix of microalgae species and silt. Furthermore, these studies were carried out in South Africa where cold south Atlantic currents are predominant; in contrast, the Brazilian coast has warm waters brought by central Atlantic currents. Such differences in temperature and productivity, and consequently in food availability and its organic content, will be reflected in ecophysiological differences of these filter feeders.

The aim of this study is to generate a model to predict food uptake by *P. perna* in culture areas of Southern Brazil, based on measurements of the filter-feeding process using natural seston,

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The model reproduce the sequential passage of food through the feeding steps of filtration, selection, rejection, ingestion, and absorption, and the calculation of each step is based on relationships either with quantity and quality of seston or with some of the preceding steps on the food processing sequence. Mussel aquaculture is a fast growing industry in Brazil and problems regarding the environmental capacity of this industry may occur in the near future. This research will have the capability to deliver information that can be incorporated into models of energy budget and growth as a function of stocking density, for use in planning and managing strategies of growing areas.

METHODS

Feeding experiments were conducted at three sites within mussel farms in Southern Brazil; Brito Cove (48°37'W, 27°46'S), Porto Belo (48°33'W, 27°8'S), and Armação de Itapocoroi (48°38'W, 26°58'S). Rope-cultured *P. perna* were collected from mussel farms at each site immediately before the experiments. All experiments were done on one to three occasions at each site and were exposed to natural differences in concentration and organic content of seston at each site and time (Table 1). Each site was arbitrarily classified as turbid or clear, based on total particulate matter (TPM). The clear site had TPM <5 mg L^{-1} (Porto Belo), while the turbid sites had TPM between 10–40 mg L^{-1} (Brito Cove and Armação do Itapocoroi).

The experiments were conducted on a raft containing a tray with 10 individual 350-mL plastic chambers. Eight individual mussels, cleared of epibiotic growth, were placed in separate chambers, with two chambers left empty to act as blanks. Seawater was pumped into the chambers with flow rates in each compartment between 150 and 200 mL min^{-1}; these were adjusted at the beginning of the experiment. A baffle between the mussel and the inflow water provided a homogeneous distribution of water flow inside the feeding chambers (Fig. 1). The mussels were initially left undisturbed for 1 h to acclimate, after which time all biodeposits on the bottom of the chambers were removed. Once the experiment started the mussels were allowed to feed for four hours, during which time all feces and pseudofeces for each mussel were separately collected using a pipette immediately after being released. For each individual mussel the feces and pseudofeces collected in each hour were stored in separate test tubes on ice. A 2-L sample of inflow seawater was collected every 20 min for the determination of seston concentration and organic content. Water

temperature and salinity were monitored every hour during the experiment.

After 5 hours of feeding the experiment was terminated and the mussels and samples were transported back to the laboratory on ice. The biodeposit samples were homogenized by repeat pipetting and filtered onto pre-ashed and weighed Whatman glass microfibre (serie C) 1.2 μm (GF/C) filters (25 mm or 47 mm diameter). The samples were rinsed with 15 mL distilled water to remove salts and dried at 60°C for 48 h before re-weighing and calculation of the total sample dry weight. Each sample was then ashed at 450°C for 4 h prior to final weighing, allowing calculation of both of the ash (inorganic) and ash-free (organic) mass of each filtered sample. To account for settled material in the chamber, the mean organic and inorganic weight of sediment material collected from the blank chambers was subtracted from the mean organic and inorganic weight of the collected feces and pseudofeces. To determine seston concentration and organic content, three 300–400 mL samples from the 2 L of inflow seawater collected were filtered onto pre-ashed and weighed Whatman GFC filters (25 mm diameter) and dried, ashed, and weighed in the same way as the biodeposit samples. The mean of the three values was calculated. The seston concentration and organic content for each hour was calculated as an average of the three 2-L samples taken during that hour.

To determine the lag time between when the mussels consumed food and when feces and pseudofeces production occurs, mussels starved for one day in the laboratory were fed green microalgae. Green feces were observed within an hour of feeding therefore we assumed the gut transit time to be 1 h. Green pseudofeces were seen within minutes of the microalgae being added. Therefore, in the analysis of the field data the quantity and content of the feces was correlated with seston concentration and organic content in the preceding hour. No time lag was assumed in correlation with pseudofeces production. Feeding and absorption parameters were defined and calculated (Table 2) using procedures outlined in Hawkins et al. (1996a, 1998b), and using the mean of the hourly feeding rate obtained for each mussel throughout the experiment. For the regression analysis, seston concentration and organic content were the means of the hourly values obtained during each experimental run.

From each mussel used in the experiments, total length was measured and soft tissue removed, dried at 60°C for 48 h, and weighed. To standardize findings and allow comparison of results with other studies, feeding responses were expressed per 1 g dry weight using $Y_s = (W_s/W_e)^b * Y_e$, where Y_s is the corrected

TABLE 1.

Summary of environmental parameters and mussel size range for each day the experiments were run. Data of environmental characteristics are the mean ± SD. TPM: total dry particulate mass; POM: total particulate organic matter; OCS = organic content of TPM; ND = no data.

Experiment Days	Location	Environmental Characteristics n = 12					Mussels	
		TPM (mg L^{-1})	POM (mg L^{-1})	OCS (fraction)	Temperature (°C)	Turbidity (NTU)	Shell Length (cm)	Dry Weight (g)
14/03/01	Brito's Cove	29.6 ± 11.9	4.7 ± 3.7	0.15 ± 0.05	25.7 ± 0.5	ND	5.05–8.90	0.398–3.522
14/04/01	Brito's Cove	12.4 ± 3.0	1.2 ± 0.3	0.10 ± 0.02	25.5 ± 0.5	7.7 ± 1.7	5.70–8.16	0.485–2.034
05/06/01	Brito's Cove	9.8 ± 3.1	1.0 ± 0.1	0.11 ± 0.03	22.2 ± 0.3	4.5 ± 1.6	5.72–8.27	0.628–2.517
07/02/01	Porto Belo	1.7 ± 0.3	0.7 ± 0.3	0.41 ± 0.17	29.0 ± 0.4	0.5 ± 0.2	5.74–8.28	1.177–3.257
31/03/01	Porto Belo	1.6 ± 0.4	0.3 ± 0.1	0.20 ± 0.08	26.5 ± 0.4	1.0 ± 0.1	5.05–9.22	0.618–3.103
07/07/01	Porto Belo	1.2 ± 0.3	0.4 ± 0.1	0.36 ± 0.09	18.3 ± 0.0	0.3 ± 0.1	4.11–8.22	0.343–2.757
26/05/01	A. Itapocoroi	4.6 ± 0.7	2.3 ± 0.4	0.10 ± 0.08	21.3 ± 0.2	2.8 ± 0.8	6.00–8.49	0.857–3.087
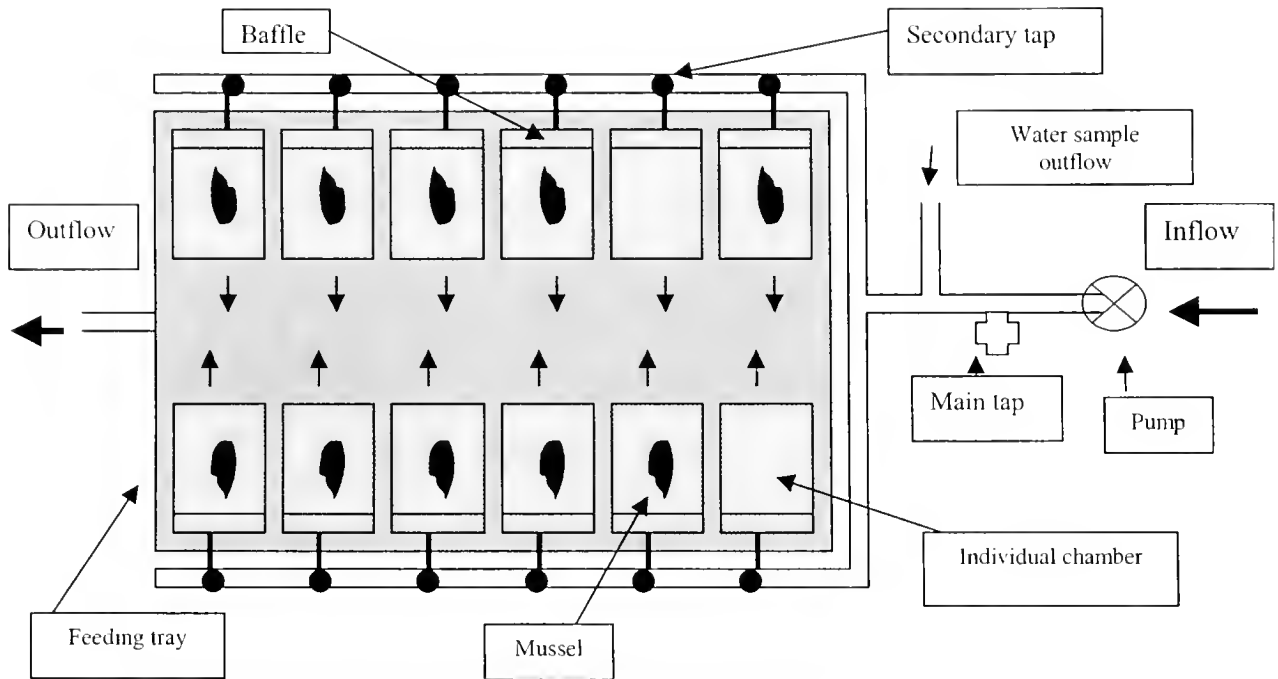
Figure 1. Schematic diagram of the feeding tray used in the biodeposition experiments.

parameter, W_s is the standard weight (1 g), W_e is the weight or length of the experimental animal, Y_e is the uncorrected parameter, and b is the average size exponent (Hawkins et al. 2001). However, given the absence of spawning synchronicity (Marques et al. 1991), there is high variability in mussel dry weight within the same population in every time of the year. Therefore, we used the shell length equivalent of 1 g dry weight (6.26 cm) and the power exponent that scales the feeding rates with SL (b ≈ 1.85). The power exponent has previously been used for *Mytilus galloprovin-*

cialis (Pérez Camacho & Gonzáles 1984, Navarro et al. 1996) and for *P. perna* (Berry & Schleyer 1983).

All statistical analysis was done using SPSS for Windows, Version 10 (SPSS Inc., Chicago, IL) and Sigma Plot. Multiple regression models were fitted using the step-wise technique, entering the most significant independent variable at the first step and then adding or deleting independent variables until no further variables could be added to improve the overall fit. The coupling of the equations to produce an integrated feeding model and the posterior

TABLE 2.

Definitions and descriptions of the calculation of separate components of feeding behavior.

Parameter	Acronym	Units	Calculation
Particulated inorganic matter	PIM	mg L^{-1}	Ash free dry weight of TPM
Particulated organic matter	POM	mg L^{-1}	TPM-PIM
Organic content of seston	OCS	fraction	POM/TPM
Clearance rate	CR	l h^{-1}	(mg inorganic matter egested both as true feces and pseudofeces h^{-1} ÷ (mg inorganic matter available l^{-1} seawater)
Total filtration rate	FR	mg h^{-1}	(mg inorganic matter egested both as true feces and pseudofeces h^{-1}) ÷ (1-OCF)
Organic filtration rate	OFR	mg h^{-1}	CR × mg total particulate organic matter l^{-1} seawater
Inorganic filtration rate	IFR	mg h^{-1}	CR × mg total particulate inorganic matter l^{-1} seawater
Organic content of filtered matter	OCF	fraction	OFR ÷ FR
Rejection rate	RR	mg h^{-1}	mg total pseudofeces egested h^{-1}
Inorganic rejection rate	IRR	mg h^{-1}	RR–ash free mg total pseudofeces egested h^{-1}
Organic rejection rate	ORR	mg h^{-1}	RR–IRR
Net organic selection efficiency	NOSE	fraction	[1-(organic fraction within pseudofeces) ÷ (OCS)]
Ingestion rate	IR	mg h^{-1}	FR–RR
Organic ingestion rate	OIR	mg h^{-1}	OFR–ORR
Inorganic ingestion rate	IIR	mg h^{-1}	IFR–IRR
Net organic ingestion rate	NOIR	mg h^{-1}	[FR × (OCS)]–[RR + (organic fraction within pseudofeces)]
Organic content of ingested matter	OCI	fraction	NOIR ÷ (FR–RR)
Net absorption efficiency from ingested organics	NAEIO	fraction	NOAR ÷ NOIR
Net organic absorption rate	NOAR	mg h^{-1}	NOIR–[(mg total true feces egested h^{-1}) × (organic fraction within true feces)]

sensitivity analysis was done using STELLA research software (High Performance Systems, Inc., Hanover, USA).

RESULTS

Organic content of seston (OCS) decreased as TPM increased (Fig. 2, Table 3). Clearance rate of mussels decreased from 10 to 5 L h^{-1} as TPM increased from <5 to 30 mg L^{-1} and OCS increased from <0.15 to 0.40. The parabolic relationship (Fig. 3A), suggests that $P.\ perna$ pumps more water under low TPM (<10 mg L^{-1}) and OCS (<0.20) conditions.

Filtration rate (FR, mg h^{-1}), rejection rate (RR, mg h^{-1}), ingestion rate (IR, mg h^{-1}), and net organic absorption rate (NOAR, mg h^{-1}) were all related to TPM and OCS (Table 3, Fig. 3B, C, D, and E). The maximum filtration rate measured was 156.7 mg h^{-1} when TPM was 33.9 mg L^{-1} and OCS was 0.18. Rejection rate had a strong positive relationship with TPM and inverse relationships with OCS. The maximum rejection rate recorded was 124.1 mg h^{-1}, which represented 83% of filtered matter, and was measured under the same seston conditions as the maximum filtration rate. Pseudofeces production was observed when TPM levels were as low as 2 mg L^{-1}, suggesting a very low threshold for pseudofeces production in this species.

Net organic selection efficiency (NOSE, fraction) was controlled by the proportion of particulated organic and inorganic matter in the water (POM, mg L^{-1} and PIM, mg L^{-1} respectively). Higher NOSE values were observed on the lower and higher extremes of PIM. Negative NOSE values, a minimum of −0.56, was recorded at intermediate values of PIM and POM, and positive values were recorded when POM was below 3 mg L^{-1} and PIM above 15 mg L^{-1}. Maximum NOSE was 1.71 when PIM was 1.02 mg L^{-1} and POM was 0.67 mg L^{-1} (Fig. 3F, Table 3). Organic content of ingested matter (OCI, fraction) had a positive relationship with NOSE and it was not strongly affected by TPM. Maximum OCI was 1.24 when TPM was 33.9 mg L^{-1}, OCS was 0.18, FR was 151.3 mg h^{-1}, and NOSE was 1.30 (Fig. 4A, Table 3). The net organic ingestion rate (NOIR, fraction) was below 10 mg h^{-1} when mussels were feeding on TPM levels below 5 mg L^{-1}, but this increased to 25 mg h^{-1} when TPM was above 30 mg L^{-1} and ingestion rate was ca. 50 mg h^{-1} (Fig. 4B, Table 3).
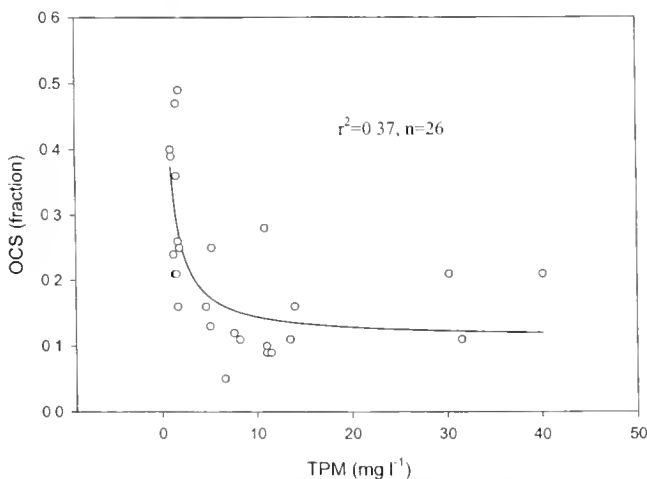
Figure 2. Relationship between the average organic content (OCS, fraction) and average total particulate mass (TPM, mg L^{-1}) of seston within the experimental feeding conditions. Data are the mean of three replicate determinations per condition. Refer to Table 3 for equation.

TABLE 3.

Equations describing the relationships between the different steps of the filter-feeding mechanism in $P.\ perna$ and the environmental variables TPM (mg L^{-1}) and OCS (fraction). Refer to Table 2 for acronyms and rate calculations.

Variables	Equation	Equation	Statistical Information
OCS, TPM	OCS = 1/(2.55 + 0.47 × TPM)	(1)	r^2 = 0.37, F = 14.85, df = 1.25, P < 0.001
CR, TPM and OCS	CR = 13.83(±2.91)−0.65(±0.17) × TPM − 47.85(±22.82) × OCS + 0.011(±0.004) TPM^2 + 83.81(±43.02) OCS^2	(2)	r^2 = 0.27, F = 7.57, df = 4.80, P < 0.0001
FR, TPM and OCS	FR = 68.77(±21.45)−0.12(±1.28) × TPM − 370.10(±167.72) × OCS + 0.07(±0.03) TPM^2 + 565.81(±330.89) OCS^2	(3)	r^2 = 0.70, F = 45.85, df = 4.80, P < 0.0001
RR, TPM and OCS	RR = 52.43(±14.90)−0.97(±0.89) × TPM − 362.47(±116.54) × OCS + 0.02(±0.02) TPM^2 + 589.79(±229.92) OCS^2	(4)	r^2 = 0.72, F = 52.16, df = 4.80, P < 0.0001
IR, TPM and OCS	IR = 24.14(±6.96)−0.62(±0.41) × TPM − 117.37(±54.46) × OCS + 0.03(±0.01) TPM^2 + 203.97(±107.45) OCS^2	(5)	r^2 = 0.54, F = 23.83, df = 4.80, P < 0.0001
AR, TPM and OCS	AR = −1.93(±2.42)−0.006(±0.14) × TPM − 13.35(±18.97) × OCS + 0.01(±0.003) TPM^2 −3.62(±37.44) OCS^2	(6)	r^2 = 0.79, F = 75.85, df = 4.80, P < 0.0001
NOSE, PIM and POM	NOSE = 0.30(±0.08)−0.21(±0.05) × PIM + 1.03(±0.24) × POM + 0.01(±0.004) PIM^2 −0.20(±0.08) POM^2	(7)	r^2 = 0.40, F = 13.41, df = 4.80, P < 0.0001
OCI, TPM and NOSE	OCI = 0.13(±0.03)−0.001(±0.005) × TPM + 0.27(±0.06) × NOSE + 0.0002(±0.0001) TPM^2 + 0.19(±0.05) NOSE^2	(8)	r^2 = 0.75, F = 62.58, df = 4.80, P < 0.0001
NOIR, TPM and IR	NOIR = 1.37(±0.50)−0.23(±0.06) × TPM + 0.11(±0.06) × IR + 0.01(±0.001) TPM^2 + 0.004(±0.001) IR^2	(9)	r^2 = 0.92, F = 223.74, df = 4.80, P < 0.0001
NOAR, OCI and FR	NOAR = −2.62(±0.55)−0.012(±0.011) × FR + 15.73(±0.06) × OCI + 0.0006(±0.0001) FR^2 −9.22(±1.93) OCI^2	(10)	r^2 = 0.90, F = 196.31, df = 4.80, P < 0.0001
NAEIO, OCI	NAEIO = 2.08(±0.33) OCI/(1 + 0.22(±0.26) OCI)	(11)	r^2 = 0.42, F = 57.64, df = 4.80, P < 0.0001
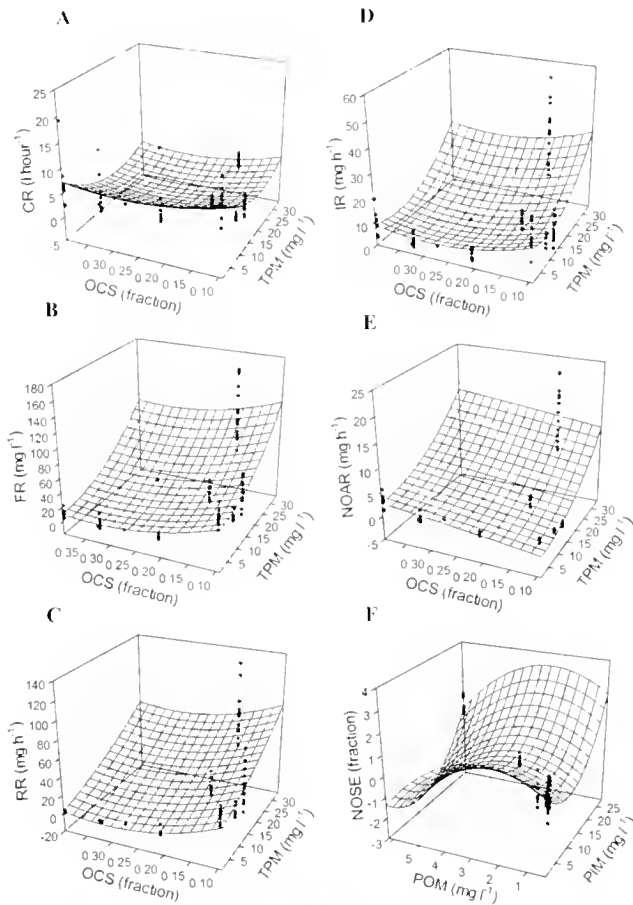
Figure 3. *Perna perna*. The relationship between total particulate matter (TPM, mg L^{-1}) and organic content of seston (OCS, fraction) and (A) clearance rates (CR l h^{-1}), (B) filtration rate (FR, mg h^{-1}), (C) rejection rate (RR, mg h^{-1}), (D) Ingestion rate (IR, mg h^{-1}), (E) net organic absorption rate (NOAR, mg h^{-1}). Net organic selection efficiency (NOSE, fraction) is plotted against particulated organic and inorganic matter (PIM and POM, mg L^{-1}) (F). Refer to Table 3 for equations and statistics.

Both the net absorption efficiency of ingested organics (NAEIO, fraction) and the net organic absorption rate (NOAR, mg h^{-1}) had a hyperbolic relationship with the organic content of ingested matter (Fig. 4C and 5, Table 3). NOAR was essentially controlled by quantity (filtration rate) and quality (OCI) of food passing through the digestive system (Fig. 4C, Table 3). The absorption rate across the experiments varied from 21.84 mg h^{-1} (TPM 33.18 mg L^{-1}, OCS 0.18) to −0.69 mg h^{-1} (TPM 10.09 mg L^{-1}, OCS 0.10).

The differential equations, logical functions, and starting values of the state variables used to couple the equations describing the filter-feeding processes for *P. perna* in STELLA are listed on Table 4. We produced a robust model with relatively low complexity and specificity. Figure 6A depicts the conceptual diagram of the *P. perna* feeding process as a function of TPM and OCS. The sub-model inserted inside the "ingested matter" variable (Fig. 6B) reproduces the absorption of organic matter and the passage of inorganic matter as inert material through the gut. As the model was based on natural seston in both turbid and clear environments and feeding rates measured in these environments, we believe that it has incorporated feeding adaptations by *P. perna* for both kinds

of environments. Model predictions and observed data of FR, RR, IR, NOSE, OCI, and AR of mussels in a range of TPM between 2 and 40 mg L^{-1}, are shown in Fig. 7A, B, C, D, E, and F, respectively, showing that predicted values satisfactorily reproduce the main trends of feeding behavior observed in *P. perna*.

As bivalve feeding behavior is mainly controlled by concentration and organic content of seston (Hawkins et al. 1998b), it is likely that this model is sensitive to these forcing functions (TPM and OCS). To verify the model sensitivity to changes in the coefficients of the equation that predicts OCS as a function of TPM, we ran the model three times, varying the coefficients values. Each coefficient (EQ. (1), Table 3, Fig. 2) was varied by ±10% from its standard value, and the sensitivity was measured by the following equation:

$$S = [\partial x / x] / [\partial P / P]$$

where (S) is a measure of sensitivity, x refers to model outputs at the end of the integration period in the standard model, and ∂x is the change in the value of x brought about by varying the model
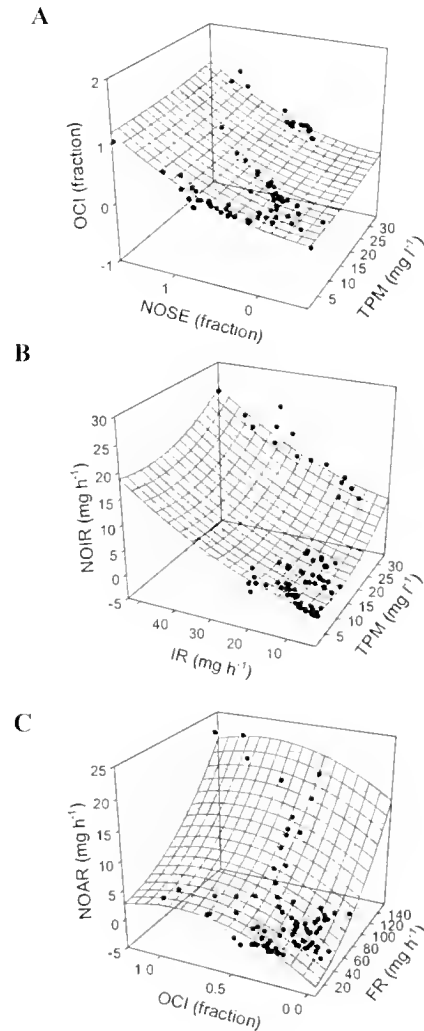
Figure 4. *Perna perna*. The relationship between (A) net organic selection efficiency (NOSE, fraction), total particulate matter (TPM, mg L^{-1}) and organic content of ingested (OCI, fraction); (B) ingestion rate (IR, mg h^{-1}), TPM and net organic ingestion rate (NOIR, mg h^{-1}); (C) net organic absorption rate (NOAR, mg h^{-1}), filtration rate (mg h^{-1}) and OCI. Refer to Table 3 for equations and statistics.
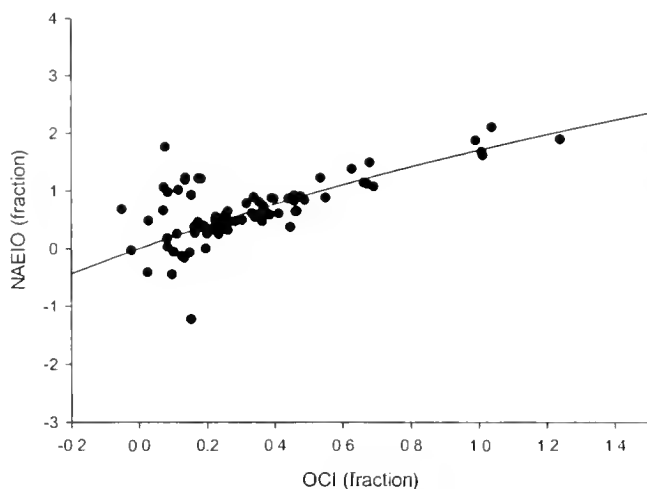
Figure 5. *Perna perna.* **The relationship between the organic content of ingested (OCI, fraction) and the net absorption efficiency from ingested organics (NAEIO, fraction). Refer to Table 3 for equations and statistic.**

coefficient. Similarly, the denominator measures the variation in the coefficient of interest divided by its standard value. This equation compares the percentage change in the model outputs with a given percentage change in one of the model parameters. The value of (S) was averaged for positive and negative variations and the results of the model outputs (absorbed matter, pseudofeces, and feces produced) for the coefficients relating TPM and OCS are shown in Table 5. The output most sensitive to variation in the relationship between seston TPM and OCS was pseudofeces production, as a result of increased or decreased rejection rate.

DISCUSSION

This study showed that *P. perna*, like other mussels, controlled its feeding mechanisms to achieve an optimum organic absorption rate independent of fluctuations in seston concentration and quality. It is important to note that the range of TPM recorded was within normal values during the year for other bivalve aquaculture locations in Southern Brazil (Suplicy, unpub. data). Therefore, the TPM range experienced in the experiments and included in the model are directly applicable to Brazilian shellfish farms conditions. Although seasonal changes in feeding physiology were not examined in this study, time series data of TPM, POM, and OCS from 1998 to 2002 do not suggest strong seasonal changes in food availability in the sub-tropical waters of Santa Catarina, (Suplicy et al. unpublished data). Similarly, the condition index of *P. perna* does not follow a seasonal trend, as seen in *Mytilus edulis* (Navarro & Iglesias 1995), because spawning occurs throughout the year with small peaks in summer, autumn and spring (Marques et al. 1991). Therefore, we believe that the findings reported here can be used to predict feeding physiology throughout the year.

Food availability (TPM and OCS) was the main forcing function of the models produced, therefore characterizing the available seston is of primary importance to generate a model to predict food uptake by *P. perna*. Data for Southern Brazil showed that the organic content of available food decreased as TPM increased, a common pattern in many estuaries and sheltered bays both in temperate and tropical waters (Hawkins et al. 1996a, 1998b). This reduction of the organic proportion is a function of the dilution of organic particles when resuspended silt increases particulate inor-

ganic matter on the water column (Fréchette & Grant 1991, Widdows et al. 1979)

The methods used in this study to estimate clearance rates of filter feeders were less accurate than the methodology proposed by Hawkins et al. (1998a, 1999) for measurements using natural seston. The most appropriate method to accurately measure clearance rates by bivalves is controversial (Cranford 2001, Riisgård 2001, Widdows 2001). As new methods are being developed, new models about how these animals control their food uptake are being produced. It is agreed that mussels do not always filter at their maximal rate in their natural environment (Riisgård 2001, Widdows 2001). This may be due to a regulation of feeding processes in response to changes in quantity and quality of suspended particles, salinity, temperature, and the presence of pollutants in the water (Widdows 2001). In this study only 27% of the variation clearance rates of mussels using TPM and OCS as independent variables was explained, and the significant proportion of the remaining variance in clearance rate in POM was not. In their experiments, however, Hawkins et al. (1999) increased the amount of the variability in clearance rate explained from 13–53% when they included Chl and TPM as independent variables instead of only POM. Although all precautions proposed by Iglesias et al. (1998) in the use of the biodeposition method for suspension-feeding

TABLE 4.

Equations used in the formulation of feeding physiology model in STELLA.

$TPM = GRAPH$ (time-series)
$OCS = 1/(2.55 + 0.47 * TPM)$
$PIM = 0.22 + 0.81 * TPM$
$POM = TPM\text{-}PIM$
$FR = 68.77\text{-}0.12\ TPM\text{-}370.10\ OCS + 0.07\ TPM^2 + 565.80\ OCS^2$
Filtered matter $(t) =$ Filtered matter $(t - dt) + (FR - RR - IR) * dt$
INIT Filtered matter $= 219.81$
$RR = 52.43 + 0.97\ TPM\text{-}362.47\ OCS + 0.02\ TPM^2 + 589.79\ OCS^2$
Pseudofeces $(5) =$ pseudofeces $(t - dt) + (\text{rejection}) * dt$
$IR =$ filtration-rejection
Ingested $(t) =$ ingested $(t - dt) + (\text{ingestion}' - NIIR - NOIR) * dt$
INIT ingested $- 36.46$
$NOIR = 1.37 - 0.23\ TPM + 0.11\ IR + 0.01\ TPM^2 + 0.004\ IR^2$
$NIIR =$ ingested-NOIR
Inorganic $(t) =$ inorganic $(t - dt) + (NIIR - IM\ \text{on gut}) * dt$
Organic $(t) =$ organic $(t - dt) + (NOIR - OM\ \text{on gut}) * dt$
OM on gut $=$ organic
Im on gut $=$ inorganic
INIT organic $= 13.17$
INIT inorganic $= 23.29$
Ingested matter $=$ food on gut $+$ organic $+$ ingested $+$ inorganic
Food on gut $(t) =$ food on gut $(t - dt) + (OM\ \text{on gut} + IM\ \text{on gut} - \text{absorption}' - \text{egestion}') * dt$
INIT food on gut $= 0$
$NOSE = 0.30 - 0.21\ PIM + 1.03\ POM + 0.01\ PIM^2 - 0.20\ POM^2$
$OCI = 0.13 - 0.001\ TPM + 0.27\ NOSE + 0.0002\ TPM^2 + 0.19\ NOSE^2$
$NOAR = -2.62 + 0.012\ RF + 15.73\ OCI + 0.0001\ FR^2 - 9.22\ OCI^2$
Absorption$' = NOAR$
Absorption $=$ absorption$'$
Absorbed matter $(t) =$ absorbed matter $(t - dt) + (\text{absorption}) * dt$
INIT absorbed matter $= 0$
Egestion$' = IM\ \text{on gut} + (NOIR\text{-}NOAR)$
Egestion $=$ egestion$'$
Feces $(t) =$ feces $(t - dt) + (\text{egestion}) * dt$
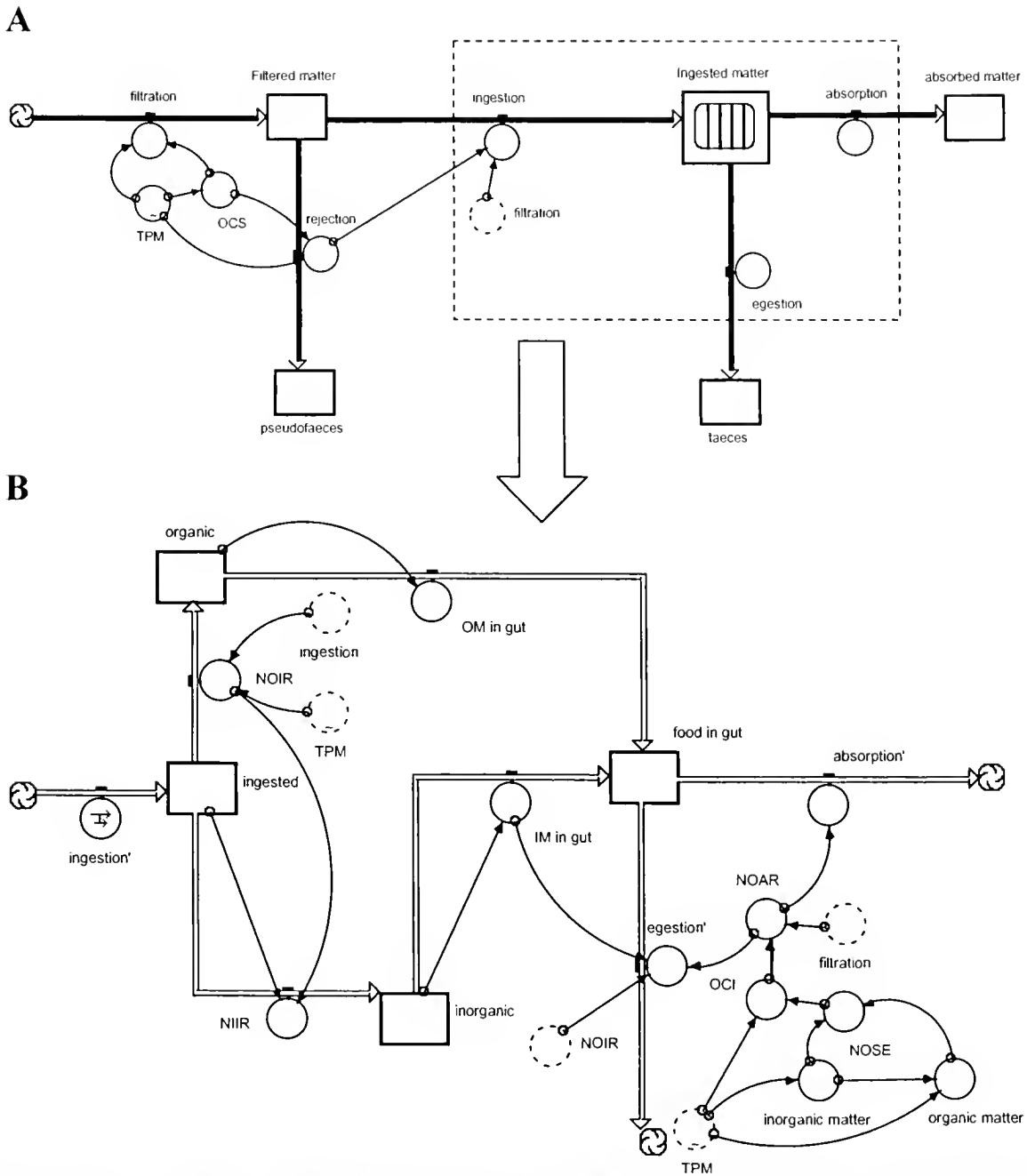
Figure 6. (A) Diagram of the feeding processes of a general filter-feeding bivalve, used on the modeling of *P. perna* feeding physiology. (B) Diagram of the sub-model of a mussel gut showing the absorption of organic matter and feces production. Refer to Tables 2 and 3 for variables and acronyms and Table 4 for logical and differential equations.

measurements were taken in this study, it seems that the new methodology proposed by Hawkins et al. (1998b, 1999) is more appropriate for studies using natural seston. It seems that qualitative features of seston may be just as important as availability of food in mediating feeding responses (Hawkins et al. 1998b). The general trend for decreasing clearance rates as seston concentrations increase, however, is seen in other studies (Hawkins et al. 1999, Hawkins et al. 1998b, Wong & Cheung 2001). There are many methods to quantify concentration and organic content of seston in feeding experiments. Most use mass measurements of total particulate matter available in the seston (TPM, mg L^{-1}),

particulate organic matter available in the seston (POM, mg L^{-1}), and the ratio between these two variables, which is the organic content of seston (OCS, fraction). Recent findings suggest that clearance rate is primarily dependent on seston availability measured in terms of total volume, rather than mass. This helps to explain the confusing variation in clearance rate reported by many studies and stresses a need to consider volumetric constrains in bivalve feeding studies (Hawkins et al. 2001). More detail about the seston organic fraction can be obtained if the carbon:nitrogen ratio is measured, which can vary from <4 to >26 (Bayne & Hawkins 1990). The measurement of the biologically available
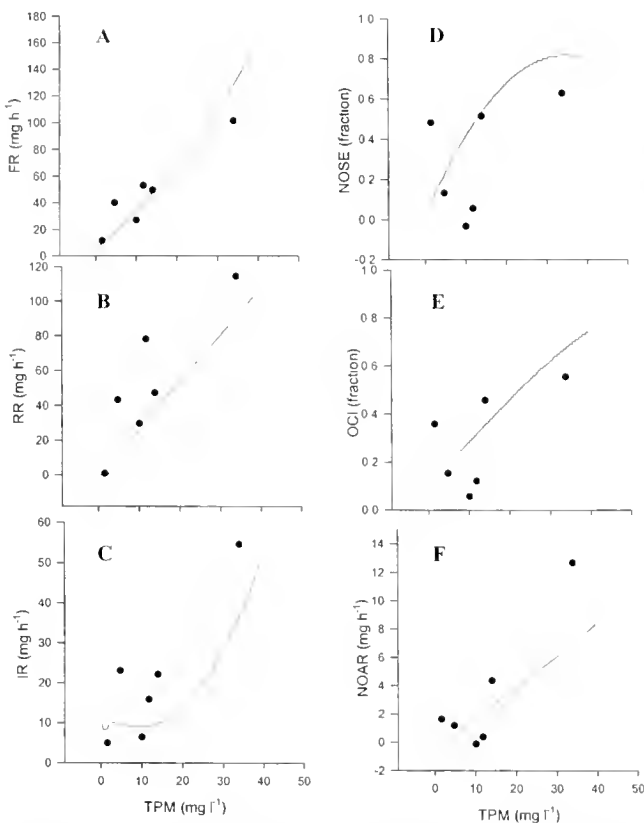
Figure 7. Predictions of the *P. perna* filter-feeding model produced on STELLA. (A) filtration rate (FR, mg h^{-1}), (B) rejection rate (RR, mg h^{-1}), (C) ingestion rate (IR, mg h^{-1}), (D) selection efficiency (NOSE, fraction), (E) organic content of ingested matter (OCI, fraction), and (F) net organic absorption rate (NOAR, mg h^{-1}), in the range of total particulate matter (TPM, mg L^{-1}) observed in this study.

organic carbon and nitrogen in the water and in associated biodeposits can provide, not only more accurate measurements of the clearance rate, but also important information about the absorption of these elements by filter feeders.

The biodeposition approach demands that the gut residence time is correctly calculated to generate accurate physiologic feeding rates. As starved animals were used to estimate gut passage time this may have over-estimated the normal passage time. However, our estimates are comparable to those from other biodeposition studies using *Perna canaliculus*, in which the gut passage time for non-starved mussels was 80 min, and no delay time was assumed for *Perna viridis* (Hawkins et al. 1998a).

Perna perna appeared to selectively enrich the organic content of ingested matter by rejecting particles of higher inorganic con-

TABLE 5.

Sensitivity analysis of absorbed matter, pseudofeces and feces production for the coefficients a and b in the equation OCS = 1/(a + b * TPM).

	Absorbed Matter	Pseudofeces	Feces
a	0.202	0.637	0.118
b	0.232	0.734	0.136

tent before ingestion. This selection efficiency was a function both of filtration rate and the proportion between inorganic and organic particulated matter available in the water. The increase in selection efficiency at higher filtration rates is important, because this helps to maintain nutrient acquisition independent of fluctuations in seston organic content (Hawkins et al. 1998a). Extreme values of net organic selection efficiency measured in this study (NOSE >1 or <0) must be considered with caution as they are probably measurement errors associated inadvertently with collecting settled sediment when collecting biodeposits. This would effectively alter the organic ratio of pseudofeces. Extreme values were observed in 15% of measurements. Nevertheless, NOSE values recorded in this study (>0.7) suggest that *P. perna* is efficient in selecting organic particles available in the seston. Hawkins et al. (1996a) recorded NOSE values of up to 0.5 in *M. edulis*, and Hawkins et al. (1998b) report maximum NOSE of 0.7 for *P. viridis*.

Maximum net organic ingestion rate (NOIR) recorded for *P. perna* was 24.05 mg h^{-1} and occurred when TPM was 33.93 mg L^{-1} and OCS was 0.18. This is similar to values obtained for *P. canaliculus* in New Zealand, that showed maximum organic ingestion rate of 27.3 ± 6.3 mg h^{-1} (Hawkins et al. 1999), and for *P. viridis* in Malaysia with a recorded rate of 24.8 ± 3.6 mg h^{-1} (Hawkins et al. 1998a). These rates are considerably higher than the maximum organic ingestion rate of 6.5 mg h^{-1} reported for *M. edulis* (Hawkins et al. 1997). The growth rates of *P. perna* in southern Brazil are among the fastest reported for mussels in the *Perna* genus, reaching commercial size (80 mm) in 8–10 mo (Suplicy, unpub. data). This rapid growth is probably related to higher weight-specific rates of energy acquisition and higher water temperatures in the sub-tropical waters of southern Brazil.

Data from this study suggested that *P. perna* takes advantage of the abundant organically rich seston available in Brazilian waters throughout the year by maintaining high ingestion rates. There is evidence that when ingestion rate is high absorption efficiency is high and gut residence time is short (Bayne et al. 1988). Furthermore, the proportion of gut volume occupied by ingesta may vary, thereby facilitating an increase in absorption efficiency with little change in the gut passage time (Bayne et al. 1987). Widdows et al. (1979) report that absorption efficiency declines as ingestion rate increases and food progresses from the digestive gland to the intestine. However, this pattern may be counterbalanced by elevated organic content of ingested matter due to selection processes (this study, Hawkins et al. 1999) that positively increase the absorption efficiency and ultimately the absorption rate. Similarly to the considerations raised for NOSE values, negative absorption rate values are not biologically meaningful and must be considered with caution as these could be caused by collection of inorganic sedimented material together with mussel feces. Negative absorption rates were measured in 7% of measurements.

The integration of all equations from Table 4 with STELLA software resulted in a reductionistic and deterministic non-linear model that reproduces the feeding processes of *P. perna* in both clear and turbid environments. The general conceptualization of the diagram was based on the description of the bivalve filter-feeding process provided at the TROPHEE workshop (Bayne 1998, Hawkins et al. 1998b), and final equations were based on intensive measurements that enabled calibration of the outputs. This feeding model may not be a perfect reproduction of the bivalve feeding process, but the objective is to provide a useful tool to understand and predict feeding processes of this species. The model includes a complete sequence of steps in the feeding process

that may cause an accumulation of predictive error (Grant & Bacher 1998). Its value lies in the ability to provide an understanding of the interaction between a mussel farm and the environment, for example, the amount and organic content of biodeposits released into the water column and sediment beneath the farm.

Sensitivity analysis indicated that model predictions of absorbed matter and feces production were less affected by changes in the relationship between TPM and OCS than model prediction of pseudofeces production. This analysis suggests that predicted absorption would stay reasonably invariable if the model is applied to environments with different seston concentration and organic content. Therefore, mussels maintain a reasonably constant organic ingestion rate in varying seston conditions by compensating for low organic content of the seston through adjusting selection efficiency and rejection of inorganic matter as pseudofeces.

This feeding model can be used as an important tool for the understanding of how *P. perna* interact with the culture environment. Current studies are under way to integrate this feeding model with energy budget and population dynamics of *P. perna*. Further coupling of the *P. perna* biologic models with physical models of seston hydrodynamics and models of primary production are also planned, and this approach will allow the development of carrying capacity analysis for suspended mussel culture in sub-tropical environments like the southern Brazilian coast.

ACKNOWLEDGMENTS

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PHENOTYPES OF THE CALIFORNIA MUSSEL, *MYTILUS CALIFORNIANUS*, CONRAD (1837)

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ABSTRACT The morphological variability of *Mytilus edulis* complex species has been the subject of a variety of studies. However, the morphological variability of *Mytilus californianus* has not been studied. We found that there are some *M. californianus* without some of the shell characteristics mentioned by Conrad (1837) in the original description of this species. The most remarkable difference was the absence of radial ribs on the exterior of the shell; thus, we tested the presence of at least two phenotypes in *M. californianus*. Six hundred ninety five *M. californianus* of different sizes were collected from the locations La Mina del Fraile, La Bufadora, and La Salina in Baja California. For comparison, 58 *M. galloprovincialis* were collected from an aquaculture facility at Rincón de Ballenas in Bahía de Todos Santos, Baja California. Fourteen morphometric measures and the weight of the shell were measured and a principal component analysis (PCA) and a logistic regression (LR) were carried out to find differences between mussels studied and for obtaining a prediction to assign the phenotypes. The presence of ribs, small ligament margin, a narrow posterior byssal retractor muscle scar, and shell weight were the discriminating characters between two groups in *M. californianus*. These findings confirm the presence of at least two phenotypes in this species, in all mussel sizes and the studied locations. The LR correctly assigned 99.28% of the shells to each phenotype, and it considered only eight out of the fifteen morphometric measures. The PCA showed a clear morphologic difference between both phenotypes of *M. californianus* and *M. galloprovincialis*. The original description of this species by Conrad in 1837 was done taking into account only the phenotype with ribs.

KEY WORDS: *Mytilus californianus*, *Mytilus edulis* complex species, morphological variability, phenotypes

INTRODUCTION

The marine mussels of the genus *Mytilus* are widely distributed in boreal and temperate waters of the Northern and Southern Hemispheres (Soot–Ryen 1955). Prior to protein separation and molecular genetics, about nine species of the genus *Mytilus* were recognized (Gosling 1992). Today, about five species are considered belonging to this genus: *Mytilus californianus*, *Mytilus coruscus* (Gould 1861), *Mytilus edulis* (Linné 1758), *Mytilus galloprovincialis* and *Mytilus, trossulus* (Gould 1850) (Seed 1992). The three later species are considered to be the *M. edulis* complex species because they are very close in their external shell morphology. These species have caused a variety of studies for their differentiation, taking into account shell morphology, allozyme, and molecular genetics (Beaumont et al. 1989, Figueras & Figueras 1983, McDonald & Koehn 1988, Koehn 1991, McDonald et al. 1991, Geller et al. 1994, Inoue et al. 1995, Rawson & Hilbish 1995, Ohresser et al. 1997). *Mytilus californianus* has never been questioned as a separate species from the *Mytilus edulis*-complex because of its characteristic radiating ribs, strong growth lines, and heavy shell in larger specimens; these characters allow easy differentiation from the other species in adult stage (Soot–Ryen 1955, Koehn 1991). During a field study of *Mytilus californianus* in an exposed rocky shore of the West Coast of Baja California, México, we found some specimens with typical external characteristics of the shell described by Conrad in 1837. Other individuals, however, showed a smooth shell without coarse ribs, similar to the *M. edulis* complex form, but with heavy shells. A question arises from this observation, are there two or more phenotypes of *M. californianus*? This study focused on answering this question.

MATERIALS AND METHODS

In March 1997, 129 *M. californianus* (size range from 16.8–113.5 mm, mean size 59.1 mm) were collected from an exposed rocky shore along the intertidal zone during low tide in La Mina del Fraile, B. C. México. In August 2000, 278 mussels were collected from La Salina (size range from 27.6–98.1 mm, mean size 56.9 mm) and 288 from La Bufadora (size range from 44.7–88.1 mm, mean size 54.1 mm), B. C. México, both areas exposed rocky shores, and the mussels were collected during low tide along the intertidal zone. Additionally, 58 *M. galloprovincialis* were obtained from culture long-lines placed at Bahía de Todos Santos, B.C. (size range from 47.2–85.3 mm, mean size 61.4 mm) and they were used to compare the morphological characteristics with *M. californianus* (Fig. 1).

The shells of all mussels were cleaned with a brush and water stream and dried in an oven at 40°C overnight. The following morphometric dimensions were measured for differentiation among mussel groups and species (Fig. 2): number of ribs on the external shell (rib), maximum shell length (sl), height (sh) and width (sw), the position of maximum shell width (a) along the dorso-ventral axis, the maximum dimensions of the anterior (aams) and posterior (pam) adductor muscle scars, the maximum length (lbr) and width (wbrs) of the posterior byssal retractor muscle scar, the location of the center of the posterior adductor muscle scar along both the anterior-posterior (pam-pm) and dorso ventral (pam-vm) axes, the size of the hinge plate (hp) and number of hinge teeth, the distance between the palial line and the ventral shell margin (pl-vm) midway along the shell, and ligamentary margin (lm). All measurements were taken with an electronic digital caliper to the nearest 0.1 mm and were in accordance with those taken by Beaumont et al. (1989). The dry shell weight (w) was also measured for all mussels and it was included in the analyses. A principal component analysis (PCA) was carried out to discriminate between phenotypes, followed by a logistic regression (LR)

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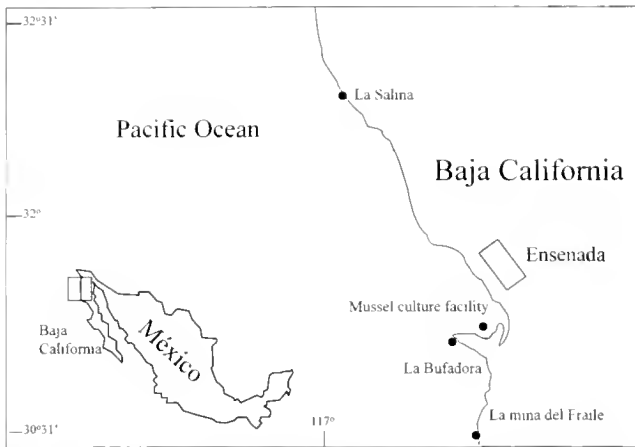
Figure 1. Map showing the three exposed rocky shore localities where *Mytilus californianus* was collected: La Mina del Fraile, La Bufadora, and La Salina. The blue mussel *Mytilus galloprovincialis* was collected from a culture facility at Bahía de Todos Santos, Baja California, México.

(Sokal & Rohlf 1995) to fit the mussel phenotypes. A two way ANOVA was used to determine possible differences between mussel size among locations and phenotypes. A comparison through the PCA between both phenotypes of *M. californianus* with *M. galloprovincialis* was carried out. These analyses were done using the JMP statistical package by SAS Institute Inc.

RESULTS

Fifteen principal axes were extracted from the morphological and shell weight data of *M. californianus* (Table 1). The first component explained 76% of total variance and was considered as a size axis. A low correlation of size with number of ribs suggests that the number of ribs does not change with mussel size. The second component accounted for 7% of the variation indicating

morphological differences. Mussels with a high number of ribs were correlated with this second component separating two groups (Fig. 3). Also, in the second component, mussels with higher shell weight (w), but with small ligament margin (lm) and a narrow posterior byssal retractor muscle scar (wbrs) were correlated. The rest of the components had eigen values smaller than the unit accounting for about 13% of the total observed variance and thus, no further explanation is necessary (Table 1). These data provide statistical support to validate the presence of two phenotypes in *M. californianus*: A (with ribs) and B (without ribs), and they were visually differentiated in mussels of different sizes (Fig. 4). After separating both groups in all locations, 68% of the total mussels belong to phenotype A and the rest to phenotype B.

Both phenotypes of *M. californianus* were present in the three locations. The two way ANOVA showed size differences among mussel from different locations, ($F_{2,689} = 6.58$, $P = 0.001$), but the phenotype mean size was similar ($F_{1,689} = 0.02$, $P = 0.892$) without interaction ($F_{2,689} = 2.11$, $P = 0.122$).

Once the PCA differentiated two phenotypes, the LR (Sokal & Rohlf 1995) was used to determine whether it was possible to assign any *M. californianus* to a particular phenotype, taking into account morphological variables, excluding the number of ribs. The LR considered only eight morphological measures from the original fifteen to assign any mussel to a particular phenotype, ($\chi^2_{(8\ d.f.)} = 184.73$, $P < 0.0001$; Lack of fit: $\chi^2_{(686\ d.f.)} = 684.5$, $P = 0.51$). The coefficients of the eight morphometrical variables were positive for: shell length (sl $= 0.104$) and height (sh $= 0.247$), posterior adductor muscle scar (pam $= 0.483$), the distance between the palial line and the ventral shell margin (pl-vm $= 0.708$), and weight (w0.145); while the shell width (sw $= -0.281$), the position of maximum shell width (a $= -0.333$), and the ligamentary margin (lm $= -0.348$) were negative. After applying the LR we found that 99.28% were correctly assigned to each phenotype. Thus, the visual, PCA and LR confirm the presence of two phenotypes in the Californian mussel.

Results of the PCA between morphometric data and weight of
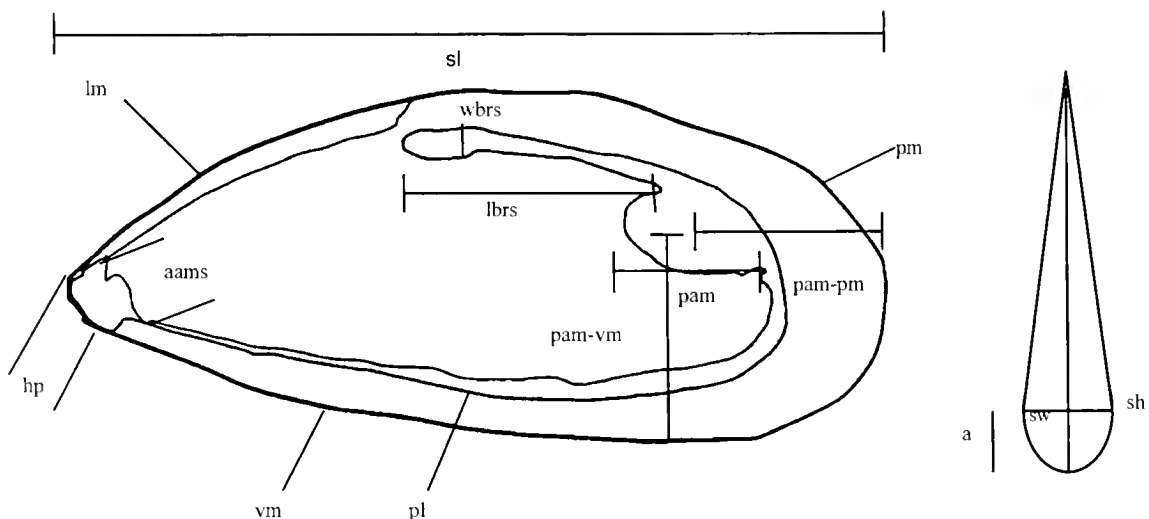
Figure 2. Morphometric dimensions measured for *Mytilus californianus* and *Mytilus galloprovincialis*: number of ribs on the external shell (rib), maximum shell length (sl), height (sh) and width (sw), the position of maximum shell width (a) along the dorso-ventral axis, the maximum dimensions of the anterior (aams) and posterior (pam) adductor muscle scars, the maximum length (lbr) and width (wbrs) of the posterior byssal retractor muscle scar, the location of the center of the posterior adductor muscle scar along both the anterior-posterior (pam-pm) and dorso ventral (pam-vm) axes, the size of the hinge plate (hp), and number of hinge teeth, the distance between the palial line and the ventral shell margin (pl-vm) midway along the shell and ligamentary margin (lm).

TABLE 1.

Eigenvalues, explained variance (%), cumulative explained variance (%) and eigenvectors (rounded to two decimal places) from the principal component analysis of *Mytilus californianus* morphometric data from the Pacific coast of Baja California.

	Principal Components														
	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15
Eigenvalue	11.45	1.07	0.59	0.45	0.29	0.25	0.22	0.18	0.14	0.11	0.08	0.08	0.05	0.03	0.02
Variance(%)	76.31	7.12	3.93	3.02	1.94	1.69	1.46	1.17	0.96	0.71	0.51	0.50	0.35	0.21	0.12
Cum. var.(%)	76.31	83.43	87.36	90.37	92.31	94.00	95.46	96.64	97.59	98.3	98.81	99.32	99.67	99.88	100.00
	Eigenvectors														
rib	0.02	0.95	0.17	-0.02	0.02	-0.02	-0.06	0.06	0.20	0.07	0.04	0.09	0.04	-0.03	0.01
sl	0.29	-0.04	0.02	-0.15	-0.06	-0.10	-0.10	-0.03	0.20	0.03	-0.13	-0.12	0.19	0.16	-0.86
sh	0.28	0.05	-0.11	-0.01	0.04	0.01	0.03	0.32	0.07	-0.23	-0.16	-0.12	-0.82	0.15	-0.03
sw	0.29	-0.06	0.04	-0.09	0.02	-0.12	-0.06	-0.12	0.04	-0.01	0.24	0.17	-0.17	-0.86	-0.11
a	0.27	-0.06	-0.27	0.09	0.14	-0.09	0.15	0.62	0.22	-0.30	0.07	0.26	0.42	-0.02	0.12
aams	0.26	0.01	0.05	-0.11	-0.04	0.92	-0.14	0.03	-0.13	-0.09	0.06	0.01	0.10	-0.04	-0.01
pam	0.27	0.05	0.14	-0.33	0.29	-0.08	0.48	0.17	-0.39	0.34	0.24	-0.35	0.06	0.05	0.04
lbr	0.28	-0.04	0.00	0.03	-0.08	-0.05	-0.20	-0.13	0.45	-0.04	0.00	-0.70	0.16	-0.07	0.35
wbrs	0.21	-0.13	0.76	0.59	0.04	-0.01	0.07	0.10	0.00	0.01	-0.05	0.06	0.00	0.04	-0.01
pam-pm	0.28	0.04	0.14	-0.31	0.00	-0.13	0.03	-0.17	-0.24	-0.20	-0.75	0.13	0.15	-0.08	0.22
pam-vm	0.28	0.00	0.15	-0.22	-0.04	-0.21	-0.17	-0.33	-0.12	-0.45	0.51	0.20	-0.01	0.38	0.11
hp	0.25	0.05	-0.34	0.35	0.61	0.09	0.16	-0.48	0.11	0.09	-0.07	0.13	-0.02	0.10	-0.01
lm	0.27	-0.18	0.04	-0.23	-0.16	0.00	-0.13	0.04	0.33	0.64	-0.01	0.41	-0.10	0.21	0.23
pl-vm	0.26	0.10	-0.27	0.32	-0.07	-0.17	-0.57	0.16	-0.54	0.23	0.00	-0.08	0.06	0.02	0.02
w	0.25	0.12	-0.25	0.28	-0.69	0.02	0.51	-0.20	-0.08	0.01	0.03	0.02	0.02	0.01	0.00

M. californianus and *M.galloprovincialis* are shown in Table 2. The fist component explained 71% of the total variance and was also considered a size axis. The number of ribs had a low correlation with this axis. The second component explained 10% of the total variance. The number of ribs (rib) and the shell width (sw) were positively correlated with this component whereas the width of the adductor muscle scar (wbrs) was negatively correlated. Components 3 to 15 accounted for 19.4% of the total variance, but their eigen values were smaller than one and they are not explained further. The graphic presentation of the component scores shows a clear difference between phenotypes of *M. californianus* and among these phenotypes and *M. galloprovincialis* (Fig. 5).

DISCUSSION

Figure 4 shows different shell characteristics among *M. californianus* specimens, and the PCA and LR support this visual perception confirming that there are two phenotypes in *M. californianus*, one with ribs and the other with a smooth shell, and Figs. 4 and 5 show a morphological differentiation between both phenotypes of *M. californianus* and *M. galloprovincialis*.

The original description by Conrad (1837) for *Mytilus californianus* was done from specimens collected by Thomas Nuttal in upper California. Conrad describes "shell ovate elongated, inflated; anterior margin straight; posterior side emarginated; ribs not very numerous, slightly prominent broad, rounded; lines of growth very prominent". This description agrees with phenotype A studied here, where the rib number goes from 4 to 14 and they are very prominent. In phenotype B, however, the ribs are not distinguishable and the growth lines are very prominent. Intraspecific differences in shell sculpture on specimens from different habitats have been noted in several gastropod species from the genus *Littorina* (Struhsaker 1968, Johannesson et al. 1993, Rugh 1997). These differences have been related to the degree of wave exposure—extreme ribbed and with nodes forms live on dry raised benches, not generally subject to horizontal water swash; while extreme smooth forms predominate on low, moist benches subject to strong wave swash. It is probable that a similar relation occurs among *M. californianus* phenotypes and wave action or their position along the intertidal zone. We are carrying out a field study to explore this. The presence of ribs has been correlated with shell strength; the ribbed mussel *Geukensia emissa* has a stronger shell than *M. edulis*, this strength was correlated with shell mass, shell curvature and valve thickness (Majewski 1995). This could also be
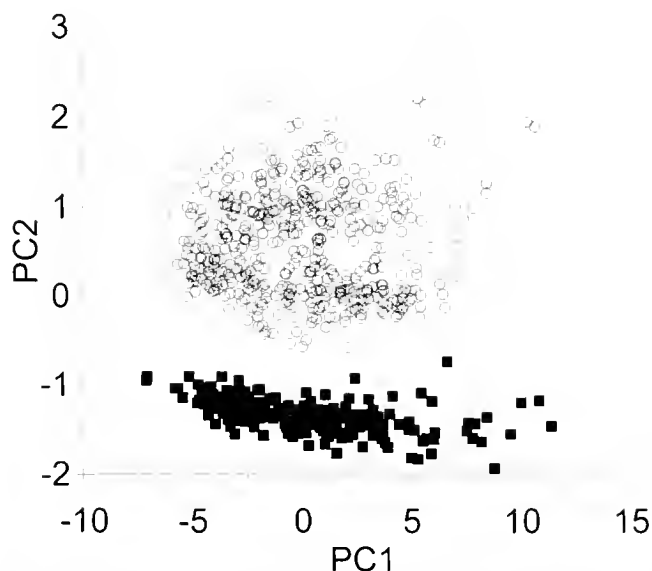
Figure 3. Principal component scores plots between PC2 vs. PC1 for *M. californianus*. Phenotype A, open circles; Phenotype B, bold squares.
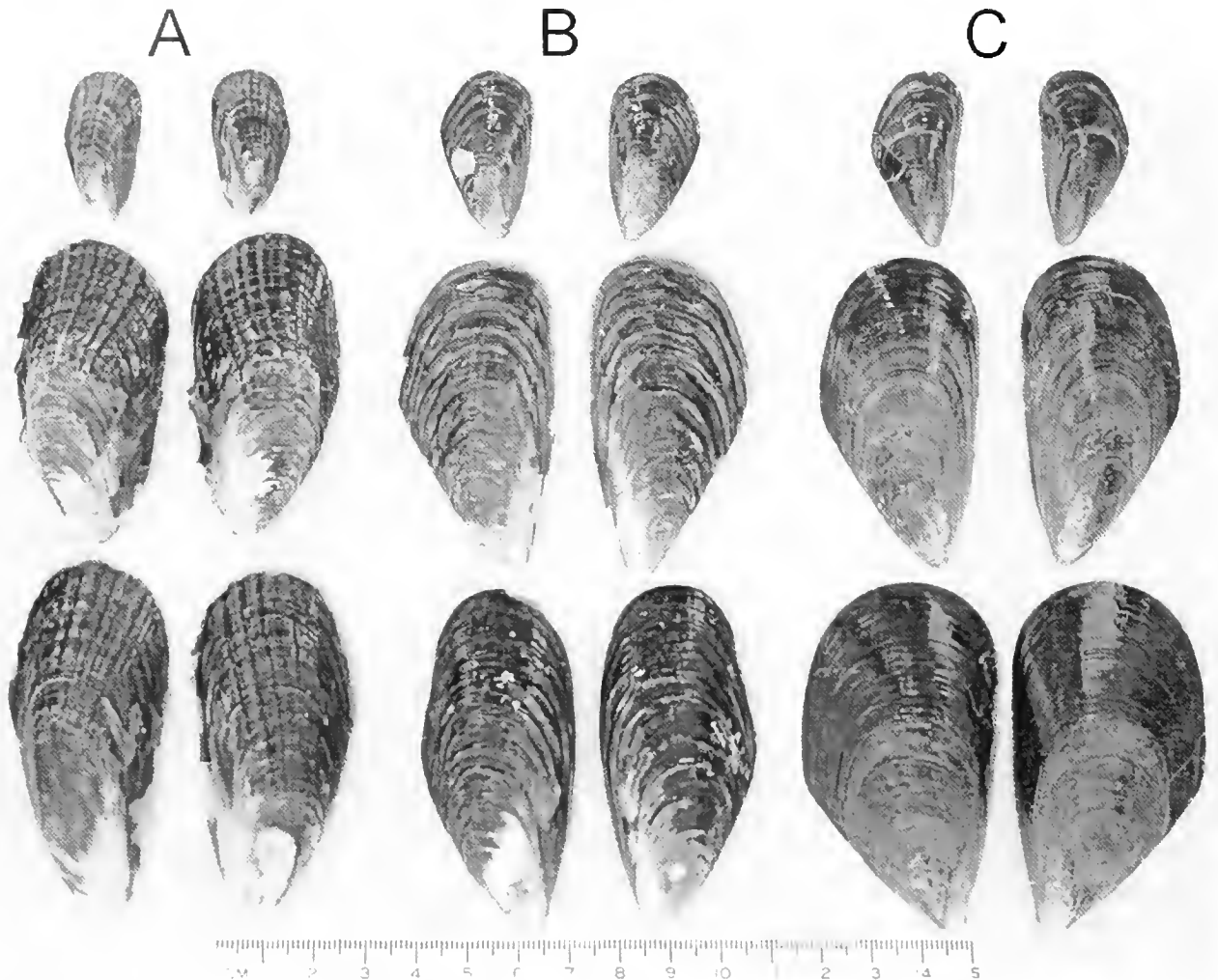
Figure 4. *Mytilus californianus* of different sizes showing the two phenotypes found in this study: (A) with ribs and (B) no ribs. For comparison, *Mytilus galloprovincialis* of similar sizes also were included in figure (C). Note that different phenotypes appeared since young specimens.

TABLE 2.

Eigenvalues, explained variance (%), cumulative explained variance (%) and eigenvectors from the principal component analysis between *Mytilus californianus* and *Mytilus galloprovincialis* morphometric data from the Pacific coast of Baja California.

	Principal Components														
	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15
Eigenvalue	10.587	1.502	0.751	0.459	0.311	0.277	0.263	0.216	0.173	0.143	0.100	0.079	0.074	0.045	0.018
Variance (%)	70.581	10.013	5.009	3.063	2.075	1.844	1.755	1.442	1.155	0.956	0.667	0.527	0.496	0.299	0.118
Cum. var (%)	70.581	80.594	85.603	88.665	90.741	92.584	94.339	95.781	96.937	97.893	98.560	99.086	99.582	99.882	100.000
	Eigenvectors														
rib	0.015	0.621	0.737	0.001	−0.044	−0.011	−0.030	−0.077	0.020	0.215	0.052	0.062	0.086	−0.032	0.013
sl	0.301	−0.043	−0.010	−0.139	−0.017	0.014	−0.118	−0.125	−0.101	0.169	0.007	−0.004	−0.161	−0.209	−0.866
sh	0.278	−0.205	0.182	0.182	−0.268	−0.142	−0.265	−0.074	0.171	0.016	−0.034	−0.077	−0.156	0.764	−0.047
sw	0.229	0.394	−0.339	−0.291	0.279	0.269	0.277	0.040	0.000	0.126	−0.211	0.077	0.273	0.467	−0.074
a	0.281	0.016	−0.163	0.220	−0.222	0.073	−0.169	0.028	0.588	0.294	−0.383	0.166	0.198	−0.322	0.113
aams	0.266	0.000	0.036	−0.102	−0.114	−0.722	0.592	0.046	0.087	−0.100	−0.094	−0.010	0.019	−0.065	−0.006
pam	0.275	0.051	0.074	−0.334	−0.228	0.298	0.048	0.454	0.317	−0.264	0.344	−0.388	−0.080	−0.092	0.036
lbr	0.292	0.037	−0.083	−0.023	0.144	0.022	0.030	−0.179	−0.216	0.459	−0.098	−0.480	−0.467	−0.110	0.353
wbrs	0.184	−0.476	0.428	0.145	0.582	0.237	0.287	0.094	0.185	0.051	−0.020	0.102	0.021	−0.004	−0.016
pam-pm	0.286	0.007	0.091	−0.326	−0.061	0.107	−0.122	−0.002	−0.170	−0.318	−0.257	0.580	−0.432	−0.073	0.222
pam-vm	0.276	−0.201	0.190	−0.166	−0.074	0.034	−0.197	−0.211	−0.344	−0.258	−0.309	−0.317	0.573	−0.109	0.098
hp	0.259	0.030	−0.035	0.553	−0.373	0.294	0.336	0.222	−0.453	0.031	0.093	0.135	0.055	−0.019	−0.012
lm	0.281	−0.169	−0.094	−0.223	−0.007	−0.128	−0.136	−0.138	−0.075	0.341	0.652	0.329	0.283	−0.016	0.210
pl-vm	0.258	0.253	−0.144	0.325	0.187	0.074	0.044	−0.585	0.215	−0.477	0.268	−0.053	−0.062	−0.074	0.016
w	0.253	0.223	−0.101	0.274	0.437	−0.339	−0.432	0.516	−0.147	−0.129	0.027	−0.018	0.037	−0.040	0.000
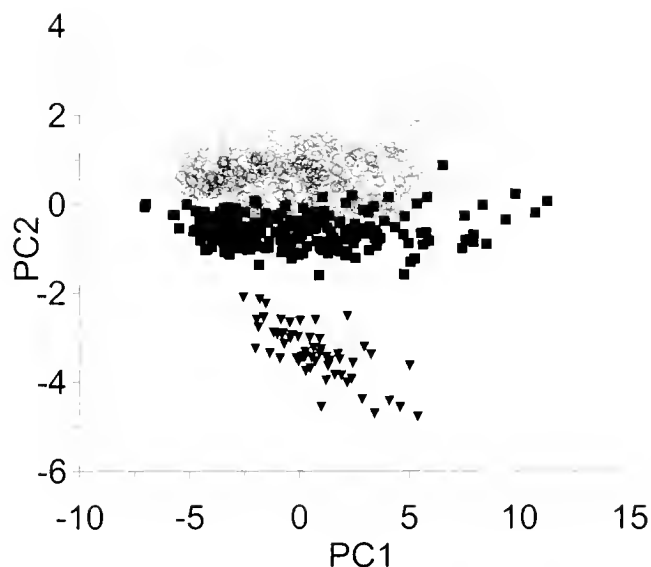
Figure 5. Principal component scores plots between PC2 vs. PC1 for *M. californianus* Phenotype A, open circles; Phenotype B, bold squares; and *M. galloprovincialis* bold triangles.

the case for *M. californianus* where the presence of ribs might indicate a stronger shell.

In accordance with Seed (1968), variations in the *M. edulis* shell form can be attributed to differences in age, habitat, growth rate, and density. Old mussels have heavier shells, down-turned divergent umboes, and varying degrees of incurvature of the ventral shell margin than the young ones do. In this study, small and large individuals showed similar morphometric characteristics; therefore, the age or size of these mussels (which grow in the same habitat) seems to have little influence on the variability of the studied morphological characters. In relation to the habitat, Seed (1968) comments that in areas free of predators (like the upper shore) old individuals are common, whereas in areas where the mussel turnover is rapid there is a predominance of young mussels. Also, the presence of predators can affect shell morphology. *M. edulis* has been found to have a smaller shell length, height and width with larger posterior adductor muscle, thicker shell, and more meat per shell volume when a starfish was present (Reimer & Tedengren 1996). In the Baja California region, *M. californianus* is the dominant species where there is high wave action, whereas *M. galloprovincialis* is the dominant species in protected bays with thinner shell and more meat than *M. californianus* (Harger 1970, Harger 1972). It has been observed that shore level has an influence on the morphology and physiology of *M. galloprovincialis* in the Adriatic see (Dalla Via et al 1987). Low shore level mussels have higher and narrower shells and a higher dry weight ratio whereas high shore mussels have a higher oxygen consumption rate. When cultivated *Mytilus edulis* was transplanted between two different locations there were some morphological differences that were considered to be due to genetic variation (Stirling & Okumus, 1994). The same characters found in parents of distinct ecotypes also occurred in progeny raised in the laboratory thereby indicating that the phenotypic differences have a genetic basis (Struhsaker 1968). The presence of two phenotypes and similar morphometric characteristics of the shell in small and large *M. californianus* in all three locations indicates not only some similarity among environments but it also strongly suggests that the presence of ribs is genetically produced. To our knowledge, there is no record on hybridization between *M. californianus* and *M. galloprovincialis*, which could result in a heavy shell without ribs. Our morphological results showed a clear difference between both phenotypes of *M. californianus* and *M. galloprovincialis*, which may suggest that phenotype B of *M. californianus*, is not the result of hybridization with *M. galloprovincialis*. Further studies using genetic markers will help to discard whether there has been any degree of introgression between these two species due to hybridization, which has been found in other *Mytilus* species (Geller et al 1994).

ACKNOWLEDGMENTS

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ADJUSTMENTS OF *LIMNOPERNA FORTUNEI* (BIVALVIA: MYTILIDAE) AFTER TEN YEARS OF INVASION IN THE AMERICAS

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ABSTRACT   *Limnoperna fortunei* (Dunker, 1857) or golden mussel invaded South America through the Río de la Plata estuary in 1991. Ten years later, the golden mussel lives in the main rivers of the Plata Basin. The gonadal cycle and the population settlement in a temperate climate are discussed in this article. This basic knowledge is needed to assist industries that may suffer the effects of macrofouling and also increment the ability to predict potential invasions of other countries. The study of population density and reproductive cycle was performed in Rio de la Plata estuary, Argentina. The temporal variation of population density from data of settlement and age structure collected between 1991 and 2001 is presented. The reproductive cycle between August 1998 and March 2000 was analyzed. Through the analysis of oocyte percentages four gonad spawning events were observed. The spawning events appear regulated by temperature changes. After the initial increase in population density following the invasion, there was a decrease. The population appeared stabilized at one third of the initial peak.

KEY WORDS: invasion, *Limnoperna fortunei*, freshwater, bivalve, reproductive cycle, Neotropical Region

INTRODUCTION

Limnoperna fortunei (Dunker 1857), or golden mussel, is a freshwater invasive bivalve, from the southeast of Asia. It invaded South America in 1991, through the Río de la Plata estuary. This represents the first record of *L. fortunei* for the American continent. Ten years later, the golden mussel lives in the main rivers of one of the most important Basins of the Neotropical Region (Bonetto 1994), the Plata Basin (the Río de la Plata, and the Uruguay, Paraná, and Paraguay rivers). Since 1999, this species invaded the Guaiba Basin in the south of Brazil (Mansur et al. 1999). The golden mussel spreads 240 km/year, upstream along the Plata Basin, (Darrigran & Ezcurra de Drago 2000).

The golden mussel attaches to every available hard substrate. This lifestyle (epifaunal) is atypical in local freshwater bivalves. The attachment capability and the great adaptability and reproductive capacity of these mussels make this species very effective invaders (Darrigran 2000). The mussels impact on the natural environment (displacement of native species—Darrigran et al. 1998b, Darrigran et al. 2000—or change of native fish diet—Penchaszadeh et al. 2000) as well as on human activities (macro fouling in fresh water (Darrigran 2000, Darrigran & Ezcurra de Drago 2000).

Detailed information about the life cycle of this harmful invasive species provides a basis for the development and application of control strategies. The impact caused by this species in human activities (plugging of water intake for industrial cooling, power generation, and potable water) resembles what happened in the north hemisphere with the zebra mussel *Dreissena polymorpha* (Pallas, 1771). The study of reproductive cycle, age structure and temporal density variation, is essential to generate sustainable techniques for golden mussel prevention and control.

Details of the reproductive cycle, and the population settlement in temperate climate are discussed in this article. This type of knowledge is not only essential to assist biologists and ecologists in the industries which may suffer from this new economic-environmental problem in the Neotropical Region, but it is also necessary for predicting potential invasions of other countries in the north hemisphere such as USA (Ricciardi 1998) and southern Europe.

TABLE 1.

Date and number of specimens histologically processed per sample.

Date	N	Size range (mm)	Males	Females
23/08/98	27	0.6–2.5	17	10
25/09/98	30	0.6–2.6	23	17
30/10/98	29	0.4–2.5	18	11
27/11/98	17	0.5–2.6	14	13
23/02/99	14	0.5–2.9	13	11
19/04/99	20	0.8–2.2	7	13
15/05/99	24	0.7–2.2	14	10
30/06/99	29	0.7–1.9	13	16
26/07/99	25	0.7–2.1	10	15
27/08/99	28	0.5–1.8	19	9
21/10/99	32	0.6–2.1	22	10
27/11/99	34	0.5–1.7	23	11
16/12/99	31	0.5–1.7	14	17
26/01/00	27	0.6–2.1	16	11
22/02/00	35	0.7–2.1	25	10
12/03/00	29	0.6–2.2	18	11
Total	431		266	195
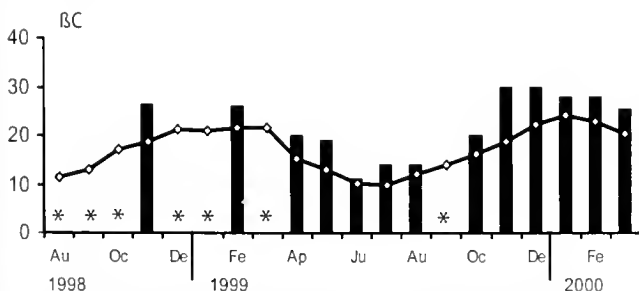
Figure 1. Monthly variation of mean air temperature (line) and water temperature (bars) during sampling period in Bagliardi Beach, Río de la Plata. *Without data.
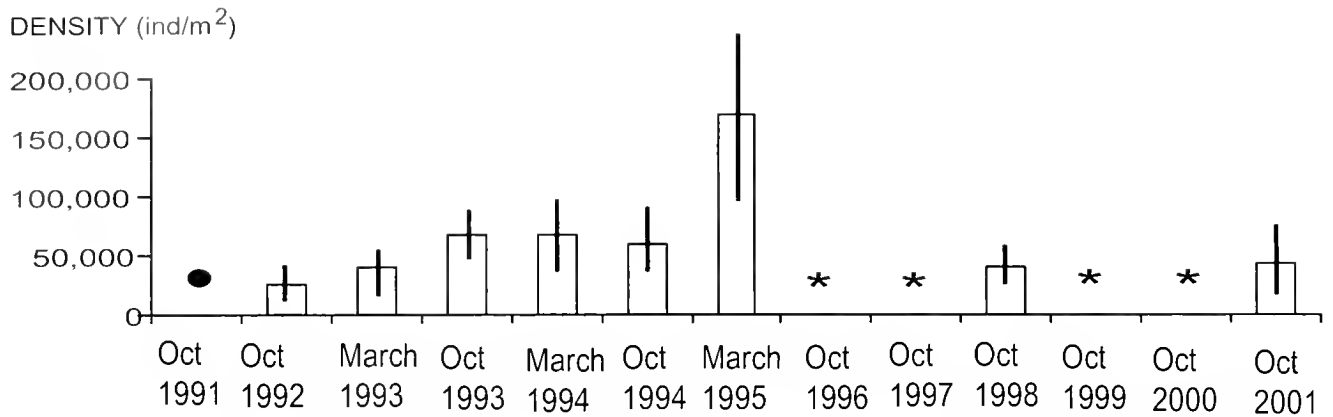
DENSITY (ind/m^2)

Figure 2. Temporal variation of mean density (bars) and standard deviation (lines) of *Limnoperna fortunei* in Bagliardi Beach, Río de la Plata. ● 4–5 ind/m^2. *Without data.

MATERIAL AND METHODS

To study the golden mussel population density and reproductive cycle, samples were collected along the rocky banks of Bagliardi Beach (34°55'S; 57°49'W), Río de la Plata estuary. Argentina, South America,—where the mussel was found for the first time in 1991 (Pastorino et al. 1993). The water temperature in this locality has a temperate regimen ranging from approximately 11°C to 31°C (Fig. 1). The physicochemical features of the Río de la Plata may be found in Darrigran (1999). The density data were obtained partly from Darrigran, et al. (1998b) and through sampling carried out *ad-hoc* (October 1998 and October 2001) in Balgliardi Beach. Samples of mussels were collected for density analysis from the fringes with macrobenthos from a rectangular
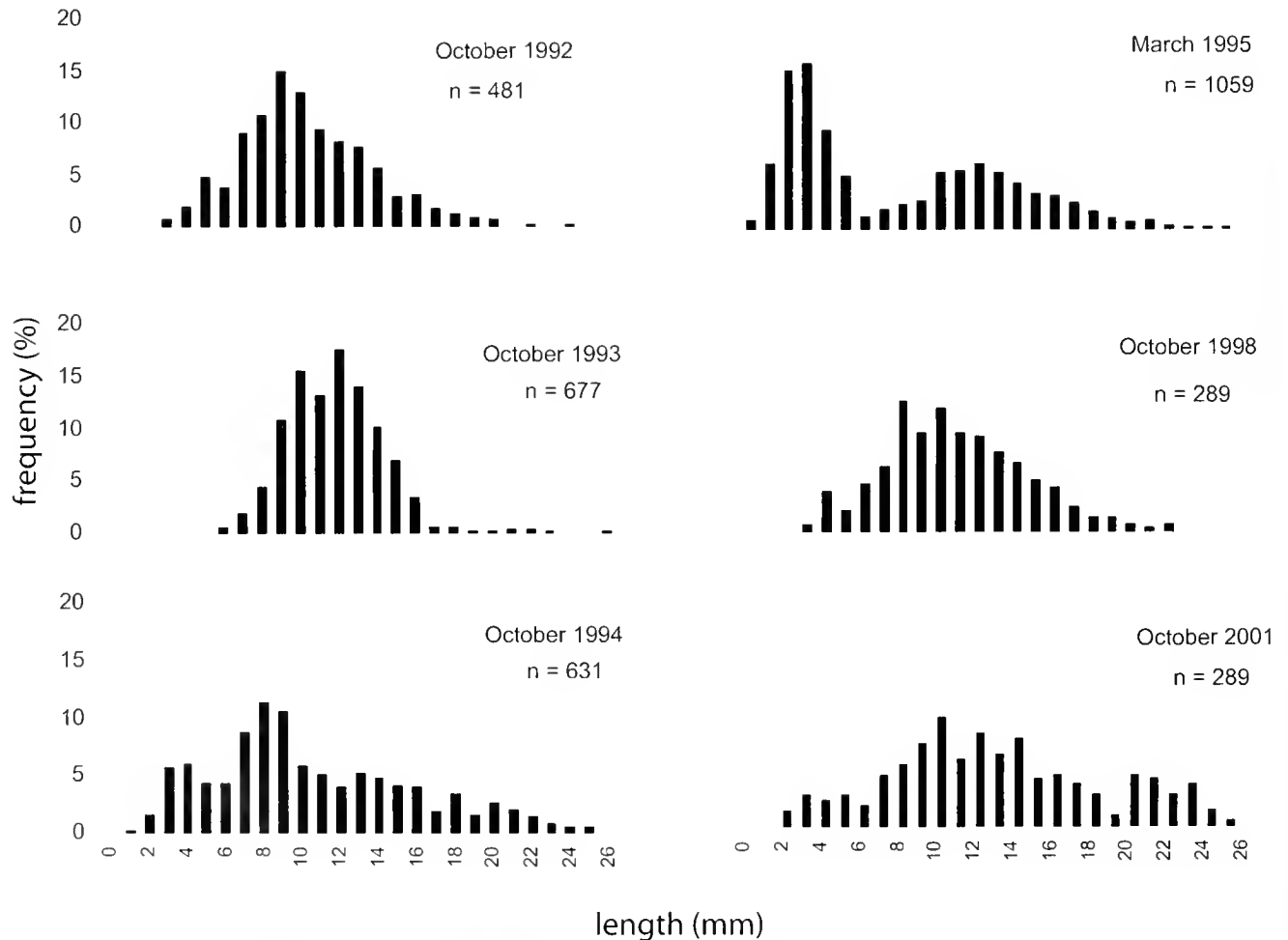
Figure 3. Size frequency (%) of *Limnoperna fortunei* in Bagliardi Beach, Río de la Plata.

area, variable in size, according to Darrigran et al. (1998b). For the age structure analysis, the maximum shell length was measured and the length frequency distribution was made at 1 mm class intervals (see Fig. 3 later).

The dates of sampling for reproductive cycle analysis, performed at low tides, may be observed in Table 1. The maximum shell length of the 431 collected individuals was taken. The material was fixed in Bouin solution and the histologic processing was performed according to Darrigran et al. (1999).

Approximately 25 oocytes with conspicuous nucleolus, both free in the follicular lumen and attached to the follicle wall, for each gonad were measured. The percentage of males with spermatozoids and the percentage of follicular occupation on the mantle were calculated for each sample. The latter was calculated using magnification (×200) in three different sections of the mantle, (upper, middle, and lower) through the visual estimation of field. The lysis periods were determined by microscopical analysis.

RESULTS

The temporal variation of population density found on the rocky litoral zone of Bagliardi Beach between 1991 and 2001 is given in Figure 2. From 1991 to 1995, the density increase was remarkable (from four to five individuals/m^2 to over 100,000 ind/m^2). The population density then decreases and stabilizes at approximately 40,000 ind/m^2. In Figure 3 it is shown that since 1994 the population has had an age structure where most size class intervals are represented.

The female and male follicles grow in the mantle and in the visceral mass. During this study 0.25% hermaphrodite specimen, with female, male, and mixed follicles were recorded.

The gonad growth is characterized by growing follicles. In this stage the follicles are small and there exists an abundant connective tissue between them. A more developed stage shows young oocytes on the wall, many stalked oocytes (Fig. 4A) and abundant spermatogoniums in the males (Fig. 4D). In a later stage the follicles are bigger and the follicular lumen contains abundant oocytes half-grown and also almost fully grown oocytes (60–80 μ). When fully mature, the female and male follicles reach the maximum size. Male follicles are packed with spermatozoa (Fig. 4E) and females' follicles with fully-grown (80–100 μ) oocytes.

When the gonads are spent and partially spent, the follicles contain large spaces. Partially spent gonads retain genital products.
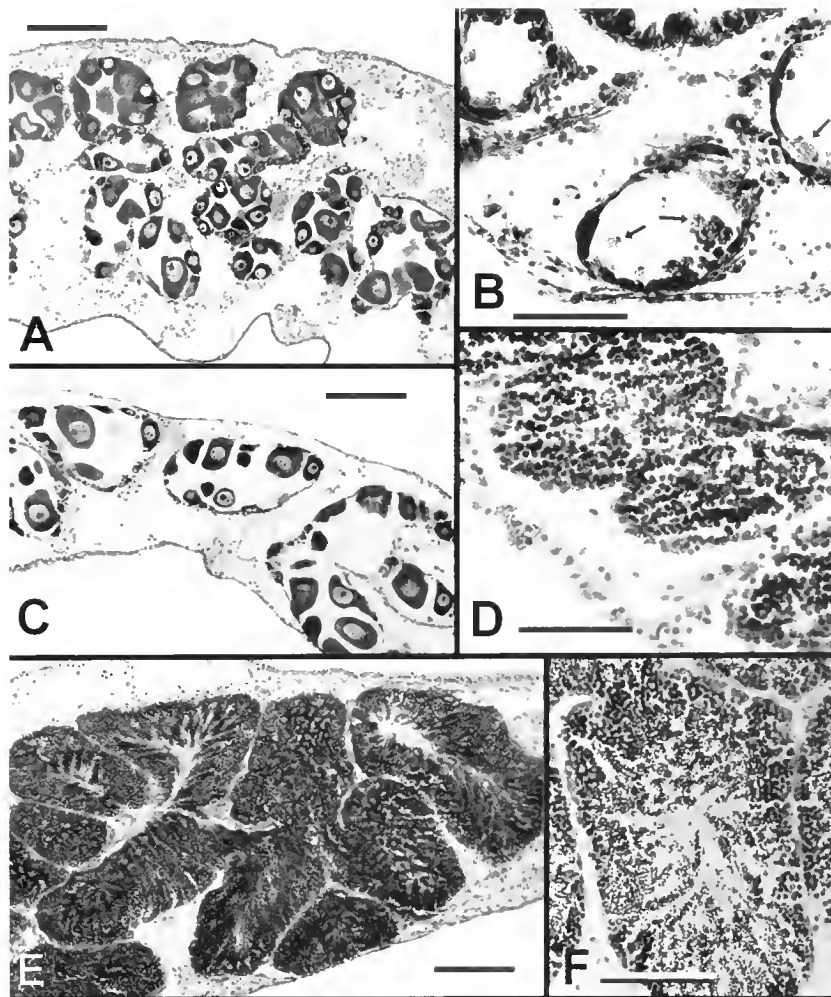
Figure 4. Female and male follicles in different development stages. (A) Female follicle partially grown with young oocytes on the wall and many stalked oocytes, scale bar = 100 μ. (B) Spawned female follicles with abundant yellow bodies (arrows), scale bar = 50 μ. (C) Female follicles partly spawned, scale bar = 100 μ. (D) Developing male follicles, scale bar = 50 μ. (E) Fully developed male follicles, scale bar = 100 μ. (F) Male follicles partly spawned, scale bar = 50 μ.
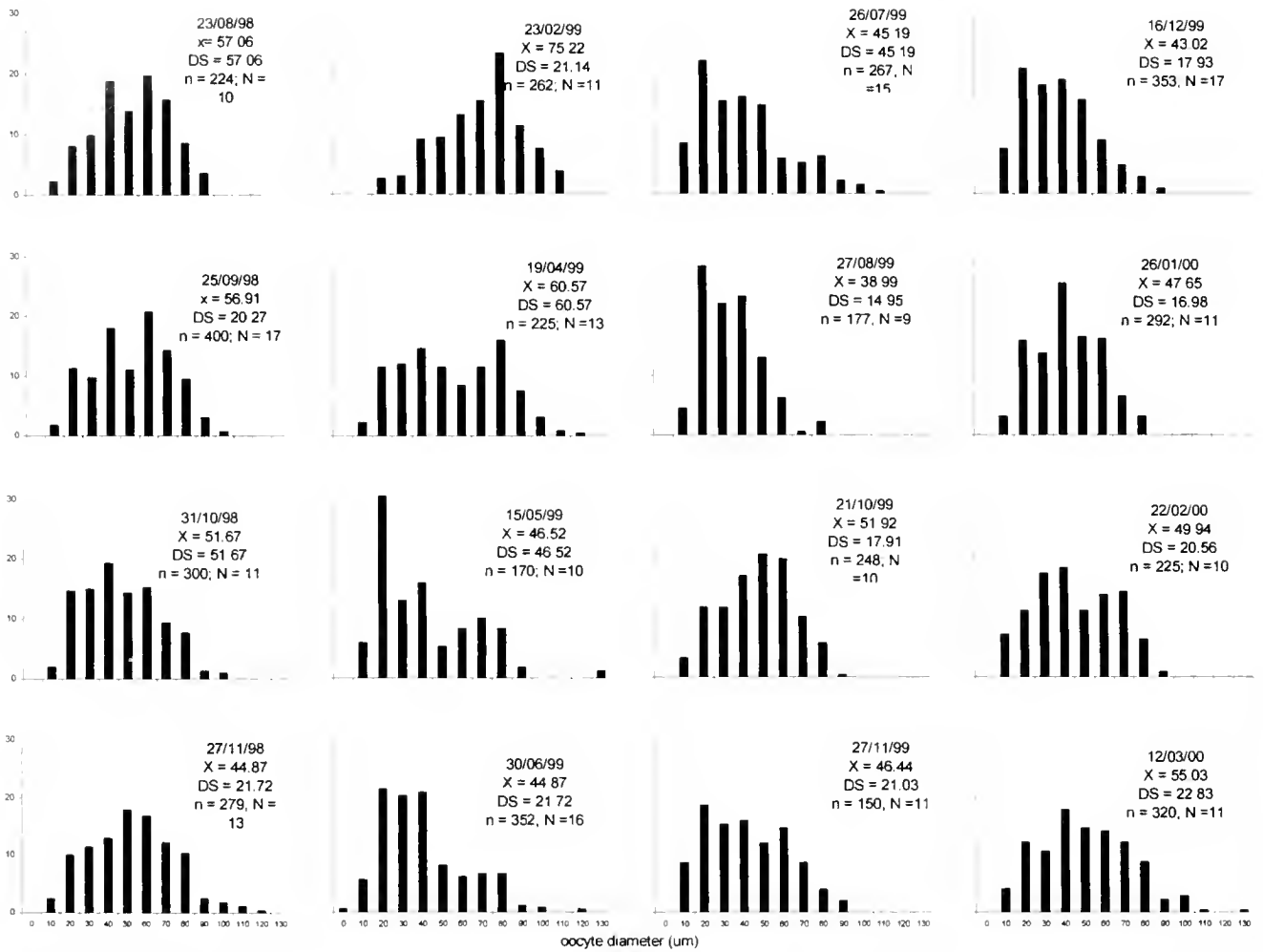
Figure 5. Frequency (in percentage) of oocyte sizes (µ) in different samplings. x, mean oocyte size; DS, standard deviation; n, number of oocytes; N, number of females.

In males spermatozoids and spermatocites are observed (Fig. 4F). Partly developed oocytes, oogonies, and young oocytes are retained on the female follicle walls (Fig. 4C). Oocitary lysis phenomena (Fig. 4B), with yellow bodies are evident for a short time after spawning is completed.

The body length at which the follicle, either female or male, development is completed, varies seasonally. The smallest shell length at which follicles differentiate is 5.5 mm, for both males and females (Fig. 5). During this study (August 1998 to March 2000), oocyte growth was always recorded. From May 1999 until August 1999, the oocytes smaller than 20 µ were 30% of the total oocytes examined.

The change in frequency of oocytes <20 µ and >60 µ indicates two reproductive peaks each year. The first peak occurs at the end
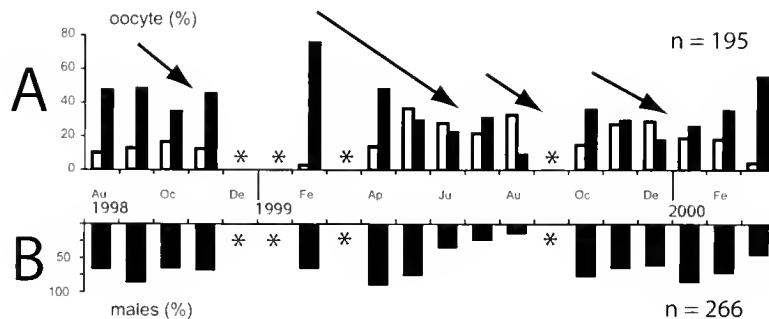
Figure 6. Temporal variation. (A) Percentage of oocytes bigger than 60 µ (full bars) and smaller 20 µ (empty bars). The arrows indicate moments of gamete liberation. (B) Percentage of males with sperm. *Without data. n, number of male individuals.
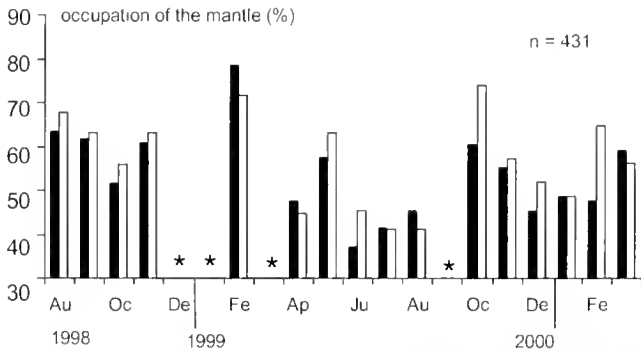
Figure 7. Temporal variation of mantle occupation. Female follicles (full bars) and males (empty bars). n, total number of considered males and females. *Without data.

of winter or beginning of spring (August to September of 1998, October to November of 1999) and the second peak is recorded during the summer (February of 1999, March of 2000). During these periods in the female follicles the oocytes bigger than 60 μ dominate, while smaller oocytes are scarce (<20%). During the period of study gonad recuperation were observed (October 1998 and May to June of 1999). Through the analysis of oocyte percentages present in the gonad, four spawning events were observed (Fig. 6A):

(1) From September to October 1998.
(2) February 1999 to May 1999. It is the most important for its duration and magnitude.
(3) in July to August 1999, the least important.
(4) between October and December 1999.

Figure 6B shows the percentage of males with sperm throughout the period considered. The pattern agrees in general with that observed for females.

The spawning pattern mentioned is similar to the follicular occupation of the mantle (Fig. 7). The percentage of occupation decreases during the spawning periods and stays low during the recuperation period (June, July, and August 1999).

Lysis phenomena were observed in several samples (Fig. 8). They are more important during May to August 1999, and coincide with recuperating follicles or in partial evacuation.

DISCUSSION

The bivalve sexual processes are generally related to ambient temperature (Lubet 1983). The results presented here for a population of L. *fortunei*, as well as those observed in the first study (Darrigran et al. 1999), those performed for a Hong Kong population (Morton, 1982), and the analysis of larvae density in the Río de la Plata (Cataldo & Boltovskoy 2000) show the strong relation-

ship between ambient water temperature and the reproductive cycle. The spawning events are regulated by changes in temperature, and increases and decreases of temperature rule the gametogenesis in this species.

During the initial study (Darrigran et al. 1999), we found that oocytes were always present in the mussels even during the resting period. Periods of scarce proliferation were recorded from December 1993 to May 1994. This study was performed a short time after the first record of L. *fortunei* in the Americas (Pastorino et al. 1993). The analysis of reproductive biology at that time differentiated numerous spawning events (five were recorded), many of them of low magnitude. Between September 1992 and January 1993 (the first period), two spawnings of reduced intensity were recorded and between February 1993 and November 1994 (the second period) three spawnings were recorded (two of these of higher magnitude). During the first period, the oocytes bigger than 60 μm and those smaller than 20 μm are always present and their proportion is similar (about 30%), the spawnings are low in magnitude but the proportion of oocytes bigger than 60 μm is always larger than 20%. During the second period, the spawnings are more intense and result in a diminution of the bigger oocytes proportion (by 10%). In contrast to the first period, the oocytes bigger than 60 μm reach more than 60% (Darrigran et al. 1999).

The population analyzed here shows a predictable reproductive pattern. Only two major spawnings are observed throughout the year, one when summer temperatures are recorded and the other with spring temperatures. A small winter spawning is also observed. This pattern, after 10 years of settlement in America, is similar to that described by Morton (1982) for the population of Hong Kong where the spawnings take place between May to June and November to December. The pattern shown during the first study [only after a year of settlement in the location considered (Darrigran et al. 1999)] could be due to the recent invasion.

Morton (1982) describes short spawnings for a month in spring and a month in autumn. In this study in South America, mainly in autumn, the evacuation continues from April 1999 to May 1999. The presence of larvae in the Río de la Plata, between August and April (Cataldo & Boltovskoy 2000), also indicates that the spawning periods are longer than those described by Morton (1982).

Similar to what was found in the first study of the golden mussel reproductive cycle (Darrigran et al. 1998a) 0.25% of the population was hermaphrodite.

According to the variation of population density, this species, at the beginning of the invasion in temperate climate, presents a noticeable increase of density. Then, it decreases its density to a third part and stabilizes. At the same time, it presents an age structure with most class intervals represented. These facts would indicate a stable settlement of the population to the environment.
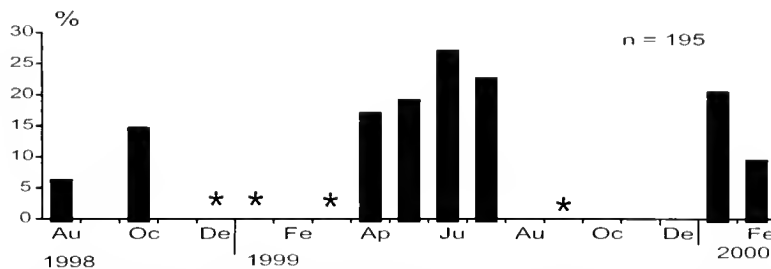
Figure 8. Percentage of females with follicles where lysis phenomenon occur. *Without data. n, number of considered females.

The initial increase recorded in a temperate climate could also be observed in a subtropical climate. Despite the preliminary studies of this species invasion in the south of Brazil, subtropical climate (Mansur et al. 1999), the golden mussel presents an increase in its population density similar to that observed in this study. Two years after its first record (Mansur et al. 2001a, Mansur et al. 2001b), the maximum density is 62,100 ind/m^2.

The golden mussel, like other invasive species, is opportunistic. This fact makes it difficult to relate the reproductive pattern with environmental variables and to determine the different facts that might be modified in the reproductive cycle. *L. fortunei*, for its great adaptability and reproductive capacity, increases its distribution permanently by occupying environments of particular features.

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QUANTITATIVE EVALUATION OF THE DIET AND FEEDING BEHAVIOR OF THE CARNIVOROUS GASTROPOD, *CONCHOLEPAS CONCHOLEPAS* (BRUGUIERE, 1789) (MURICIDAE) IN SUBTIDAL HABITATS IN THE SOUTHEASTERN PACIFIC UPWELLING SYSTEM

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ABSTRACT Landings of *Concholepas concholepas*, a carnivorous gastropod and valuable fishery resource, appear disproportionately high compared with herbivores or suspension feeding mussels. The species has been previously described as feeding on a great variety of prey, the most important being barnacles, mussels, and tunicates. To quantitatively evaluate published information on the diet of *C. concholepas*, an analysis of the stomach contents of 925 individuals was performed, representing a wide size-range, broad geographical distribution (29°30'S to 32°08'S), and different community types (variety of potential prey choices). The diet was based principally on suspension feeders, such as barnacles (*Balanus laevis* and juveniles of *Austromegabalanus psittacus*) (75%) and the ascidian *Pyura chilensis* (16%). An additional sampling, in which abundance of prey in the habitat and microhabitats occupied by the gastropod was determined, showed that the gastropod positively selects these prey species, the ascidian being the most preferred. The rest of the diet was made up of *Calyptraea trochiformis* and mytilid bivalves. According to the literature, intertidal individuals of this species only feed at night. To confirm this behavior for subtidal populations, two 24-h samplings (analyzing digestive tract contents) were performed at a single location. No distinct circadian cycle of feeding for subtidal populations was found, most animals feeding most of the time. This, together with the characteristics of diet, made mainly by suspension feeders, which transfer energy from primary productivity in the water column which varies along the coast, to benthic carnivores, help to explain the high productivity of the gastropod and its variability along the coast of Chile.

KEY WORDS: feeding behavior, circadian rhythm, selectivity, carnivorous gastropod, Chile, subtidal, upwelling system

INTRODUCTION

The muricid gastropod *Concholepas concholepas* (Bruguière 1789) ("Chilean abalone") is distributed from 12°S to 55°S along the Peruvian and Chilean coasts and is an important predator occupying rocky shores (Castilla 1981, Castilla & Paine 1987). It is a valuable product in artesanal fisheries (Castilla & Jerez 1986) along its distribution. In Chile, the highest landings ranged between 6,369t and 25,000t between 1978 and 1988 whereas the fishery was unregulated; the maximum value was recorded in 1980 (SERNAP 1999). In region IV (between 29°30'S and 32°08'S, 320 km coast) in the period between 1985 and 2000 landings fluctuated between 258 and 2,219t for this carnivorous gastropod species. In the same period and along the same stretch of coast the herbivorous gastropods *Fissurella* spp. (eight different species that are fished) and *Tegula atra* (Lesson), which share the habitat with *C. concholepas*, together registered landings between 695 and 1525t. The aim of this work was to investigate what kind of food sustains the comparatively important production of this high trophic level carnivore, the ecological position to which *C. concholepas* is usually assigned. Stotz (1997) has shown that within management areas the abundance of *C. concholepas* is related to the amount of food, the species overexploiting its food source when not fished, and then migration to other areas. Thus, the knowledge of diet and feeding behavior is also of importance in developing a management strategy of the species within management areas.

According to published literature, *C. concholepas* has been observed feeding on a variety of prey, the most often mentioned being barnacles, mussels, and tunicates (Viviani 1975, Castilla & Cancino 1979, Castilla & Guisado 1979, Castilla et al. 1979, DuBois et al. 1980, Castilla 1981, Guisado & Castilla 1983, Som-

mer 1991, Sommer & Stotz 1991). But quantitative feeding information is scarce; the number of published observations for individuals feeding in their natural subtidal habitats was less than 96, observed at two localities (Castilla et al. 1979, Guisado & Castilla 1983, DuBois et al. 1980, Sommer 1991). These did not represent the entire spectrum of subtidal communities in which the gastropod lives. There are also qualitative observations (Viviani 1975, Castilla et al. 1979, Castilla 1981) that increase the data regarding the prey diversity of *C. concholepas* but do not allow evaluation of the relative dietary importance of the different prey species of this gastropod.

The published quantitative information on food types consumed by *C. concholepas* was obtained by feeding behavior observations (Castilla et al. 1979). DuBois et al. (1980) stated "an individual is feeding when one observes an unusual extension of the foot over a potential prey species or when the individual shows movements to remove a prey." This includes lifting individuals to check for empty shells, direct observations of ingestion of prey, empty spaces on the substrate in front of the mouth or of the "shell teeth", which the species has on the anterior border of the shell, proboscis introduced into the prey, or prey held by the propodium and directed to the mouth (Castilla et al. 1979). This method gathers information on the specific prey being consumed at the moment of observation. Thus, those prey species that are more difficult to consume and for which the process of ingestion lasts longer will have a higher probability of being observed. Also, in order not to disturb animals and thus record observations of natural feeding behavior, observations have been limited to individuals found on open surfaces. Feeding by individuals found in crevices or on the undersides of boulders, including most juveniles and medium-sized individuals of *C. concholepas* (Castilla & Cancino 1979, Guisado & Castilla 1983, Sommer 1991, Stotz & Lancellotti 1993) cannot be easily observed. Thus, observations of *C. concholepas* on open surfaces will focus only its feeding on prey abundant on

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such places and food composition described using this method may not necessarily reflect the relative importance of the different prey species in the diet of *C. concholepas*.

In contrast, the analysis of digestive tract contents provides a quantitative measure of food consumption over a certain time interval, representing the range of prey species and their relative importance in the diet of the predator. Only in case digestion rates for different prey species differ greatly, some bias may occur. This is the first work in which feeding of *C. concholepas* has been studied through the analysis of the contents of the digestive tract.

According to published information, *C. concholepas* feeds only at night (Castilla & Guisado 1979, Castilla & Cancino 1979, Castilla et al. 1979, Guisado & Castilla 1983). However, this has been concluded mainly from laboratory experiments mostly using individuals collected in the intertidal zone. Only DuBois et al. (1980) have made observations in the subtidal, recording the feeding activity of 96 individuals of this species.

Intertidal gastropods search out and consume food mainly at night to avoid desiccation (Underwood 1979, Branch 1981, Hawkins & Hartnoll 1983, Lowell 1984). Subtidal populations of *C. concholepas*, not exposed to this stress, may feed mainly at night for other reasons: (1) to avoid visual predators active during daytime (Castilla & Cancino 1979) and/or (2) to capture prey that respond to visual stimuli and may be able to escape predation by *C. concholepas* during the day.

Visual predators, which are known to include *C. concholepas* in their diet, such as the sea-otter *Lutra felina* (Molina) (Castilla & Bahamondes 1979), the sea lion *Otaria flavescens* (Shaw) (Aguayo & Maturana 1973) and the fishes *Pimelometopon maculatus* (Perez) and *Sicyases sanguineus* Müller & Troschel (Viviani 1975), do not figure prominently in the mortality of this gastropod species. *L. felina* has been suggested to be highly specialized on fish and crustacea as prey (Sielfeld 1990); *O. flavescens* does not appear to prey on gastropods firmly attached to substrates, as is the case for *C. concholepas* (George-Nascimento et al. 1985); and the fish species prey mainly on juveniles of *C. concholepas* which, according to our observations, are hidden in crevices in the subtidal. Prey selection is an unlikely factor promoting night time feeding, as the main prey of *C. concholepas* are sessile species, such as the barnacles *Austromegabalanus psittacus* (Molina), *Balanus laevis* Bruguière, and *Jehlius cirratus* (Darwin); the tunicate *Pyura chilensis* (Molina); the mitilid *Perumytilus purpuratus* (Lamarck); and the hemisessile gastropod *Calyptraea trochiformis* (Born) (Castilla & Guisado 1979, Castilla et al. 1979, DuBois et al. 1980, Guisado & Castilla 1983, Castilla & Durán 1985, Moreno et al. 1986, Sommer 1991, Sommer & Stotz 1991). Therefore, there appears to be no strong argument that subtidal populations of *C. concholepas* feed exclusively at night. Nevertheless, this needs to be investigated, which is one aim of this work.

This work reports food composition and feeding behavior (circadian feeding rhythm and food selection) for *C. concholepas* based on the analysis of the food content in the digestive tract. A greater variety of habitats than in previous studies were sampled, including open surfaces, crevices, the undersides of boulders, holdfasts of the subtidal kelp *Lessonia trabeculata* (Villouta & Santelices), and under the canopy of this algae along an extensive stretch of coast from 29°30'S to 32°08'S (ca. 320 km). On one site the sampling and analysis of the digestive tract contents of a large number of individuals collected over a 24-h cycle was conducted. For some of the individuals sampled along the coast and in different communities, the abundance of potential prey in the environment is quantified to establish to what degree the food in the gut represents the availability of prey. This allows us to study whether there is some kind of preference for some prey species.

MATERIALS AND METHODS

Study Sites

Individuals of *C. concholepas* were collected at several sites along the ca. 320 km of coast of the Coquimbo Region, between Pichidangui (32°08'S) and Punta Choros (29°30'S) (Fig. 1). The sites were chosen considering accessibility and being representative of different coast and community types. A qualitative description of subtidal communities of each sampling site is provided in Table 1. Quantitative data of communities in which the gastropod was sampled are provided in Tables 3 and 4. For the 24-h sampling the site at Punta Lagunillas (30°05'S; 71°26'W), located ca. 15 km south of Coquimbo, was chosen. It is a rocky point forming the northern border of Bahía Guanaqueros (Fig. 1). Although it is an exposed coast, it has an irregular configuration that creates sheltered ponds that allow for safe diving through the surf and at night. The substrate is formed by different sized boulders that are covered by a dense kelp forest formed by small and bushy (many blades, short stipes) individuals of *Lessonia trabeculata*. It corresponds to community type 1 (Table 1). Quantitative data for the community at this site are given in Table 3. Larger individuals of *C. concholepas* are found mostly within the kelp forest, whereas smaller individuals are mainly hidden in crevices or on the undersides of boulders.
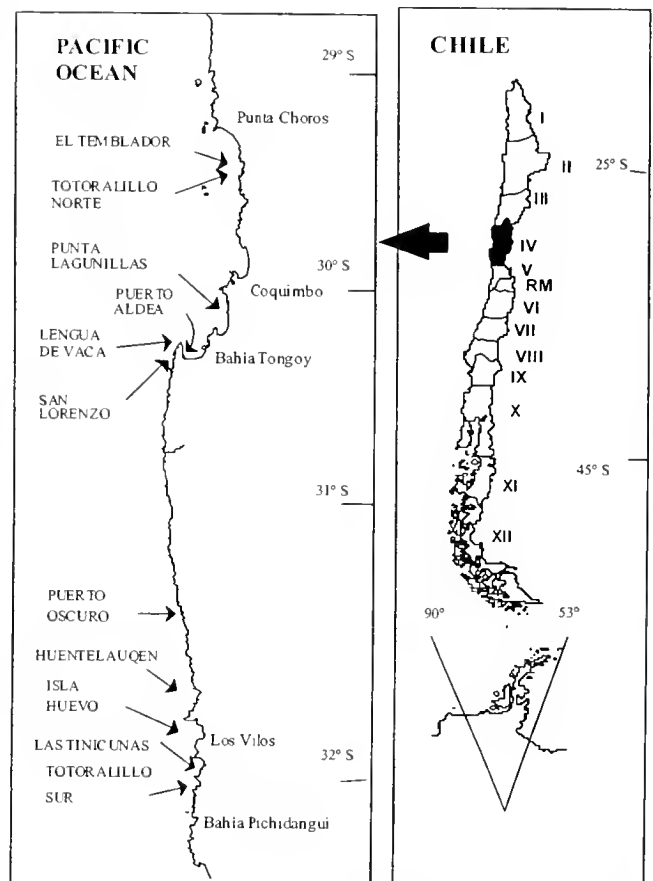
Figure 1. Location of the study sites along the coast in the region of Coquimbo (region IV).

TABLE 1.

Subtidal communities where *C. concholepas* was collected a general description of each community is given.

Type	Communities	Localities
I	Kelp bed of *Lessonia trabeculata*	El Temblador, Punta Lagunillas, Punta Lengua de Vaca, San Lorenzo, Caleta Las Conchas, Totoralillo Sur (isle and bay)
II	Barren ground	Toralillo Norte (rock), Puerto Oscuro
III	Barnacles and seaweeds	Totoralillo Norte (isle)
IV	Colonies of *Pyura chilensis*	Puerto Aldea

General description of the subtidal community types

Type	Community	General Description
I	Kelp bed of *Lessonia trabeculata*	Community characterized by the kelp *Lessonia trabeculata*. Under the canopy, dense patches of barnacles (e.g., *Balanus laevis*) and to a lesser extent the ascidian *Pyura chilensis* are found. In crevices and on the underside of boulders are observed aggregations of the gastropod *Calyptraea trochiformis*, sponges and small patches of barnacles.
II	Barren ground	Community characterized by an high cover of calcareous crustose algae and high densities of the black urchin *Tetrapygus niger*. In crevices and on the underside of boulders are observed aggregations of *Pyura chilensis*, of *C. trochyformis* and patches of barnacles.
III	Barnacles and seaweeds	Community dominated by extensive patches of barnacles, specially by *Austromegabalanus psittacus*, which can be covered by a dense mat of the red algae *Gelidium chilense*. Also aggregations of the ascidian *Pyura chilensis* may be present in crevices.
IV	Colonies of *Pyura chilensis*	Community formed mainly by aggregations of the ascidian *Pyura chilensis*, which covers most of the surface. The ascidians could be partly covered by the algae *Gigartina chamissoi*. On the underside of boulders aggregations of *Calyptraea trochiformis* can be observed.

Sampling of *C. concholepas* Along the Coast to Describe Diet

Individuals were collected by Hookah diving from the intertidal down to a maximum depth of 25 m. At each site two divers collected all *C. concholepas* that they were able to find within approximately 1 h of diving, which allows the inspection of an area of about 200–500 m². Individuals of all sizes were collected and the searches included the undersides of boulders. Table 2 summarizes the number and size range of individuals collected at each site of the samplings undertaken between January 1994 and December 1995.

Experiments for the Identification of Prey and Food Retention Time in the Gut

The identification of each prey item was aided by a simple experiment in which known prey were offered to individual *C. concholepas*. Three groups of 10 adult individuals (70–110 mm peristomal length) were collected at Punta Lagunillas and maintained in tanks with running seawater. Each group was offered one of the most important prey items described in the literature (Sommer & Stotz 1991): the barnacles *Austromegabalanus psittacus* and *Balanus laevis*, the gastropod *Calyptraea trochiformis*, and the

TABLE 2.

C. concholepas: Number of individuals collected in the field, number of entire digestive tracts analyzed in the laboratory, size range, number of individuals with food in their tracts, and number of individuals with recognizable prey in their digestive tracts are given.

Localities	Sample Size Field (Nᵃ)	Sample Size Labor. (Nᵃ)	Size Range (mm)	Individuals with Food No.	Individuals with Food %	Recognizable Prey No.	Recognizable Prey %
Playa El Temblador	76	74	24–122	54	73.0	47	87
Totoralillo Norte (rock)	21	21	37–93	13	61.9	10	76.9
Totoralillo Norte (isle)	9	8	15–122	4	50.0	4	100.0
Punta Lagunillas (August)	166	166	21–121	122	73.5	110	90.2
Punta Lagunillas (January)	282	260	7–125	235	90.4	225	95.7
Puerto Aldea	13	13	102–129	13	100.0	11	84.6
Punta Lengua de Vaca	52	45	51–116	33	73.3	22	66.7
San Lorenzo	188	158	16–126	105	66.5	93	88.6
Puerto Oscuro	7	7	59–100	5	71.4	5	100.0
Isla Huevos	54	54	24–131	52	96.3	52	100.0
Totoralillo Sur (isle)	95	72	69–47	65	90.3	62	90.4
Totoralillo Sur (bay)	51	47	26–125	40	85.1	39	97.5
Total	1014	925	7–131	741	80.1	680	91.8

ascidian *Pyura chilensis*. Individuals were maintained continuously with food, sampling after the initial 48 h, and then daily, two individuals. Sample animals were dissected and their stomach and gut contents examined. The physical characteristics of each prey item after ingestion by *C. concholepas* were recorded and then used as a reference in the analysis of stomach and gut contents from individuals sampled in nature.

To measure the time the food is held in the digestive tract, a field experiment was performed at Punta Lagunillas on October 25–26, 1995. Therefore, all the individuals collected during a 30-min period at 1800 h and again at 0600 h of the next day were maintained in a mesh bag in the water in the study site, without food. Every 2 h, six individuals of this mesh bag were sampled and sacrificed, fixing the visceral mass in 10% saline formalin. In the

laboratory, the proportion of individuals with food in the stomach or gut in each sample was determined.

Samplings to Compare Diet with the Food Available in the Environment

At seven sites (El Temblador, Punta Lagunillas, Punta Lengua de Vaca, Huentelauquen, Isla Huevos, Tinicunas, Totoralillo sur) (Fig. 1), between January 1996 and March 1997, samplings were repeated, but this time recording also abundance of prey in the environment. For each *C. concholepas* individual collected, the density and percent cover of species present on the spot, was recorded. A 0.25-m² quadrant with 100 regularly distributed points
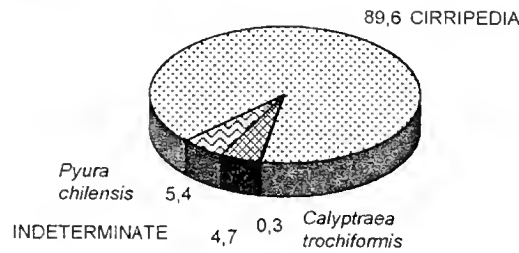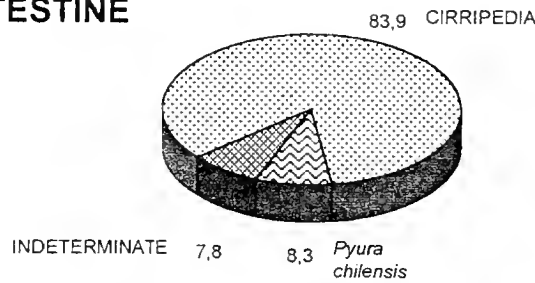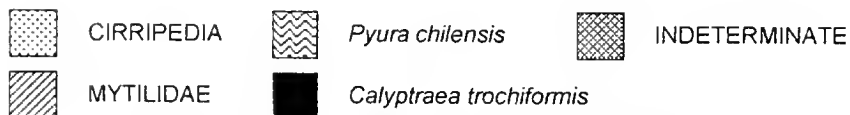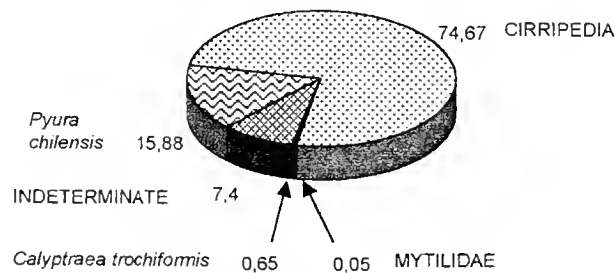
Figure 2. Dietary composition of *Concholepas concholepas*.

was used. The quadrant was located with its center on the spot were the *C. concholepas* individual was captured.

For four of these seven sites (Isla Huevo, Punta Lengua de Vaca, Punta Lagunillas, and El Temblador) (Fig. 1), a general quantitative description of communities present on the site was done. A 50-m long and 2-m wide transect was placed parallel to the coastline. For less frequent species their abundance in the entire transect area (100 m²) was counted, whereas for smaller, more frequent species five 0.25-m² quadrants, distributed regularly along the transect, were used. To quantify the laminarian algae *Lessonia trabeculata*, the transect was divided into 25 areas of 2 ×

2 m, estimating percent cover within each of these areas. Within these same areas the percent cover of each substrate type was estimated in those cases in which the bottom was a mixture of sand and rocks. This estimate was used to correct abundance and percent cover estimates of species, in order that they refer only to rocky bottom.

24-h Sampling at Punta Lagunillas

The 24-h sampling was accomplished twice: on October 24 and 25, 1994 and August 5 and 6, 1996. Dives took place at 1700,
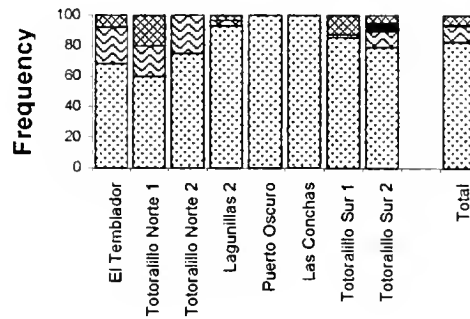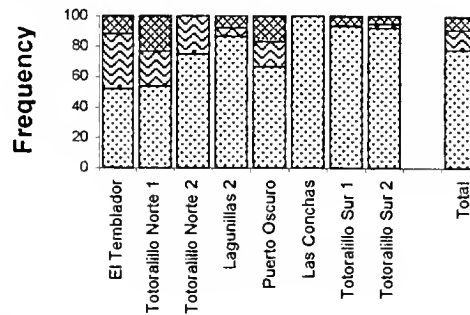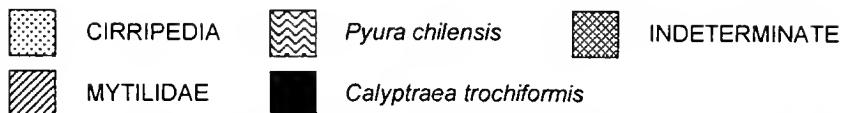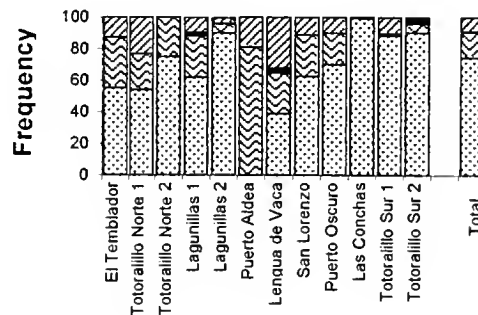
Figure 3. General dietary composition of *Concholepas concholepas* from each sampling site.

2100, 0100, 0500, 0900, and 1300 h. On each dive, two divers sampled the subtidal at depths between 4 and 10 m, collecting each *C. concholepas* they were able to find within a half-hour dive. Searches were concentrated beneath the canopies of *L. trabeculata* and included the undersides of boulders. At night searches were conducted using underwater flashlights. Diving was conducted using a compressor on the beach that provided air to the divers through a hose (Hooka diving). In the 1996 sampling, the individuals collected by each diver were considered as replicate samples.

Processing of Samples

All samples of *C. concholepas* were processed immediately after collection. Peristomal length of individuals were measured with calipers and grouped into seven size classes from <30 mm to >130 mm (see Figs. 4 and 5). Each specimen was taken out of the shell and the visceral mass dissected and fixed in 10% saline formalin. Visceral masses of all individuals from each size class were stored together in a single container and transported to the laboratory.

In the laboratory, the digestive tract of each individual was dissected; the contents emptied separately for stomach and gut in two Petri dishes, diluted with tap water, and spread on the bottom of the dish. The relative abundance of each prey item was recorded for each individual using a dissecting microscope. Therefore the dish was put over a point matrix, recording the food item over each point, and calculating its proportion to all the points covered by the sample. Also the presence of prey species, which were present, but not registered over any point, were annotated.

For *C. concholepas* from the 24-h sampling a measure of fullness was recorded. Fullness and digestion level was determined using the following scale:

Fullness:

Full: contents occupy ca. 100% of the volume of the stomach or gut.

Medium: contents occupy around 50% of the volume of the stomach or gut.

Presence: contents occupy around 10% of the volume of the stomach or gut.

Empty: no contents registered.

Digestion level:

Some digestion: entire structures are observed, such as pieces of cirri, gills, muscles, etc.
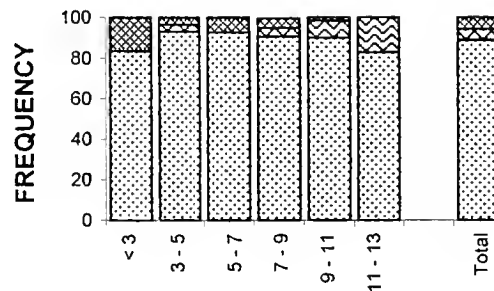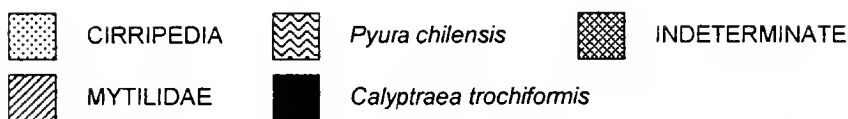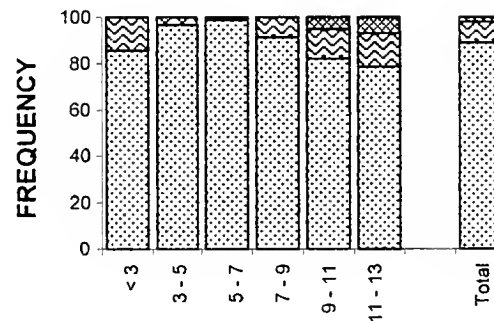
Figure 4. Dietary composition of *Concholepas concholepas* in different size classes (length of peristomal opening).

STOMACH INTESTINE
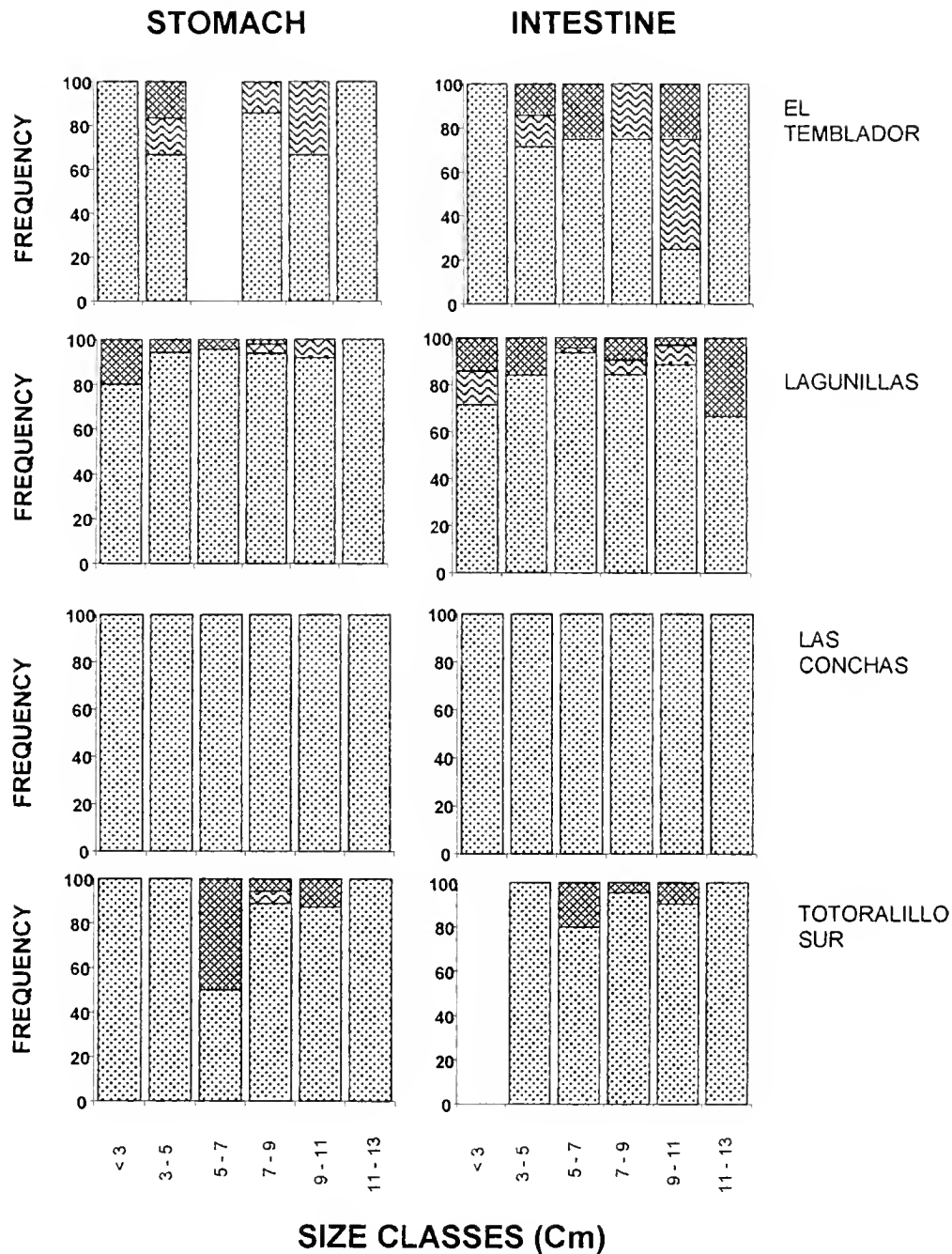
Figure 5. Dietary composition of *Concholepas concholepas* in different size classes (length of peristomal opening) from four sampling localities.

Medium: structures could still be identified, but already with some digestion.

Total digestion: soft parts are completely digested, only pieces of shells or hard skeletons can be identified.

Prey Selection Analysis

To determine the degree of selection of prey by *C. concholepas* an index proposed by Pearre (1982) was used. This allows the estimation of the selection index *C*, but also using a χ^2 test with

one degree of freedom (Sokal & Rolf 1969, Pearre 1982) to estimate significance levels, using the following relations:

	Species A	Other spp.	Total
In the diet	A_d	B_d	$A_d + B_d = C$
In the environment	A_a	B_a	$A_a + B_a = D$
	$A_d + A_a = A$	$B_d + B_a = B$	$A_d + A_a + B_d + B_a = N$
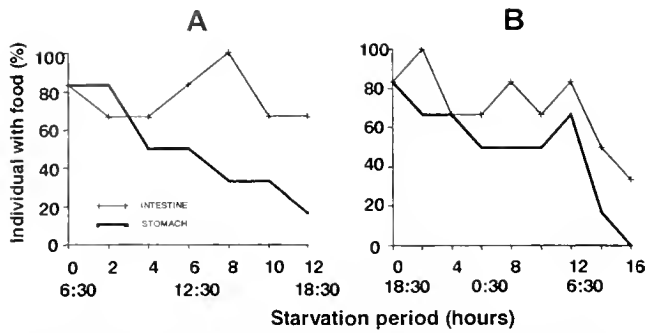
Figure 6. Percentage of individuals with contents in the stomach and intestine during the starvation periods beginning in the morning (A) and in the afternoon (B).

Where:

A_d = Proportion of species A in the stomach
A_a = Proportion of species A in the environment
B_d = Proportion of the rest of species in the stomach
B_a = Proportion of the rest of species in the environment

The index "C" is obtained from the following relation:

$$C = \pm \left(\frac{X^2}{N}\right)^{1/2}$$

Where:

$$X^2 = \frac{N\left[(A_d \cdot B_a - A_a \cdot B_d) - \frac{N}{2}\right]^2}{(A \cdot B \cdot C \cdot D)}$$

The index C varies between −1 and +1. A significant positive value indicates that the prey species was preferred and rejected with a significant negative value. Values around zero means that the prey species is consumed in the same proportion it appears in the environment.

For estimation of the index only those species found in the diet of *C. concholepas* where considered. For the calculations, the density of invertebrates present in the quadrant was transformed into percent cover to have all the values on the same scale. For this, the area occupied by an average individual was estimated, calculating its proportion within the 2,500 cm^2 of the sampled area. This proportion was multiplied by the number of sampled individuals, thus obtaining their percent cover.

Once this proportions where estimated, a correction for poten-
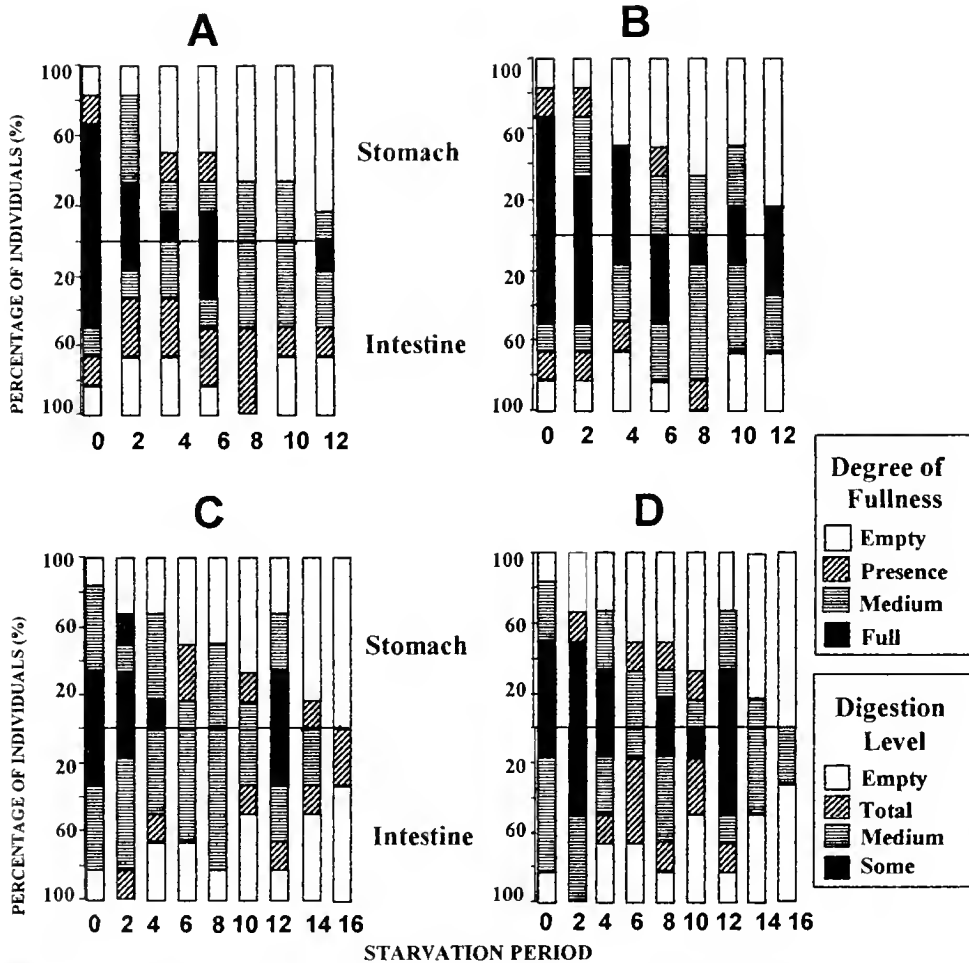
Figure 7. Prey digestion level (first column: A, C) and degree of fullness (second column: B, D) of stomach and intestine during the starvation periods beginning in the morning (first line: A, B) and in the afternoon (second line: C, D).

MORNING SAMPLE

Cirripedia (principally *Balanus laevis*)

Mollusca

Indeterminate Totally digested

Pyura chilensis

Calyptraea trochiformis

AFTERNOON SAMPLE

Cirripedia (principally *Balanus laevis*)

Mollusca

Indeterminate Totally digested

Pyura chilensis
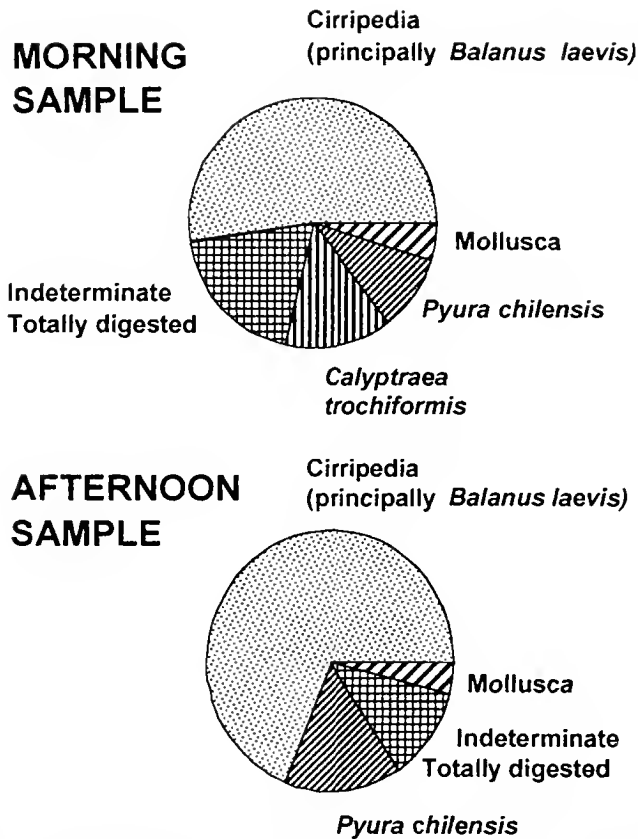
Figure 8. Prey composition of *Concholepas concholepas* in the starvation experiment at Punta Lagunillas.

tial prey species was done. Therefore, the percent cover values for algae and empty space was eliminated, calculating a new proportion considering that potential prey species cover 100% of the substrate.

For these analyses, only the content of the stomach was used because this represents the most recently ingested food, most probably from the sampled spot. Also, empty or destroyed stomachs were not considered.

RESULTS

Diet

Of the 1,014 individuals of *C. concholepas* collected at nine sites (Table 2) visceral masses of 925 individuals were examined. Of these, only 741 individuals (80.1%), covering a size range from 7–131 mm peristomal length, had food in their digestive tracts (Table 2).

Only 8.2% of the digestive tracts had contents that could not be identified because the process of digestion was already too advanced (Table 2). About 98% of the individuals examined fed on one prey type. Only 18 individuals (2%) had more than one prey item in the digestive tract.

The most important prey items were barnacles, representing 89.6% of the stomach contents, and 83.9% of intestinal contents (Fig. 2). The second most important prey item, the ascidian *P. chilensis*, represented 5.4% and 8.3% of the stomach and gut contents, respectively. The remainder of the prey was *Calyptraea trochiformis*, mitilids, and unidentified materials. Differences be-

tween stomach and intestine were produced by more advanced digestion in the latter. That favored recognition of the ascidian in the intestine because its remains were recognized mainly by color, which was not affected by digestion. *C. trochiformis* was not found in the intestine. But these different digestion rates of the various prey did not change the general dominance of barnacles in the diet.

The dietary importance of barnacles was most pronounced at Caleta Las Conchas, where they represented the only prey. In contrast, at Puerto Aldea, where *C. concholepas* was introduced by fishermen, barnacles were entirely replaced by *P. chilensis* (Fig. 3). With only two exceptions (Puerto Aldea and Lengua de Vaca), in all sites the barnacles were the predominant prey (Fig. 3), even though the basic community structure varied (Table 1).

Prey composition did not differ among the different size groups within the pooled sample, where barnacles were always the dominant prey item (Fig. 4). The same analysis made at selected sampling sites, also showed in general, with only two exceptions (El Temblador 9–11 cm; Totoralillo Sur 5–7 cm) (Fig. 5) that the barnacle was the predominant prey. Although in all cases the smallest and the biggest individuals only fed on barnacles, intermediate-sized individuals showed a slightly more varied diet (Fig. 5)

Identification of Prey and Food Retention Time

The feeding experiments with known prey items allowed general descriptions of the prey after ingestion by the gastropod. Skeletal plates, cirri, and eggs were observed in the stomach and gut when *C. concholepas* fed on barnacles. When the ascidian *Pyura chilensis* was the prey, an orange or red mass sometimes containing syphons was observed. In the case of *Calyptraea trochiformis*, white-colored muscular tissue and egg capsules could be recognized. Comparison of these characteristics with those observed in the digestive contents of individuals collected in the field allowed the identification of most prey items.

Regarding food retention, the percentage of individuals with content in the digestive tract is highest (83.3%) in the morning (0630 h) and in the evening (1830 h) when just sampled. As the starvation period increases, the proportion of individuals with content in the digestive tract fluctuates, decreasing after 12 h of starvation (Fig. 6). The decrease is more evident and regular for the stomach, not so much for the intestine. The stomach appears completely empty after 16 h of starvation. Accordingly, the percentage of full stomachs or those with the content showing some digestion decreases as the starvation period increases (Fig. 7). Nevertheless, the tendency is not that clear, close to the end of the experiment appearing again individuals with full stomach or intestine, and showing just some digestion (Fig. 7). This suggests that some contamination of the experiment may have occurred. The problem probably stems on the fact that the shells of the individuals put together in the mesh bag were not cleaned. Thus the barnacles, which normally are attached to the shell, might have been consumed by some of the experimental individuals. Considering this possible contamination, the experiment suggests that the retention time in the stomach is around 6 h, whereas in the intestine the food seems to be retained up to 16 h. The prey species the experimental individuals had ingested were the same as described above for the individuals sampled along the coast (Fig. 8).

Prey Selection by C. concholepas

The most important prey species are not the most abundant species in the habitat (Table 3). Barnacles appear in small patches,

TABLE 3.

Abundance of macroalgae and invertebrates (percent cover and density, mean and standard deviation) in the rocky subtidal in which *Concholepas concholepas* was collected at four sites.

	Temblador	Lagunillas	Lengua de Vaca	Isla Huevo
Percent cover (%)				
Algae				
Rhodophyta	19.8 ± 25.07	45.6 ± 27.57	57.0 ± 19.46	49.8 ± 23.22
Mesophyllum sp.		0.4 ± 0.55		0.4 ± 0.55
Corallina officinalis	4.6 ± 10.29		4.6 ± 4.67	
Gelidium chilense	5.0 ± 11.18	4.0 ± 6.42	6.2 ± 8.90	10.0 ± 20.20
Calcareus algal crusts	2.4 ± 2.51	10.0 ± 11.16	1.2 ± 1.79	13.8 ± 13.18
Phaeophyta				
Glossophora kunthii	68.0 ± 28.72	70.0 ± 15.55	60.8 ± 21.78	49.2 ± 28.12
Lessonia trabeculata		2.2 ± 4.92		1.0 ± 1.73
Porifera annellida				
Phragmatopoma sp.	29.8 ± 17.04	3.6 ± 5.68		
Romanchella pustulata	0.2 ± 0.45	1.6 ± 2.30	0.2 ± 0.45	
Spionidae	5.6 ± 12.52			
Crustacea				
Balanus laevis	0.2 ± 1.79			17.4 ± 13.92
Balanus flosculus		10.6 ± 10.67		
Austromegabalanus psittacus			6.6 ± 7.47	
Bryozoa				
Bugula sp.		0.4 ± 0.89		
Briozoa indeterminated	0.8 ± 1.79		3.0 ± 6.71	
Hemichordata				
Pyura chilensis	9.8 ± 10.43	1.2 ± 1.64	6.0 ± 7.04	0.4 ± 0.89
Free space	21.2 ± 13.81	20.8 ± 23.86	15.2 ± 15.32	7.2 ± 16.10
Density (ind.m^{-2})				
Mollusca				
Nassarius gayii	15.2 ± 25.52	0.6 ± 1.34	20.8 ± 29.04	124.8 ± 265.85
Crassilabrum crassilabrum	1.6 ± 2.19	1.6 ± 3.58		
Tegula sp.	0.8 ± 1.79		4.8 ± 10.73	164.0 ± 257.74
Mitrella unifasciata		1.0 ± 1.00	0.8 ± 1.79	125.6 ± 265.38
Crepidula sp.		0.4 ± 0.89		
Tegula tridentata			1.6 ± 2.19	26.4 ± 36.40
Calyptraea trochiformis			0.8 ± 1.79	
Density (ind. 100m^{-2})				
Cnidaria				
Anemonia alicemartinae		95	1	
Phymactis clematis	20	2	25	2
Phymanthea pluvia			23	4
Mollusca				
Concholepas concholepas	4	7	6	
Fissurella costata	6	2	1	3
Fissurella cumingii				2
Crustacea				
Paraxanthus barbiger	2		1	
Taliepus dentatus			5	
Homalaspis plana			2	
Rhynchocinetes typus	361	55	584	26
Echinodermata				
Aetionidium chilensis (Holothuroidea)	16	1		
Meyenaster gelatinosus	2	3	6	2
Stichaster striatus		17		
Heliaster helianthus		1		1
Tetrapygus niger		5		

mostly associated to the area immediately around the holdfast of *Lessonia trabeculata*, where fronds do not wipe the rock. *Pyura chilensis* is mostly restricted to crevices. Percent cover of both prey species together fluctuates between 10 and 20% cover. But *C.* *concholepas* within the habitat selects microhabitats in which his prey species, mainly barnacles, are more abundant. In those microhabitats percent cover of barnacles may increase up to almost 80% (Table 4). The polychaeta *Phragmatopoma* sp., which con-

TABLE 4.

Proportion (%) of potential prey in the different microhabitats in which *Concholepas concholepas* was captured on seven study sites.

	El Temblador	Lengua de Vaca	Huentelauquén	Isla Huevo	Totoralillo Sur	Las Tinicunas	Lagunillas
Main prey species							
Pyura chilensis	14.34	6.16		1.01			1.96
Cirripedia	24.75	8.52	21.14	68.04	74.63	79.82	38.34
Phragmatopoma sp.	45.66	43.74		27.68	4.88		1.75
Other potential prey							
Porifera annellida	8.76	6.88	8.13	2.52	14.63	6.88	5.58
Polychaeta indetermined		10.16	56.91				
Romanchella pustulata	3.68	6.47					0.67
Mollusca							
Calyptraea trochiformis	0.29	1.75				1.38	5.90
Fissurella spp			0.81	0.19	0.49	0.46	
Tonicia elegans		0.31					0.34
Brachiodontes granulata	0.22	2.26					3.66
Crassilabrum crassilabrum	0.22	0.10		0.56			0.67
Tegula spp	0.51	0.41			5.37		
Nassarius gayii		0.41				0.92	0.40
Bryozo							
Bryozoa	1.59	8.42	9.76			10.55	34.09
Cnidaria							
Hydrozoa indeterminate							6.64
Echinodermata							
Tetrapygus niger			3.25				
Hemichordata		4.41					

structs tubes of sediment attached to the rock surface, in some areas gets very important, covering together with the barnacles most part of the space in some sites (Table 4).

The digestive tracts of *C. concholepas* from the sampled sites contained mainly barnacles and *P. chilensis*. Although barnacles are the most abundant prey species in the environment, *P. chilensis* was only rarely found, mostly in very low abundance. Only in one site the ascidian was important in the environment (El Temblador, Table 4). Barnacles appear in four of the seven sites as being positively selected (Table 5, Fig. 9). In the remaining three sites barnacles are consumed proportionally to their abundance in the environment. *P. chilensis* was present only in four of the seven sites (Table 4), being always positively selected (Table 5). On one site (Las Tinicunas) *P. chilensis* did not appear registered in the environment (its proportion less than 1%), but was in the digestive tract of the gastropod. When the data from all the sites are grouped and analyzed together, it is shown that only *P. chilensis* is posi-

tively selected, the rest of preys being consumed proportionally to their abundance in the environment (Fig. 9H).

Circadian Feeding Rhythm

A total of 275 individuals were collected, representing a size range between 29 to 120 mm of peristomal length in the first 24-h sampling period. For the second period 88 and 84 individuals were sampled by each diver, representing a size range between 20 to 119 mm of peristomal length (Table 6). Numbers collected during individual sampling hours varied from 13 individuals at 2100 h to 71 individuals at 1300 h in the first sampling period and seven individuals at 2100 h to 28 individuals at 1700 h for the second sampling period (Table 6). As some of the samples were destroyed during the transport to the laboratory, the analysis is based on 254 individuals for the first sampling period, and on 66 and 81 individuals respectively for the two replicate samples of the second sampling period.

TABLE 5.

Selection index C and χ^2 for main prey species of *Concholepas concholepas* on seven sites.

	El Temblador		Lengua de Vaca		Huentelanquén		Isla Huevo		Totoralillo Sur		Las Tinicunas		Lagunillas	
	C	χ^2	C	χ^2	C	χ^2	C	χ^2	C	χ^2	C	χ^2	C	χ^2
Pyura chilensis	0.18*	6.64	0.47*	43.89			0.26*	13.39			0.34*	20.47	0.28*	16.22
Cirripedia	0.19*	6.95	0.05	0.45	0.80*	128.54	0.10	2.15	−0.09	1.53	−0.43*	32.35	0.23*	10.37
Phragmatopoma sp.	−0.39*	30.42	−0.53*	55.98	−0.63*	79.55	−0.32*	20.33	−0.16*	5.00			−0.09	1.76
Other species	0.03	0.21	0.01	0.01	−0.34	23.30	−0.13	3.23	0.14*	4.19	0.25*	11.11	−0.40*	31.59

Values with * show significant positive or negative selection.

TABLE 6.

Date and time of 24-h samplings, number of individuals collected in the field, number of entire digestive tracts analyzed in the laboratory, and individuals with food in their digestive tracts (number and percentage).

Date	Time	Sample Size Field (No.)	Individuals Analyzed in the Lab (No.)	Individuals with Food (No.)	(%)
24 OCT 1994	17:00	44	42	31	73.8
	21:00	13	13	11	84.6
	01:00	46	40	31	77.5
	05:00	44	44	37	84.1
	09:00	57	52	41	78.8
	13:00	71	63	45	71.4
Total		275	254	196	77.2
5 AUG 1995	17:00	28	19	15	78.9
(Replicate 1)	21:00	7	5	5	100
	01:00	8	7	6	85.7
	05:00	15	7	6	85.7
	09:00	15	15	14	93.3
	13:00	15	13	11	84.6
Total		88	66	57	86.4
5 AUG 1995	17:00	21	21	21	100
(Replicate 2)	21:00	7	7	7	100
	01:00	12	12	9	75.0
	05:00	16	13	12	92.3
	09:00	15	15	13	86.7
	13:00	13	13	10	76.9
Total		84	81	72	88.9

Considering all the individuals analyzed for the entire 24-h sampling, the individuals with food in their digestive tract (stomach and/or gut), represent 77.2% for the first sampling period and 86.4% and 88.9%, respectively, for the two replicate samples for the second sampling period (Table 6, Fig. 10). During the different sampling hours the proportion of individuals with food in their digestive tract for all sampling hours represented at least 71.4%. Although no clear pattern appears, in all sampling periods, the highest values were always registered at the late afternoon and early morning, thus suggesting that feeding intensity increases during the afternoon and in the second half of the night, or at dawn and dusk. Nevertheless, no statistical difference was detected between day (individuals sampled at 0900, 1300, and 1700 h) and night (individuals sampled at 2100, 0100, 0500 h), as well as between the different replicate samples (sampling in October 94, and each diver in August 96) (3×3 G test; $\chi^2 = 11.714$; df = 7; $P > 0.1$). Neither statistical difference was detected between different hours (Contingency Table $6*3*2$; $\chi^2 = 32.7304$; df = 27; $P > 0.05$).

At the different sampling hours different degrees of fullness were observed (Fig. 10). Although no clear pattern can be identified, the stomach shows a slight tendency of greater fullness in the afternoon or late afternoon hours, decreasing during night, with the same tendency repeating during the early morning hours. For the intestine it is observed, that as the stomach empties, the intestine increases in fullness (Fig. 10). Thus, again the data suggest that intake of new prey tends to increases at dawn and dusk.

In all sampled individuals during both 24-h samplings, barnacles appear as the main prey species, with proportions ranging from 41.9% to 75.2%, with a mean value of 57.9% (Fig. 11). The second most important prey was *Pyura chilensis*, which comprised 17.7% to 40.3% of the digestive tract contents. The remaining individuals had other preys of minor importance, such as *Calyptraea trochiformis* (Fig. 11). The food composition also did not vary greatly with sampling time, and barnacles were always the dominant prey item.

DISCUSSION

Concholepas concholepas fed almost exclusively on barnacles and the ascidian *Pyura chilensis*. The similarity in diet composition among individuals from different localities and among different size classes, suggests that this is a general characteristic for subtidal populations of this species.

These data support corresponding literature data (Castilla et al. 1979, DuBois et al. 1980, Sommer 1991), but show quantitatively, that barnacles were usually the most consumed prey in the different community or microhabitat types where *C. concholepas* was found. The smaller individuals of *C. concholepas* live on the undersides of boulders or in crevices (Stotz 1997, Guisado & Castilla 1983, Sommer 1991), where potential prey is probably different from that present on the rock surfaces where larger individuals live. Nevertheless, all size groups had consumed very similar food types. This suggests a strong feeding preference for barnacles, which nevertheless seems not always supported by the analysis with the selection index. With the pooled data, *P. chilensis* appears as the most preferred prey species. However, the preference is better shown by the fact that the gastropod is always found in microhabitats in which the barnacles predominate. And within such microhabitat the index is not any more able to show a preference. Considering all the prey species described, the preference extends in general to suspension feeders. A similar behavior has been described for *Acanthina lugubris angelica*, the diet of which was restricted exclusively to sessile suspension feeders (Vermeij et al. 1994). The diet based on suspension feeders seems to be a general pattern for benthic predators, such as diverse gastropods and seastars (Table 7).

The most common barnacles in subtidal communities are *Balanus laevis* and *Austromegabalanus psittacus*. Individuals of the latter species are mostly small individuals with 0.5–1 cm basal diameter, while the species is able to growth to sizes of ca. 5-cm basal diameter. But in the region, barnacles of such big size are seldom observed.

Feeding based on barnacles that are small, sessile, and form a uniform cover on the substrate makes *C. concholepas* conceptually resemble a grazer. The feeding of *C. concholepas* is similar to the "grazing" of hydroid colonies by nudibranchs, or even to grazing gastropods, for example, the keyhole limpets *Fisurella* spp. (Moreno & Jaramillo 1983, Moreno et al. 1984, Godoy & Moreno 1989). This observation applies to many gastropods and starfishes (Table 7). It is a well-described characteristic for intertidal whelks (Dayton 1971, Paine 1966, Menge & Sutherland 1987), habitat in which the sessile suspension feeders are the main space occupiers, but less known for species living in the subtidal, where a wider variety of potential prey species may be expected. In fact, *C. concholepas* makes use of a wider variety of prey in such habitats, including mobile predators as crabs and even fishes (personal observations), but quantitatively only the suspension feeders are important.

The feeding behavior of *C. concholepas*, not showing a clear circadian rhythm, differs from what has been published previously
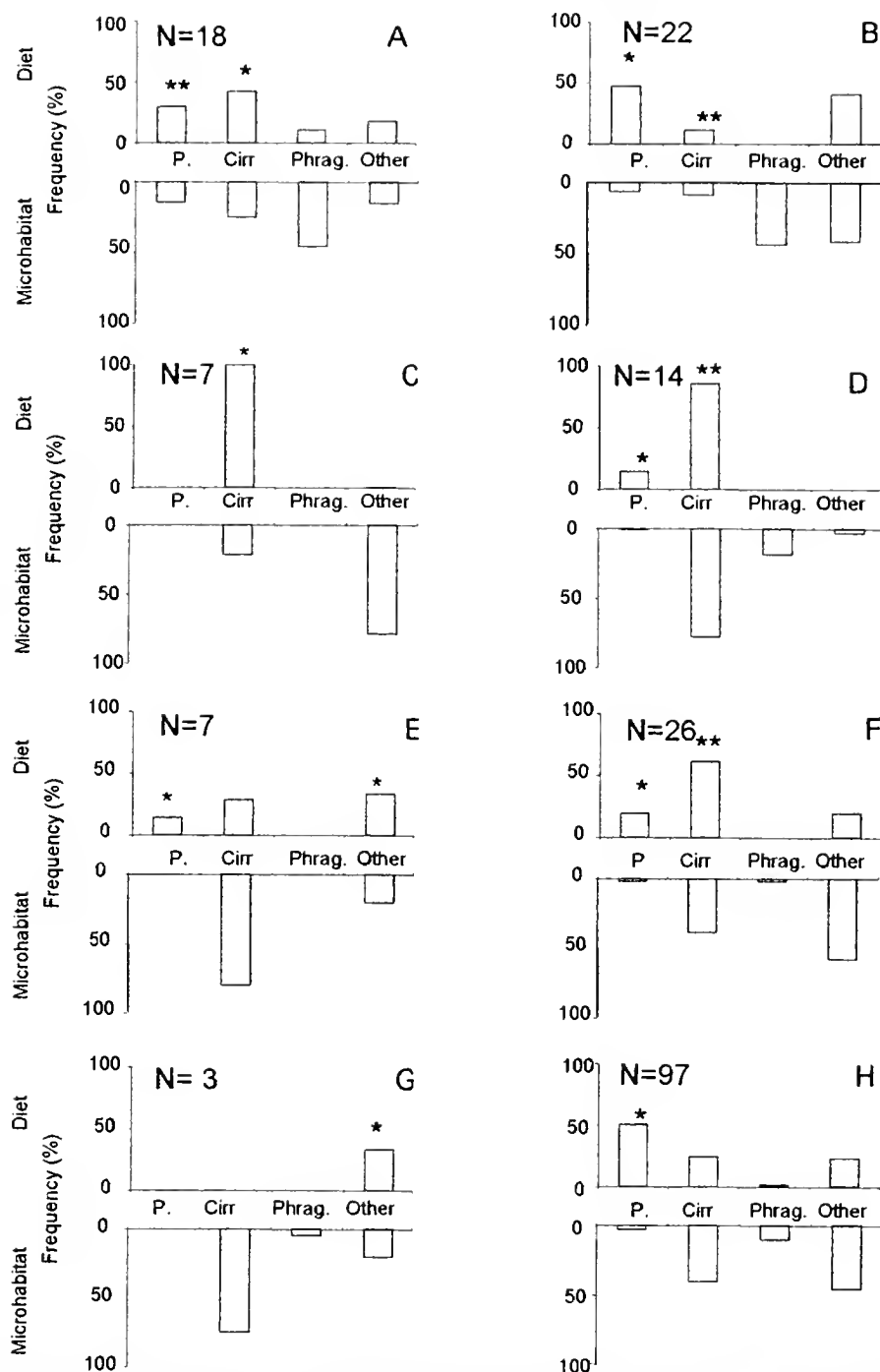
Figure 9. Frequency of prey in the diet and in the microhabitat in which *Concholepas concholepas* was captured in various localities: (A) Temblador, (B) Lengua de Vaca, (C) Huentelauquén, (D) Isla Huevo, (E) Las Tinicunas, (F) Lagunillas, (G) Totoralillo Sur, (H) Pooled Sample.

for this species by Castilla and Guisado (1979), Castilla and Cancino (1979), Castilla et al. (1979), Guisado and Castilla (1983), and DuBois et al. (1980). Differences in the methodological approach may explain this. Previous studies have been based in the intertidal zone, or in the laboratory, but using individuals collected from the intertidal. Environmental characteristics of the intertidal zone, principally dessication stress, often cause circadian rhythms, with activity periods at night and resting periods during the day (Underwood 1979, Branch 1981, Hawkins & Hartnoll 1983, Lowell 1984). Pino et al. (1993) compared the activity periods of the

intertidal gastropod *Fissurella crassa* Lamarck and the subtidal species *F. latimarginata* Sowerby and observed that the intertidal species had a distinct day–night activity cycle whereas the subtidal species did not. The novelty for *C. concholepas* is that in this case, the difference is between different populations (intertidal and subtidal) of the same species. However, DuBois et al. (1980) has also reported a day–night activity cycle for a subtidal population of *C. concholepas*.

DuBois et al. (1980), as all the published work done before on the feeding of *C. concholepas*, based his conclusion on the direct
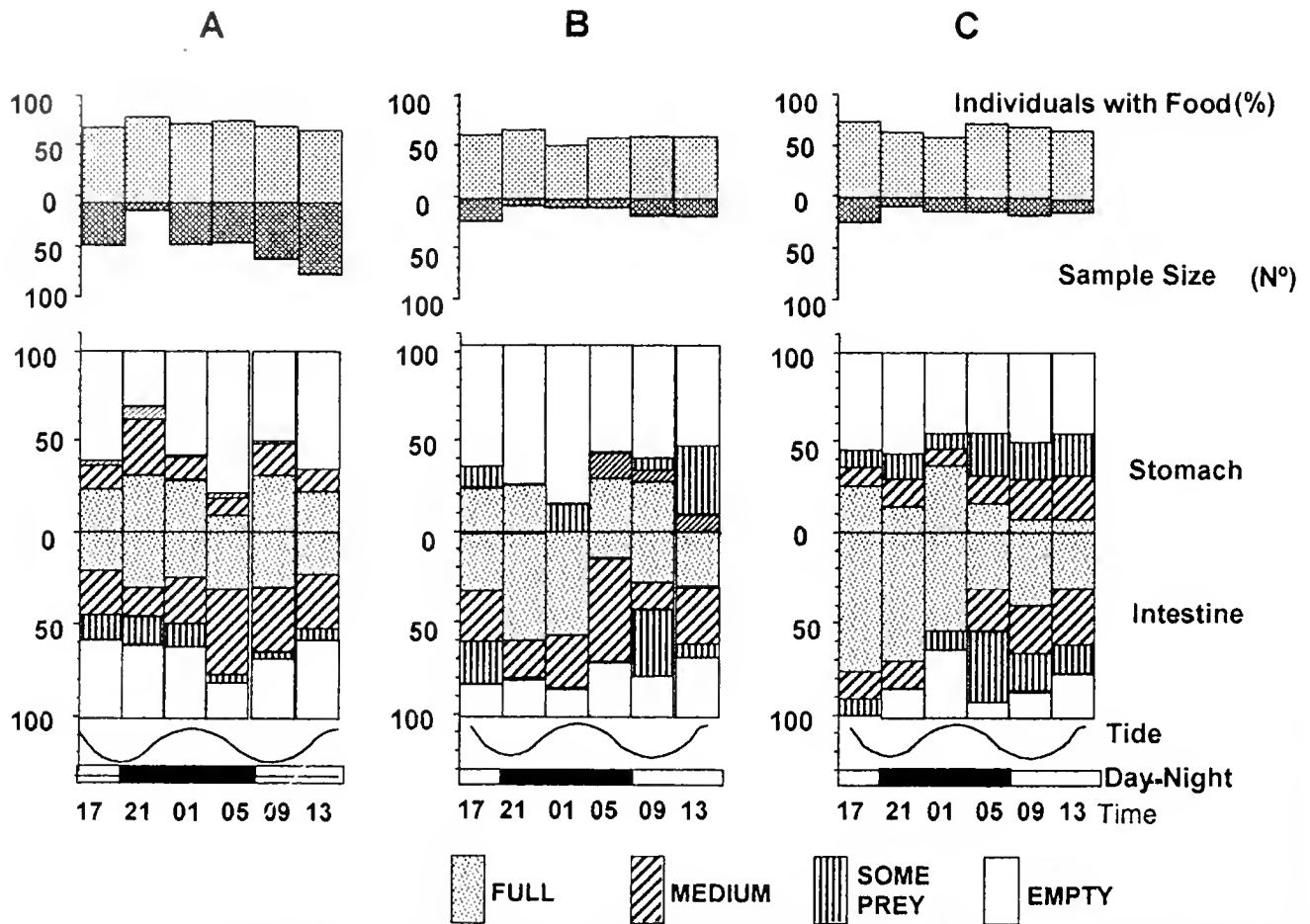
Figure 10. Circadian variations: Percentage of individuals with contents in their digestive tracts, corresponding sample sizes, and percentage of individuals with different degrees of fullness of the stomach or intestine for each of the three replicate samplings (A) October 1994; (B) August 1996, replicate 1; (C) August 1996, replicate 2.

observation of capture and ingestion, using criteria defined by Castilla (1979). If the prey is small and the predator is positioned directly over it, no sign of feeding will be seen. This may often be the case when *C. concholepas* feeds on barnacles, its main prey species. Study results may also be influenced by different conditions of observation (day and natural light, night and artificial light). For example, it is possible that at night the field observations are made mainly on more active individuals located on the surface of rocks, whereas during the day individuals found in crevices and between the algae might be included, and for these individuals it would be more difficult to establish if they were active or resting. Moreover, depending on the light conditions, animals could react differently to the presence of the diver. Finally, DuBois et al. (1980) also mention that some of the animals included in their observations from Caleta Hornos were introduced to the study site prior to the experiment. The behavior of these individuals might differ from that of resident (subtidal) animals.

In the approach used by DuBois et al. (1980), if capture and ingestion of prey occurs rapidly and is of short duration, it is less likely that observations will be recorded. The study of digestive tract contents also includes the process of digestion, thus covering a much longer time period, being less likely that a individual which has been feeding is missed. But on the other hand, the long retention time shown by *C. concholepas*, may obscure the existence of

a circadian feeding rhythm. Nevertheless, if no ingestion of food took place over the day (or over the night), at the end of the day (or night) most of the stomachs should be empty, as seen in the experiment in which the individuals where starved. And this is not
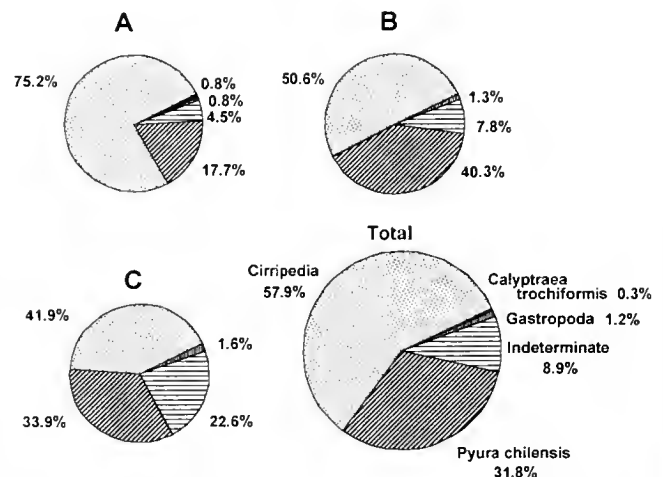
Figure 11. Prey composition of *Concholepas concholepas* sampled over 24 h at Punta Lagunillas. Composition of each replicate sampling (A) October 1994; (B) August 1996, replicate 1; (C) August 1996, Replicate 2; and total diet are shown.

TABLE 7.

Summary of prey species for several gastropods and starfish.

Predator	Main Prey	Site	Author
Gastropods			
Thais emarginata	*Balanus glandula*	Washington, USA	Paine 1966
Thais clavigera	*Tetrachta squamosa*	Cape d' Aguilar, Hong	Blackmore 2000
	Balanus amphitrite	Kong	
	Siphonaria japonica		
Thais biserialis	Barnacles	Costa Rica	Paine 1966
Acanthina brevidentata	Bivalves		
Thais emarginata	Barnacles	Washington, USA	Dayton 1971
Thais canaliculata			
Thais lamellosa			
Nucella lapillus	*Sembalanus balanoides, Balanus crenatus,*	New England, USA	Menge & Sutherland 1976, 1987
	Mytilus edulis and other bivalves		
Nucella lapillus	*Mytilus edulis, Semibalanus balanoides*	Maine, Anglesey	Hughes 1992, Dietl, 2000
Nucella emarginata	Bivalves	Canada	Gosselin & Chia 1996
	Barnacles		
Chicoseus capucinus	*Balanus amphitrite*	Singapur	Koh-Siang Tan 2000
	Modiolus sp		
Stramonita haemastoma	*Crassostrea virginica*	Gulf of Mexico	Brown & Stickle 2002
Stramonita haemastoma	*Brachiodontes pharaonis*	Israel	Rilov, Gasith & Benayahu 2002
	Barnacles		
Concholepas concholepas	**Barnacles, tunicates**	**Chile**	**This study**
Starfishes			
Leptasterias polaris	*Mytilus edulis*	Canada	Gaymer et al. 2001
Asterias vulgaris	*Mytilus edulis*	Canada	Gaymer et al. 2001
Asterias rubens	*Mytilus edulis*	German Bight, North Sea	Saier 2001
Asterias vulgaris	*Mytilus edulis*	Outer Brewster Island	Menge 1979
Asterias forbesi	*Balanus crenatus*	(Massachusetts)	
	Balanus balanoide		
Asterias vulgaris	*Mytilus edulis*	Gulf of St. Lawrence	Himmelman 1991
	Chlamys islandica		
Crossaster papposus	*Mytilus edulis*	Gulf of St. Lawrence	Himmelman 1991
	Chlamys islandica		
	Ascidea sp.		
	Didemnum albidum		
Leptasterias polaris	*Mytilus edulis*	St. Lawrence Estuary	Himmelman & Lavergne 1985
	Mya spp		
	Hiatella artica		
	Balanus sp		
	Halocynthia pyriformis		
	Ascidea sp		
Coscinas calamaria	*Chlamys asperrimus*	Rapid Bay (Australia)	Keough & Butler 1979
	Ascidaceas		
	Podoclavella cylindrica		
	Botryllioides leachii		
	Stolonica australis		
Cosmasteria lurida	*Aulacomya ater*	Puerto Toro (Chile)	Vásquez & Castilla 1984
	Balanus spp.		
	Tunicata:		
	Styella melineae		
	Colonial tunicate		
Pisaster ochraceus	Mussels	Temperate NE Pacific	Menge 1992b
Asterias vulgaris	Mussels	Temperate NW Atlantic	
Stichaster australis	Mussels	Temperate SE Pacific	
Leptasterias hexactis	**Balanus cariosus**	San Juan Island,	Menge 1974
Pisaster ochraceus	*Balanus glandula*	Washington	
	Mytilus edulis		
	Chthamalus dalii		

TABLE 7.

continued

Predator	Main Prey	Site	Author
Heliaster helianthus	*Semimytilus algosus*	Ancon Bay, Perú	Tokeshi et al. 1989
	Perumytilus purpuratus		
	Brachiodontes sp		
	Chamidae sp		
	Jehlius cirratus		
	Chthamalus scabrosus		
Pycnopodia helianthoides	*Mytilus edulis*	Torch Bay, Alaska	Duggins 1983
	Bivalves		
	Balanus spp		
Asterias vulgaris	*Mytilus edulis*	Golf of St. Lawrence	Himmelman & Dutil 1991
	Macoma spp		
	Mya truncata		
Leptasterias polaris	*Mytilus edulis*	Golf of St. Lawrence	Himmelman & Dutil 1991
	Mya truncata		
	Mya arenaria		
	Macoma spp		
Meyenaster gelatinosus	*Brachiodontes granulata*	El Francés, Chile	Vásquez 1993
	Semele solida		
	Balanus sp		
Meyenaster gelatinosus	*Aulacomya ater*	Golfo de Penas, Chile	Dayton et al. 1977
	Megabalanus sp		
	Pyura sp		

the case. Although no statistical differences was detect between individuals with food at different hours, a slight indication of the existence of greater ingestion is suggested to happen at dawn and dusk, at least in two of the three replicates.

Thus, the high percentage of individuals with stomach contents throughout the day and night, showing no distinct pattern of variation which could be associated with the circadian rhythm, suggests that most animals are feeding at all day and night hours. Thus, *C. concholepas* invests most of its time to feeding, as has been described by Bayne and Scullard (1978) for the snail *Thais (Nucella) lapillus*. They estimated that this species spends between 45 and 63% of its time feeding.

The conclusion that *C. concholepas* feeds almost over the entire 24-h cycle is important for the validation of our study of the food composition of this species because sampling time does not appear to be an important factor. Although our results show some minor variation in the prey composition with time, this can probably be attributed more to normal variability of the diet, rather than to circadian rhythms of feeding.

The high production described for *C. concholepas* (Stotz & Perez 1992) can be explained by its feeding on the lowest consumer level, which shortens the energy pathway from the primary producer level (Whittaker 1975). By feeding on barnacles and ascidians, this benthic gastropod effectively shortens the food chain. Through the consumption of suspension feeders *C. concholepas* accesses the much larger energy pool of primary production in the water column. For some coastal environments it has been calculated that 50% of the net primary production of the water column is used by benthic animals (Grahame 1987), which is the process *C. concholepas* is taking advantage of. By this feeding habit, *C. concholepas* is taking advantage of the high productivity provided by upwelling processes along the coastal zone of the southeastern Pacific coast of South America (Raymont 1980, Bakun & Nelson 1991, Thomas et al. 1994).

Upwelling processes, being localized in certain coastal areas, generate a spatial variability of primary production along the Chilean coast (Fonseca & Farías 1987, Acuña et al. 1989). The possible relation of this variability and the different production levels of *C. concholepas* along the coast, as shown by variable landings in different regions along the Chilean coast (Stotz 1997) is a hypothesis of much interest for this valuable fishery resource. Stotz (1997) showed that average landings for the period 1985–1995 along the entire coast of Chile, expressed as t per km of rocky coast, shows two patterns: (1) a general trend of decreasing landings from the south to the north, and (2) spots with higher landings than observed in surrounding areas (see Fig. 10 in Stotz, 1997). The first trend may be related to a similar trend for primary productivity described by Thomas et al. (1994), who integrated information for 8 y (1979–1986). These authors describe high primary productivity year around for the area close to the coast (0 to 100 km from the coast) in front of region X (43°S)(see Fig. 1 for location of regions). In front of region VIII (37°S) there are periods of high primary productivity only during autumn and winter. In front of region IV (29°S) the period of high primary productivity is restricted to a short period in winter. Further north primary productivity is year around low. The second pattern suggests a close relation to upwelling centers located in the regions VIII and IV. At a smaller geographic scale, for region IV, Stotz (1997) also shows a similar pattern, with the highest landings registered in the areas around the local upwelling center located in front of Punta Lengua de Vaca (Fig. 1). Variability of landings may be produced by variations in productivity of the gastropod, which, as shown by Stotz and Pérez (1992) and Pérez and Stotz (1992) differs between sites along the 320 km of coast of the Coquimbo region (region IV). Greater production of *C. concholepas* associated to upwelling would be evidence for the hypothetical alternative interaction webs in sites with differences in primary production in the water column, as postulated by Menge (1992a).

In places with higher primary production, filter feeders get more important, and consequently small carnivores, the category to which *C. concholepas* would correspond, also increase. The understanding of this variability and its causes are essential for the management of this important fishery resource. The estimation of catch quotas for different regions should consider this variability. Knowledge of the quantitative relation between primary production, production of suspension feeders and consequent production of this gastropod, would improve predictive capabilities, thus greatly aiding proper management of this resource.

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FEEDING AND GROWTH IN THE KEYHOLE LIMPET, *FISSURELLA PICTA* (GMELIN, 1791)

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ABSTRACT The feeding habits and growth relationships of the keyhole limpet ("lapa") *Fissurella picta* were analyzed in the field and under laboratory conditions. This species is of significant commercial value and considerable ecological importance in southern Chile. *F. picta* is not strictly a herbivore, although it prefers algae; the quantity of vegetable items consumed compared with animal items did not vary seasonally. The items most commonly found in the stomach of *F. picta* were the algae *Ulva sp*, *Polysophonia sp* and *Gelidium sp*. The abundance pattern of the principal items did not vary seasonally. However, there was greater diversity in the summer. The relative abundance of items in the diet was closely associated with their relative abundance in the environment. Under laboratory conditions, adults showed a higher consumption rate for the alga *Gracilaria chilensis* (artificial diet) than for *Ulva sp* (natural diet). The preferred alga is not usually found in the natural habitat of *F. picta* and has a lower calorie value than that of *Ulva* sp. *G. chilensis* proved to be the best source of energy available for growth in juveniles. Keyhole limpets feeding on the chlorophyte alga *Ulva* sp show a negative energy balance. Specimens maintained in suspended systems and fed with the artificial diet (*G. chilensis*) reached the average commercial size of 53 mm in ~3 y; the average survival rate was 90%. The results suggest that keyhole limpets prefer food with a high energetic scope for growth, although in field conditions they consume food with a lower energetic content but high in abundance. Factors such as morphology or palatability of food are more important than caloric value or presence in the natural habitats of keyhole limpets. This information is important for the culture of the keyhole limpet.

KEY WORDS: feeding, scope for growth, keyhole limpet, *Fissurella picta*

INTRODUCTION

Keyhole limpets ("lapas") of the genus *Fissurella* are grazing molluscs that consume a wide variety of macroalgae in the intertidal zone (Branch 1981, Hawkins & Hartnoll 1983). Previous studies indicate that they also ingest other types of food, such as crustaceans, small molluscs, coralline algae, ostracods, and sponges, although they remain preferentially herbivores (Ward 1966, Bretos 1978, Santelices & Correa 1985, Osorio et al. 1988).

Among Chilean species of lapas, *Fissurella crassa* is classified as a generalist herbivore, which prefers to consume foliacious algae, such as *Ulva* sp., *Enteromorpha* sp and *Porphyra* sp (Bretos 1978, Santelices et al. 1986). Data available on *F. maxima*, based on studies of its stomach contents, indicate that this species is euriphycophagous (Osorio et al. 1988). Experimental field studies on *F. picta* suggest that this species is a nocturnal herbivore, which migrates during the night to the middle intertidal zone (Jara & Moreno 1984, Moreno et al. 1984), to feed on the algae *Iridaea boryana* and *Ulva rigida*.

F. picta, has an important commercial value, and over-harvesting has resulted in the depletion of natural stocks in southern Chile (Bretos 1978, Bretos 1988). In addition, human exploitation of other species has, indirectly, had a negative effect on keyhole limpet recruitment (López et al. 1999). This species also has ecological importance given that it can modify the spatial and temporal distribution patterns of intertidal macroalgae (Moreno et al. 1984). Knowledge of the diet and dietary preferences of *F. picta* is necessary to evaluate its growth rate in artificial cultures and to interpret the ecological role of the population under field conditions.

Published literature suggests that the interaction between quantity and quality of food with factors such as pH, temperature and salinity, influences growth in mobile marine invertebrates (Newell 1979, Frantzis & Gremare 1992). The effect of type of food ingested on growth can be determined by measuring the increase in weight or size of the animals, or in terms of energy through scope for growth, established by evaluating the components of the energy balance (Paine 1971, Bayne & Newell 1983, González et al 1990, González et al. 1993, Thompson & MacDonald 1991, Navarro & Torrijós 1994, Navarro & Torrijos 1995). The aims of this study are to determine the feeding habits of the keyhole limpet, *F. picta* (Gmelin) in the field and under laboratory conditions and to establish the relationship between feeding and growth.

MATERIALS AND METHODS

Stomach Content in the Wild

The feeding habits of keyhole limpets were observed in the intertidal and subtidal zone of Metri Bay (41°36'S, 72°42'W), in southern Chile. The stomach contents of 40 *F. picta* specimens (between 32.9 and 64.8 mm total length) were analyzed per season. Specimens collected at high tide were immediately injected with formalin dissolved in seawater to stop digestion. The stomach contents were analyzed over a 100-point grid (81 mm^2). Thus, it was possible to determine (1) the relative frequency of vegetable and animal items; (2) the relative frequency of empty and full stomachs; and (3) the quantity and frequency of each item in the diet. A reference collection of all fronds of alga species present in different habitats and at different periods of the year was established to facilitate the identification of alga species consumed by lapas. Analysis was carried out under a dissecting scope. The relative abundance of sessile species present in the study area was verified during each season, based on coverage, using a 100-point grid 0.0625 m^2 along ten linear transects of 15–18 m in the intertidal zone (Burnham et al. 1980).

The statistical comparison between vegetable and animal content in keyhole limpets was carried out by the χ^2 test. The differences in dietary preference and energy consumed and lost in animals feeding on *Ulva* sp. and *G. chilensis* were analyzed with a *t*-test. Using correlation analysis, the relative abundance of algae in the diet was associated with the food supply of algae available in the environment.

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"Scope for growth" with Natural and Artificial Diets

Juveniles of *F. picta* (length between 25.0–32.3 mm) were collected from the rocky intertidal zone in Metri Bay. The animals were separated into two groups and acclimated in the laboratory at $10°C \pm 1°C$ for 20 days. During the experimental phase, each group was fed *ad libitum* with *Ulva* sp (Chlorophyta) or *G. chilensis* (Rodophyta).

All the parameters of energy balance were standardized as joules per day per gram of shell-free dry weight ($J \cdot d^{-1} \cdot gdw^{-1}$). (using 1 cal = 4.18 J) (Lucas & Beninger 1985). Animal dry weight was obtained using the regression equation for length versus dry weight, calculated for 150 keyhole limpets with lengths between 20.0–36.0 mm.

The experimental procedures for the two groups were as follows:

To evaluate the effect of natural and artificial diets on ingestion rate, 40 *F. picta* specimens of 42.2 ± 9.5 mm total length, collected in the middle and lower rocky intertidal zone of Metri Bay, were transferred to aquaria for an acclimation period of 13 days at 15°C $\pm 1°C$. The specimens were permanently submerged and the water was changed every 5–7 days. The ingestion rate of two types of macroalgae was compared: *Ulva* sp, which is the most frequent item found in the habitat of *F. picta* (natural diet) and *G. chilensis*, a rhodophycean species of alga, not present in the keyhole limpet's natural habitat (artificial diet). *G. chilensis* is the principal species used in artificial culture with an average annual production of 82119.5 ton y^{-1} (Sernap 1998). The two alga species have distinct forms: *Ulva* sp is foliaceus and *G. chilensis* is ramified. Each alga species was offered *ad libitum* to two groups of twenty animals of similar sizes kept in 1-L individual aquaria. The ingestion rate was measured gravimetrically, at 7-day intervals. An aquarium containing only alga samples was used as a control. The ingestion rate was obtained by comparing differences in alga weight at the beginning and end of the experiment, expressed in grams of dry weight of algae consumed per individual per day (gdw $\cdot$ ind$^{-1} \cdot d^{-1}$). Measurement of alga consumption was adjusted according to percentage weight variation of algae in the controls. No animal items were used as food because *F. picta* feed principally on algae and an important fraction of animal items in its diet are epiphytic organisms. The caloric contents of the *Ulva* sp and *G. chilensis* used in the experiments was measured with a Parr bomb calorimeter. Energy consumed (C) was determined using the caloric value of the algae.

The energy loss due to metabolism (R) was measured in 39 animals as the standard oxygen consumption in a 145-mL hermetic flask using a WTW-530 oxygenometer (0.01 mg O_2/l accuracy). For conversion into energy, the Thompson and Bayne (1974) oxicaloric value of 1 mL O_2 = 19.95 J was used.

The excretion rate of ammonia (U) was determined in 40 individual keyhole limpets measuring the concentration of ammonia accumulated over a period of 15 min in 200 ml aquaria, using the Solorzano method (Solorzano 1969). Conversion into energy units was carried out using the Elliot and Davison (1975) constant of 1 mg NH_4^+ = 24.85 J. The energy loss through feces (F) was measured in 15 keyhole limpets that were placed individually in 1-L aquaria containing filtered seawater (mesh size: 1 μm) that was changed daily and with a constant supply of air. The feces were collected every 12 h according to methods described by Navarro and Thompson (1996), rinsed with isotonic solution of ammonium formate, kept in containers, and dried in a Memmert

500 furnace at 75°C until a constant weight was reached. The caloric value of the feces was determined in a Parr adiabatic bomb calorimeter. Energy loss through mucus (M) was evaluated by filtering water through 120-μm mesh.

The energy values of scope for growth were calculated according to the following equation, using above average calculated values:

$$P = C - (F + R + U + M)$$

where P = scope for growth; C = energy from food consumed; F = fecal energy loss; R = metabolic energy loss; U = energy loss due to excretion and M = mucus.

Determination of Absorption Efficiency

Absorption efficiency was calculated using the Conover equation (Conover 1966):

$$AE = \frac{(F - E)}{(1 - E) \times F} \times 100$$

where AE = absorption efficiency (%); F = ash-free dry weight food/total dry weight food and E = ash-free dry weight feces/total dry weight feces.

To determine the algal and fecal organic matter content, algae and feces were carefully rinsed with distilled water and then dried in a Memmert 500 furnace at 75°C, until constant weight was reached. The samples were then incinerated in a muffle furnace at 450°C for 4 h. The organic matter was obtained by establishing the difference between the constant weight and the weight of the ash of each sample after incineration.

The results of all the above determinations were then compared (differences between animals fed with a diet of *G. chilensis* or *Ulva* sp), using one-way ANOVA after logarithmic transformation (Sokal & Rohlf 1979).

Dietary Preference—Natural and Artificial Diets

The same quantity of *Ulva* sp and *G. chilensis* (volume and weight) was supplied simultaneously to a group of 20 individuals of 46.7 ± 9.5 mm total length. The amount of algae consumed by each specimen was determined daily, based on the biomass variations, with an electronic balance ($\pm 0,01$g accuracy). A control was also set up.

Growth of Keyhole Limpets in Suspended Systems Feeding on an Artificial Diet

The direct effects of the artificial diet on keyhole limpets' growth and mortality were determined in artificial cultures.

This study was carried out over 12 months in Metri Bay. At this location, average water temperature varies between 9.6°C (winter) and 18.2°C (summer); salinity fluctuated between 28‰ and 32‰ during the study period.

Two hundred and forty specimens of *F. picta* collected from the intertidal zone were placed in trays ("linternas") that were suspended from a raft. Specimens were fed *ad libitum* with the red alga *G. chilensis*. Four size categories were used. Initial average size and the standard deviations of keyhole limpets placed in experimental growth systems ($n = 20$ per group) were: group 1: 25.9 $\pm$ 1.3 mm; group 2: 31.9 $\pm$ 1.9 mm; group 3: 37.8 $\pm$ 0.7 mm and group 4: 45.0 $\pm$ 0.9 mm. The experiments were replicated three times. Total weight and maximun length were measured monthly.
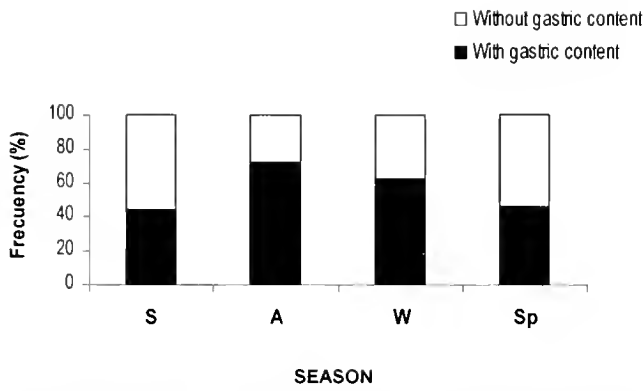
Figure 1. Relative seasonal frequency of *Fissurella picta* with and without gastric content. Summer (S); Autumn (A); Winter (W); Spring (Sp).

RESULTS

Stomach Contents in the Wild

The relative frequency of *F. picta* specimens with empty stomachs was less in autumn and winter than in summer and spring (Fig. 1). The percentage of vegetable items was always significantly higher than the animal items ($P < 0.05$), with no variation between different periods of the year (Fig. 2).

The most frequent items present in *F. picta* stomachs were the algae *Ulva* sp, *Polysiphonia* sp, and *Gelidium* sp, especially during autumn. The main animal items were cirripedes and juvenile bivalves (Fig. 3). There was a positive correlation between the relative abundance of food items present in the stomachs throughout the year and the relative abundance of these items in the environment ($r = 0.891$; $n = 65$; $P < 0.05$).

Scope for Growth

The diets used in scope for growth measurement had different energy values. The energy content of *Ulva* sp ($13,990.5$ J • gdw^{-1}) was higher than that of *G. chilensis* ($12,707.2$ J • gdw^{-1}). The type of food influenced the energy balance and the scope for growth. The scope for growth was highest when *F. picta* consumed *G. chilensis* (Table 1). The negative energy balance in specimens fed with *Ulva* sp was due to energy loss (Table 1.) The amount of energy consumed by *F. picta* juveniles did not vary significantly in animals fed with *G. chilensis* and those fed with *Ulva* sp (t =
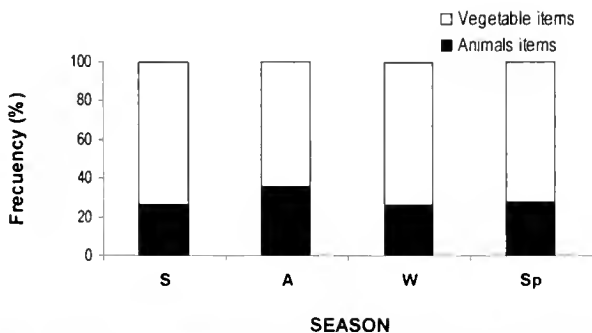
Figure 2. Relative seasonal frequency of vegetable and animal items in gastric content of *Fissurella picta*. Summer (S); Autumn (A); Winter (W); Spring (Sp).
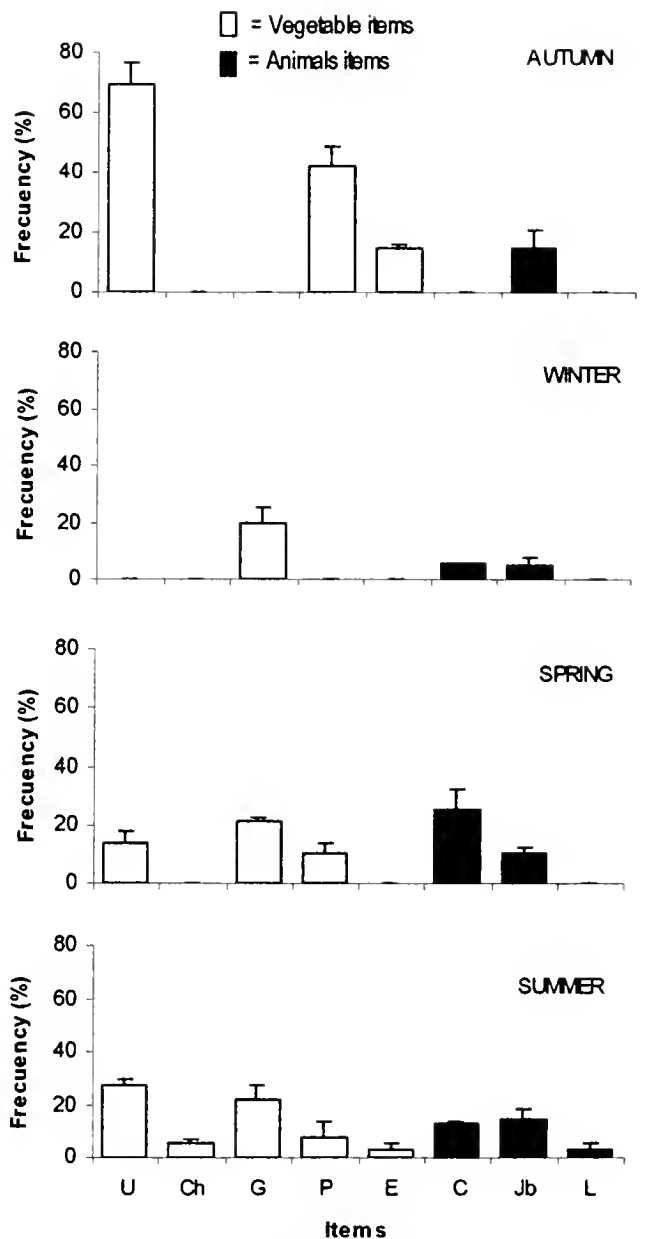
Figure 3. Seasonal frequency (average ± standard deviation) of food items in the stomachs of *Fissurella picta*. *Ulva* sp (U); *Chondrus* sp (Ch); *Gelidium* sp (G); *Polysiphonia* sp (P); *Enteromorpha* sp (E); Cirripeds (C); juvenile bivalves (Jb); *Nodilittorina araucana* (L).

0.098; df $= 28$; $P < 0.005$) (Table 1.). The quality of food affected the metabolic losses in *F. picta* (Fig. 4A). Oxygen consumption was significantly higher in animals fed with *Ulva*_sp than in those fed with *G. chilensis* (t $= 5.48$; df $= 37$; $P < 0.001$). The energy loss due to excretion was significantly higher in animals fed with *G. chilensis*, 23.0 J • d^{-1} • gdw^{-1}, than in those fed with *Ulva* sp, 5.4 J • d^{-1} • gdw^{-1} (t $= 8.10$; df $= 13$; $P < 0.001$). The fecal energy loss was also affected by the quality of food (Fig. 4B). Specimens fed *Ulva* sp had significantly higher fecal energy losses than those fed *G. chilensis* (ts $= 6.56$; df $= 13$; $P < 0.001$). Since no mucus was found in the aquaria, and given that this value would only represent 7% of the energy ingested in herbivorous molluscs (Paine 1971), energy loss through mucus (M) was not considered.

TABLE 1.

Energy ingested, energy loss and scope for growth in *Fissurella picta* juveniles fed with *Ulva* sp (natural diet) or *Gracilaria chilensis* (artificial diet) in joule/day/gram dry weight of soft parts.

Parameter	Food	
	Ulva sp	*Gracilaria chilensis*
Range of energy ingested ($J \cdot d^{-1}$ gdw^{-1})	605.3–1,509.3	740–1,920.3
Total energy loss ($J \cdot d^{-1} \cdot$ gdw^{-1})	803 ± 238.6	409 ± 140.2
Average scope for growth ($J \cdot d^{-1} \cdot$ gdw^{-1})	−10.4	390.6

Absorption Efficiency

Absorption efficiency was highest in specimens fed *Ulva* sp, 83.4%, and lowest in those fed *G. chilensis*, 74.6% ($\chi^2 = 0.49$; df = 1; 0.05).

Dietary Preference—Natural and Artificial Diets

In specimens of *F. picta*, consumption rates of *G. chilensis* (artificial diet) were higher than those of *Ulva* sp (natural diet) (t = 76.12; df = 27; $P < 0.001$) and they also presented a greater preference for *G. chilensis* than for *Ulva* sp (t = 19.89; df = 28; $P < 0.001$).

Growth of Keyhole Limpets in Suspended Systems

The alga *G. chilensis* proved to be suitable food for growth and survival in keyhole limpets. The annual average survival rate was 90% under these experimental conditions. The growth rates of the animals varied according to size. Using these data it was calculated that *F. picta* reached 26.0 mm in about 14 mo. Thus, the average commercial size of 53 mm would be achieved in approximately 3 y (Table 2).

DISCUSSION

The results obtained indicate that *F. picta* is preferentially a herbivore, as has been described for other species of this genus, (Osorio et al. 1988, Santelices et al. 1986). However, it also consumes animal items. Similarly, the high consumption of foliaceus species such as *Ulva* sp (Jara & Moreno 1984) was also confirmed. This can be associated with the food supply available in the envi-

TABLE 2.

Growth in four groups ($n = 20$) of *Fissurella picta* in suspended cultures, feeding on *Gracilaria chilensis* (artificial diet).

Group	Initial Length (mm)	Final Length (mm)	Time (Month)
1	25.9 ± 1.31	38.6 ± 4.9	12
		48.2 ± 0.1	21
2	31.98 ± 1.97	46.3 ± 5.7	12
3	37.86 ± 0.76	50.0 ± 5.3	12
4	45.03 ± 0.95	54.6 ± 1.2	8
		55.3 ± 2.3	14
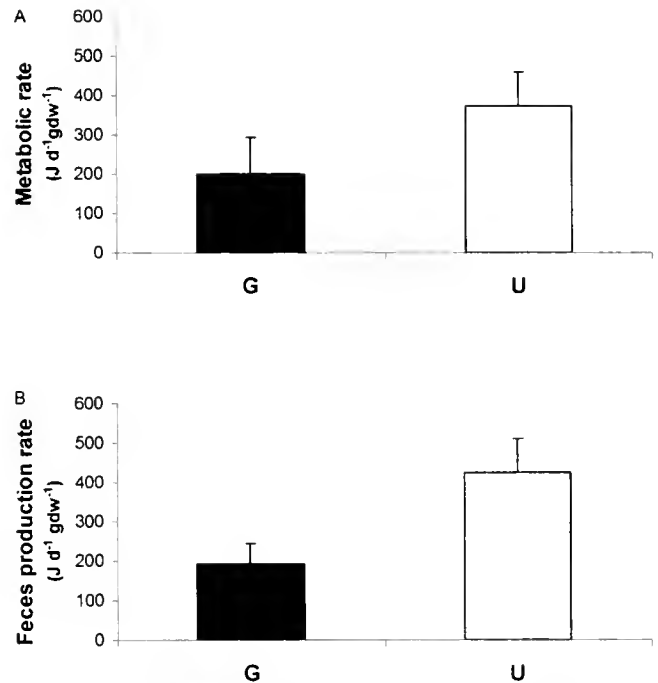
Figure 4. Energy loss through metabolism (A) and feces (B) in *Fissurella picta* feeding *Gracilaria chilensis* (G) or *Ulva* sp (U).

ronment, as has been verified in other species of *Fissurella* (Santelices et al. 1986). *Ulva* sp and *Polysiphonia* sp, the most frequent items in the keyhole limpets' stomachs, are opportunist algae species in the field. They densely colonize the intertidal zone of Metri Bay (Buschmann 1991).

The higher consumption rates, trophic preference, and scope for growth obtained with *G. chilensis*, which is not usually found in the natural habitat of *F. picta*, compared with those for *Ulva* sp, indicate that food items might not be selected due to their energy characteristics. The trophic preference is not related to the caloric value of food, given that *Ulva* sp has a higher caloric value than *G. chilensis*, and the energy budget was not associated with the food availability in the field. Although the laboratory results cannot be reliably extrapolated to the natural habitat, it can be assumed that the preference for macroalgae consumption may be associated with their digestibility, morphology, or palatability (Lowe & Lawrence 1976, Tugwell & Branch 1992). Although the chemical defenses of algae are lower than in terrestrial plants, the secondary compounds related to the plant-herbivore relationship, cannot be discarded (Hay & Fenical 1992). Further research is required to test these hypotheses.

The scope for growth in juvenile limpets varied according to the algal food offered. Specimens fed with *G. chilensis* (artificial diet) presented a positive energy balance. Considering the fact that the specimens studied were juveniles that had not yet reached sexual maturity, the balance of the energy budget can be considered as energy available for growth. In species such as the gastropod *Concholepas concholepas* (Bruguiere 1789) and the echinoderm *Loxechinus albus* (Molina 1782), it has been shown that the type of food offered greatly influences both the "sign" of energy balance and the amount of energy available for growth (González et al. 1990, González et al. 1993). These results coincide with those obtained in *F. picta*.

Keyhole limpets maintained in suspended cultures and fed exclusively on *G. chilensis*, had high survival rates. This study indicates that the type of food offered can have a considerable influence on the growth rate of juvenile *F. picta*. Our data indicate that, under artificial conditions, it can be possible to maximize reproduction and growth by selecting the food items offered. This finding could have significant consequences for cultivation of this important resource. Other factors, however, such as culture density, must be investigated to obtain higher growth rates.

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A COMPARISON OF THE DIGESTIVE CAPACITY OF BLACKLIP (*HALIOTIS RUBRA*) AND GREENLIP (*HALIOTIS LAEVIGATA*) ABALONE

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ABSTRACT In this study, the digestive capacity of blacklip abalone, *Haliotis rubra* Leach, was compared with that of the greenlip abalone, *Haliotis laevigata* Donovan. This was performed by assessing each abalone species ability to digest the protein and energy from 12 ingredients; semolina, defatted soyflour, fishmeal, casein, pregelatinized maize starch, mung beans, whey powder, skim milk powder, whole lupins (*Lupinus angustifolius* and *Lupinus luteus*), dehulled lupins (*L. angustifolius*), and bull kelp (*Durvillea potatorum*). Significant differences were found between the two abalone species in their capacity to digest the protein and energy from some of the ingredients assessed. Based on the differences observed, it was hypothesized that blacklip abalone are more efficient at digesting protein and cellulose than greenlip abalone and greenlip abalone might have a greater capacity to digest soluble nonstarch polysaccharides.

KEY WORDS: abalone, greenlip, blacklip, digestibility, protein, energy, *Haliotis rubra*, *Haliotis laevigate*

INTRODUCTION

Greenlip abalone (*Haliotis laevigata*) and blacklip abalone (*Haliotis rubra*) are the predominant species commercially farmed in Australia. Moratoriums on the collection of macroalgae for use in commercial abalone production necessitate the use of manufactured diets in these systems. To date, a significant amount of research has been completed to characterize the nutritional quality of ingredients and the nutritional requirements of greenlip abalone. It is uncertain, however, whether this information is relevant to blacklip abalone. If similarities exist between the digestive capacity of greenlip and blacklip abalone, then a large proportion of the research completed on the nutritional quality of ingredients for greenlips need not be replicated for blacklips.

Studies investigating the feeding preference of blacklip and greenlip abalone have shown that when given a choice, both species prefer to eat red algae (Hone & Fleming, unpublished data; Shepherd & Steinberg 1992, Fleming 1995). In the wild, however, abalone are forced to eat what algae is available. For example, along the coasts of Victoria blacklip abalone feed extensively on the fronds of the large kelp *Phyllopsora comosa* whereas on Tasmanian coasts they often feed on drifting blades of the giant kelp *Macrocystis pyrifera* as well as on red algae (Shepherd 1975).

The structural and storage polysaccharides present in red and brown algae are quite different. The storage polysaccharides in brown algae are mannitol, a sugar alcohol, and laminaran, a glucan, whereas the storage polysaccharide for red algae is a starch known as floridean starch. The cell wall of brown algae are two layered with an inner matrix of cellulose and microfibrils and outer layer of alginic acid and sulphated fucans (Stewart 1974). The cell walls of red algae consist of an inner rigid component made up of microfibrils and an outer more amorphous component consisting of mucilage or slime. The characteristic amorphous mucilages that make up most of the rest of the cell wall (up to 70%) are usually sulfated galactan polymers (Schweiger 1978). The two largest groups are the agars and the carrageenans.

Because they differ in their structural and storage carbohy-drates, it is reasonable to suggest that different enzymes would be required to digest red and brown algae. If, as the result of living in different habitats, blacklip abalone consume different or a broader range of algae than greenlip abalone, then it would be expected that they might have a different digestive enzyme profile. If this were so, then they may also differ in their capacity to digest the nutrients from the ingredients that are used in manufactured diets, particularly different carbohydrate sources.

Results from comparative studies conducted on other abalone have shown there are differences between species in their nutritional requirements or physiology. Mercer et al. (1993) examined the nutritional value of eight algal diets for *H. tuberculata* and *H. discus hannai* by comparing feeding rates, growth rates, and biochemical composition of the animals. The algae *A. esculenta*, *L. saccharina*, and *U. lactuca* were found to have different dietary values for the two abalone species with quite different feeding rates and feed conversion efficiency values being reported for each. Significantly different responses in growth rates were also recorded when fed particular diets. The lowest growth rates recorded for *H. tuberculata* occurred when it was fed with *L. saccharina* or *C. crispus* whereas the lowest growth rates recorded for *H. discus hannai* occurred when it was fed with *U. lactuca*. The differences in dietary values of the algae to the two abalone species were attributed to differences in their specific nutritive requirements and/or digestive physiology (Mercer et al. 1993).

Given that differences have been observed between other abalone species in their ability to use the same algal diets (Mercer et al. 1993), then it is possible that greenlip and blacklip abalone differ in their digestive capacities and/or nutrient requirements. This has important implications as feed costs represent a large proportion of farm running costs in Australia and our current manufactured diets are formulated based on results from research done on greenlip abalone. The objective of this experiment was to compare the protein and energy digestibility of a range of ingredients for blacklip and greenlip abalone and thus establish whether they differ in their digestive capacity.

MATERIALS AND METHODS

Diet Formulation and Manufacture

Twelve diets were formulated (Table 1) to evaluate the protein and energy digestibility from semolina, defatted soyflour, Tasma-

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TABLE 1.

Composition of experimental diets (g/kg, air dry basis).

Ingredient	Diet											
	1	2	3	4	5	6	7	8	9	10	11	12
Semolina	400.0	–	–	–	–	–	–	–	–	–	–	–
Defatted soyflour	–	625.0	–	–	–	–	–	–	–	–	–	–
Tasmanian fishmeal	–	–	420.8	–	–	–	–	–	–	–	–	–
Casein	–	–	–	347.6	–	–	–	–	–	–	–	–
Pregelled starch	189.4	214.4	418.6	200.0	489.4	158.7	289.4	150.0	150.0	374.8	100.0	100.0
Mung beans*	–	–	–	–	–	630.7	–	–	–	–	–	–
Bull kelp‡	–	–	–	–	–	–	500.0	–	–	–	–	–
Whey	–	–	–	–	–	–	–	600.0	–	–	–	–
Skim milk powder	–	–	–	–	–	–	–	–	600.0	–	–	–
Lupin 1‡	–	–	–	–	–	–	–	–	–	389.6	–	–
Lupin 2§	–	–	–	–	–	–	–	–	–	–	421.1	–
Lupin 3¶	–	–	–	–	–	–	–	–	–	–	–	500.0
Jack Mackerel oil	–	–	–	–	–	–	–	–	–	–	–	20.0
Mineral premix**	2.0	2.0	2.0	2.0	2.0	2.0	2.0	2.0	2.0	2.0	2.0	2.0
Vitamin premix**	3.0	3.0	3.0	3.0	3.0	3.0	3.0	3.0	3.0	3.0	3.0	3.0
Vitamin C	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5
Vitamin E	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1
Sodium alginate	–	–	–	–	–	–	–	–	–	5.0	–	–
Kaolin	400.0	150.0	150.0	441.8	500.0	200.0	200.0	239.4	239.4	200.0	448.4	369.4
Chromic oxide	5.0	5.0	5.0	5.0	5.0	5.0	5.0	5.0	5.0	5.0	5.0	5.0

* Whole *Vigna radiata.*

† *Durvillea potatorum.*

‡ Whole *L. luteus.*

§ Dehulled *L. angustifolius.*

¶ Whole *L. angustifolius.*

 Trachurus declivis (Triabunna Fish Oils, Triabunna, Tasmania).

** Vitamin and mineral premixes as described by Uki et al. (1985).

nian fishmeal, casein, whey powder, skim milk powder, whole mung beans (*Vigna radiata*), pregelatinized waxy maize starch, bull kelp (*Durvillea potatorum*), and lupins (whole *L. luteus*, whole *L. angustifolius* and dehulled *L. angustifolius*) by greenlip and blacklip abalone. The crude protein and gross energy of each of these ingredients is given in Table 2. Because of the wide range in crude protein levels of the ingredients being evaluated, it was

not practically possible to formulate the diets to be isonitrogenous. It is desirable for the diets to be isonitrogenous as it means that unbiased comparisons can be made among the different ingredients in regard to the digestibility of their protein.

Before incorporation into diets, the mung beans and lupins were crushed into a fine powder (<500 μm) using a hammermill. Each diet contained an equivalent amount of vitamin C (ascorbic acid) and E (DL-alpha tocopherol) and vitamin and mineral premixes as described by Uki et al. (1985). Sodium alginate was included in some diets to aid in binding. Kaolin and pregelatinized waxy maize starch were used in the diets as fillers. Chromic oxide was included at 0.5% for use in subsequent digestibility calculations.

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 mm × 1.5 mm. The strips were dried on mesh trays overnight in a forced draft oven at 55°C. They were then broken into three pieces before feeding.

TABLE 2.

Protein (g/kg, air-dry basis) and energy (MJ/kg, air-dry basis) content of the 12 ingredients used in the experimental diets.

Ingredient	Crude Protein ($N \times 6.25$)	Gross Energy (MJ/kg)
Semolina	104.0	15.51
Defatted soyflour	480.0	17.45
Fishmeal	713.0	18.71
Casein	863.0	22.00
Pregelled starch	3.1	15.65
Mung beans	253.7	16.54
Bull kelp	69.0	10.77
Whey	135.0	15.20
Skim milk powder	361.0	17.26
Whole *L. luteus*	385.0	18.03
Dehulled *L. angustifolius*	380.0	18.28
Whole *L. angustifolius*	320.0	17.74

Diet Allocation

Each diet was randomly allocated to three digestibility tanks to provide three replicates per diet. Because there was only 18 tanks in total, this meant that there were four separate collection periods.

Abalone and Feeding

Juvenile greenlip and blacklip abalone (shell length 40–60 mm) were used in the experiments. The abalone had been obtained from commercial hatcheries and raised on manufactured abalone feed. The abalone were preconditioned for 1 week on 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-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 regime 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 weeks. 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. After 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 reduce degradation by microbes. The feces were collected from the tubes at 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. Before analysis, the samples were freeze-dried and ground with a mortar and pestle.

Chemical Analyses

Gross energy was determined by a Parr 1281 bomb calorimeter (Parr Instrument Company, Moline, IL). Crude protein was determined by the combustion method using a LECO® CN-2000 Carbon and Nitrogen Analyser (RACI 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):

$$\text{Apparent digestibility} = 1 - \left(\frac{Cr_{\text{diet}} \times Nutrient_{\text{feces}}}{Cr_{\text{feces}} \times Nutrient_{\text{diet}}} \right)$$

where C_r is chromium content and *Nutrient* is nutrient or energy content of the diet.

Statistical Analysis

The data were analyzed by analysis of variance using a generalized linear model (SAS Institute Inc. 1988). Before analysis, residuals were plotted to establish that the data were in fact normally distributed, which was the case. Within species treatment means for nutrient digestibility of the twelve ingredients were compared by least significant difference.

RESULTS

Significant differences were found between blacklip and greenlip abalone in their apparent fecal digestibility of protein and energy of some of the ingredients evaluated (Table 3). Significant differences in protein and energy digestibility were also found among ingredients within each species (Table 3).

With respect to gross energy digestibility, blacklip abalone digested the energy from whole *L. angustifolius*, fishmeal, and skim milk powder significantly better than greenlip abalone, and greenlip abalone digested the energy from whey, bull kelp, and dehulled *L. angustifolius* significantly better than blacklip abalone (Table 3). No significant differences were found between the two species in their ability to digest energy from semolina, defatted soyflour, casein, pregelatinized maize starch, mung beans, and *L. luteus* (Table 3).

Greater differences were found between the two species in their capacity to digest protein from the ingredients with statistically similar protein digestibility values only being obtained for mung beans, whey and *L. luteus* (Table 3). Blacklip abalone digested significantly more protein from defatted soyflour, fishmeal, casein, bull kelp, and skim milk than greenlip abalone, whereas greenlip abalone digested significantly more protein than blacklip abalone from semolina and dehulled and whole *L. angustifolius* (Table 3).

Comparisons among ingredients within species showed that there were significant differences in their apparent protein and energy digestibility for both species of abalone (Table 3). Whey was the most digestible ingredient, having significantly higher protein and energy digestibility than all other ingredients evaluated for both blacklip and greenlip abalone ($P < 0.05$). Bull kelp contained the least-digestible protein for both species of abalone ($P < 0.001$), while semolina contained the least-digestible energy for both species of abalone ($P < 0.001$).

DISCUSSION

The results from the current experiment demonstrate that blacklip and greenlip abalone differ in their digestive capacity. Significant differences were found in their ability to digest the protein and energy from several ingredients.

With regard to protein digestibility it is interesting to note that blacklip abalone can digest significantly more protein from, in general, nonplant-derived proteins (excluding soyflour and bull

TABLE 3.

Comparison of the apparent faecal protein (PD) and energy (GED) digestibility coefficients obtained for 12 different ingredients fed to blacklip and greenlip abalone.

Ingredient	PD Blacklip Abalone	PD Greenlip Abalone	$F_{1,4}$	P	SEM	GED Blacklip Abalone	GED Greenlip Abalone	$F_{1,4}$	P	SEM
Semolina	0.62^h	0.84^c	441	***	0.762	0.30^h	0.34^g	5.49	NS	1.265
Defatted soyflour	0.83^f	0.82^c	18.38	**	0.730	0.83^d	0.78^s	0.73	NS	1.507
Fishmeal	0.56^l	0.46^c	27.72	**	1.382	0.63^g	0.52^f	48.09	*	1.144
Casein	0.82^g	0.77^d	27.42	**	0.624	0.79^e	0.78^d	4.02	NS	0.579
Pregelled starch	–	–	–	–	–	0.92^s	0.93^b	1.80	NS	0.647
Mung beans	0.89^d	0.91^b	5.13	NS	0.630	0.65^g	0.67^c	2.40	NS	0.986
Bull kelp	0.46^l	0.23^f	105	***	1.600	0.75^f	0.81^c	29.45	*	0.805
Whey	0.96^a	0.95^a	1.46	NS	0.373	0.99^a	1.00^a	43.20	*	0.106
Skim milk powder	0.94^b	0.85^c	510	***	0.286	0.95^b	0.89^b	1338	***	0.101
Lupin 1†	0.91^c	0.91^b	0.03	NS	0.804	0.79^c	0.83^s	2.83	NS	1.780
Lupin 2‡	0.85^e	0.92^b	723	***	0.211	0.70^f	0.82^c	66.19	**	1.169
Lupin 3§	0.84^{ef}	0.91^b	371	***	0.284	0.63^g	0.50^f	202	***	0.682

Within a species, superscripts have been used to identify significant differences among ingredients for their nutrient digestibility (within column comparisons). Between species comparisons of nutrient digestion of each ingredient are made across rows and indicated by *.

NS, not significant

* $P < 0.05$

** $P < 0.01$

*** $P < 0.001$

$^{a-g}$ Within a column, ingredient digestibility coefficients with different superscripts differ significantly ($P < 0.05$).

† Whole *L. luteus*.

‡ Dehulled *L. angustifolius*.

§ Whole *L. angustifolius*.

kelp) than greenlip abalone. In contrast, greenlip abalone can digest significantly more protein from plant-derived sources (lupins and semolina) than blacklip abalone. This finding is in agreement with that of Wee et al. (1994), who reported that blacklip abalone digested significantly more protein than greenlip abalone from a manufactured diet containing 50% fishmeal. It appears blacklip abalone may not be able to digest the soluble nonstarch polysaccharides found in terrestrial plants as efficiently as greenlip abalone and that soluble nonstarch polysaccharides may actually interfere with and reduce blacklip abalone's ability to digest nutrients (both protein and energy) from plant feedstuffs which contain them. As a consequence, use of exogenous enzymes that cleave soluble nonstarch polysaccharides may improve the digestive capacity of blacklip abalone.

Dehulling had no effect on the digestibility of protein from *L. angustifolius* when fed to blacklip abalone. Although a significant increase was found in the digestibility of its energy for blacklip abalone after dehulling it was much less than was found for greenlip abalone (0.63 to 0.70 for blacklips compared with 0.50 to 0.83 for greenlips). After removal of the hull the energy from *L. angustifolius* changed from being significantly less to significantly more digestible for greenlip compared with blacklip abalone. The hull of the lupin is composed primarily of cellulose. It appears that blacklip abalone have a greater capacity to digest cellulose than greenlip abalone given that the removal of the hull had a much smaller effect on the capacity of blacklip abalone to digest energy from this lupin compared with greenlip abalone.

Milk-based products (casein, skim milk powder, and whey) are very digestible sources of protein and energy for both blacklip and greenlip abalone. In particular, the sugar component of milk (lactose) is very digestible for abalone given the extremely high gross

energy digestibility coefficients obtained for whey (the residue from milk after removal of the casein and most of the fat). Lactose is a disaccharide composed of galactose and glucose. Thus, it is a much simpler carbohydrate than those found in many terrestrial plant-based feedstuffs, such as lupins, which are composed of complex structural and storage polysaccharides. β-galactosidase (lactase) activity, needed for the hydrolysis of lactose, has been found in abalone (Oshima 1931, Bennett et al. 1971). Obviously β-galactosidase activity in wild abalone would not be for the digestion of lactose, but probably for the breakdown of galactose, one of the major components of carrageenan which is found in the cell walls of red algae.

Pregelatinized waxy maize starch was also found to be a highly digestible source of energy for both species of abalone. Again, this is not surprising because the starch found in red algae, termed floridean starch, is essentially the same as waxy starches found in terrestrial plants in that it consists almost entirely of amylopectin. In addition Elyakova et al. (1981) found evidence for amylase-α-1,4-glucanase activity against amylopectin in extracts from the hepatopancreas of *H. asinina* and *H. varia*. The fact that the starch has been gelatinized, whereby the application of moist heat brings about swelling and rupturing of the starch granules facilitating amylolysis, would also increase energy digestibility.

The low protein digestibility of bull kelp by both species could be caused by the presence of tannins, naturally occurring polyphenols present in plants to protect them against herbivory. Their main characteristic is that they bind and precipitate proteins. *In vivo* studies have shown that protein digestibility is greatly reduced when tanniniferous feeds are part of animal diets (Reed 1995). Polyphenols are predominant in brown algae (Ragan & Glombitza 1986, Steinberg 1989). It should be pointed out that bull kelp has

a very low crude protein content (69 g/kg) and that even though it was included in the diet at a level of 500 g/kg the crude protein content of the diet was only 3.45 g/kg. Thus the endogenous N contribution would have had a much larger effect on the apparent protein digestibility of kelp than for other ingredients, resulting in these values being reduced as a result of an experimental artifact.

Neither species were able to digest the energy from semolina very well, particularly blacklip abalone. In another study semolina was found to affect the digestibility of other ingredients within a diet (Vandepeer, unpublished data). The poor digestibility of semolina and its effects on the digestibility of other ingredients is a concern given that it is currently one of the major ingredients used in manufactured diets in Australia. Further research is re-quired to establish the reasons why energy from semolina is so poorly digested, however, it is possible that the starch component significantly influences these results.

The results from this experiment demonstrate that greenlip and blacklip abalone have different digestive capacities and thus a different basis should be used for the formulation of manufactured diets. Further comparisons of the nutritional requirements of greenlip and blacklip abalone may also be justified.

ACKNOWLEDGMENTS

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REVIEW OF TECHNIQUES TO PREVENT INTRODUCTION OF ZEBRA MUSSELS (*DREISSENA POLYMORPHA*) DURING NATIVE MUSSEL (UNIONOIDEA) CONSERVATION ACTIVITIES

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ABSTRACT Because of the declines in diversity and abundance of native freshwater mussels (superfamily Unionoidea), and the potential decimation of populations of native mussels resulting from the rapid spread of the exotic zebra mussel *Dreissena polymorpha*, management options to eliminate or reduce the threat of the zebra mussel are needed. Relocating native mussels to refugia (artificial and natural) has been proposed to mitigate the threat of zebra mussels to native species. Relocation of native mussels to refugia such as fish hatchery facilities or natural habitats within their historic range, which are unlikely to be infested by zebra mussels, necessitates that protocols be developed to prevent the inadvertent introduction of zebra mussels. Several recent studies have developed such protocols, and have assessed their effectiveness on the health and survival of native mussels during subsequent relocation to various refugia. The purpose of this project is to synthesize and evaluate the current protocols and to develop a set of procedures that resource managers and researchers should consider before conducting conservation activities in zebra mussel infested waters. We found that the existing protocols have many common points of concern, such as facility modification and suitability, zebra mussel risk assessment and management procedures, and health and disease management procedures. These conservation protocols may have broad applicability to other situations and locations. A summary and evaluation of the information in these main areas, along with recommended guidelines, are presented in this article.

KEY WORDS: relocation, Unionidae, *Dreissena polymorpha*, conservation, refugia

INTRODUCTION

Native freshwater mussels of the families *Margaritiferidae* and Unionidae (superfamily Unionoidea) are one of the most rapidly declining faunal groups in North America. About 67% of the nearly 300 native species found in North America are considered vulnerable to extinction or already extinct (Bogan 1993, Williams et al. 1993). The decline of native mussel populations in North America has occurred steadily since the mid 1800s and has been attributed to overharvest, construction of dams and impoundments, sedimentation, navigation, pollution, and habitat degradation (Fuller 1974, Bogan 1993, Naimo 1995, Brim Box & Mossa 1999, Vaughn & Taylor 1999). An additional recent threat to the native fauna has come from the introduction of the zebra mussel *Dreissena polymorpha*. This species colonizes native mussels and impedes their movement, reduces the ability to feed and eliminate wastes, and competes for food and space (Mackie 1991, Schloesser et al. 1996, Strayer 1999).

Because of the declines in diversity and abundance of native mussels and the rapid and severe impacts of zebra mussels on native mussels (Gillis & Mackie 1994, Nalepa et al. 1996), a national strategy for the conservation of native freshwater mussels was developed to provide a framework for preventing further population declines and species extinction (National Native Mussel Conservation Committee 1998). This document identified a number of conservation needs and outlined goals, strategies, and tasks to address these needs. Listed among these was the recommendation to develop management options for eliminating or reducing the threat of zebra mussels to native mussels. These options included relocating native mussels to artificial and natural refugia. Although many mussel relocations have had poor success (e.g.,

Cope & Waller 1995), recent studies conducted with improved techniques, experimental design, and monitoring programs, have been successful (Dunn et al. 2000, Cope et al. 2003). Thus, with the increased likelihood of successful relocation efforts, and the continued range expansion and adverse effects of zebra mussels on native mussel populations, any relocation done to conserve native mussels necessitates that protocols be developed to prevent the inadvertent introduction of zebra mussels.

Several recent studies have developed protocols to ensure that zebra mussels would not be inadvertently introduced during native mussel conservation activities and have assessed the health and survival of native mussels during subsequent relocation (Patterson et al. 1997, Patterson et al. 1999, Gatenby et al. 2000, Nichols et al. 2000, Hallac & Marsden 2001, Newton et al. 2001). The purpose of this project was to synthesize and evaluate the current protocols and to develop a set of procedures that resource managers and researchers should consider before conducting native mussel conservation activities in zebra mussel infested waters.

RESULTS AND DISCUSSION

Almost all of the recent native mussel salvage and relocation projects have used some type of quarantine to prevent the incidental introduction of zebra mussels. The exceptions are those studies intended to remove zebra mussels from fouled native mussels and replace them back to their original location (e.g., Schloesser 1996, Hallac & Marsden 2000). By necessity, most of the quarantine protocols have been location and facility specific. For example, Gatenby et al. (2000) reviewed procedures for relocating native mussels from the Ohio River. Likewise, Newton et al. (2001) developed a specific set of procedures for relocating native mussels from the Mississippi River to artificial ponds and to fish hatchery facilities. However, these and other protocols developed for specific studies have many common points of concern, such as

TABLE 1.

Summary of collection and quarantine-related conditions and procedures, and recommended guidelines for preventing introduction of zebra mussels during native mussel conservation activities.

Condition or Procedure	Reference		Recommended Guidelines
	Gatenby et al. (2000)	Newton et al. (2001)	
Collection setting			
Time of collection	July, September, October 1995	May 1995	Early spring, before zebra mussel spawning begins (water temperatures <15 C) or mid to late fall when natives have greater energy reserves and juvenile zebra mussels are visible (>2–5 mm shell length)
Species of native mussels	*Amblema plicata, Quadrula pustulosa, Elliptio crassidens, Pleurobema cordatum, Obliquaria reflexa, Potamilus alatus*	*Amblema plicata, Fusconaia flava, Leptodea fragilis, Obliquaria reflexa, Quadrula quadrula*	
No. of native mussels	2700	768	
Native mussels analyzed for disease and pathogens before relocation	No	Yes	If possible
Air temperature (C)		6–18	Early spring or late fall temperatures; minimize differences between air and water temperature
Water temperature (C)	20–28	11–14	Early spring or late fall temperatures; minimize differences between air and water temperature
Mechanism for removing zebra mussels from native mussels	Hand scrubbed with plastic-bristled brushes	Hand scrubbed with plastic-bristled brushes under ×2 magnification	Hand scrub with plastic-bristled brushes under magnification
Method for holding scrubbed native mussels at collection site	Mesh bags in river*	Hatchery truck with aerated well water	Hold in zebra mussel-free water after scrubbing
Emersion time (min) during collection and processing	20	5	Keep to minimum, but <20
Transportation to quarantine facility	Between moist burlap in coolers with ice (no direct contact of mussels and ice)	Between moist burlap in coolers with ice (no direct contact of mussels and ice)	Between moist burlap in coolers with ice in plastic bags for transport durations <12 h; no direct contact of mussels and ice bags
Quarantine facility			
Type	Above-ground tanks, 14–500 L	Pond (0.04 ha), mussels held in 8–2720 L mesh bags	
Mussel density (no./m²)	150–250	39–159	Keep to minimum, but <150
Water source	Well water	Well water	Well water
Water temperature (C)	2–28	13–27	<28
Dissolved oxygen (mg/L)	6–14	6–20	>6
pH	7.2–8.5	7.8–10.6	6.5–9.0
Potassium (mg/L)	1.6	2.6	<4
Alkalinity (mg $CaCO_3$/L)	90	110–160	>15
Hardness (mg $CaCO_3$/L)	90	180–200	>50
Total ammonia nitrogen (mg/L)	≤1.0	0.03–0.2	<1.0
Unionized ammonia (μg/L)	2–66	2–20	<25
Total residual chlorine (μg/L)			<17
Nutrition/feeding	$\geq 1 \times 10^6$ cells/mL three times per week in quarantine; relocation ponds were fertilized with a nitrogen:phosphorous (N:P) ratio of 10:1 (1.0 mg/L N, 0.1 mg/L P) with NH_4NO_3 and $NaHPO_4$ salts	8.3 g/m³ of 10:10:10 N:P:K fertilizer added to quarantine pond 2 weeks prior to adding unionids; relocation ponds were not fertilized	1×10^5 cells/mL or 4.0 mg dry wt./L twice daily or $2.0–5.0 \times 10^4$ cells/mL or 1.9 mg dry wt./L on a continuous basis (Gatenby 2000, 2002); suitable algal species include *Neochloris oleoabundans, Bracteacoccus grandis,* and *Phaeodactylum tricornutum*

TABLE 1.

continued

| Condition or Procedure | Reference | | Recommended Guidelines |
	Gatenby et al. (2000)	Newton et al. (2001)	
Days in quarantine	Minimum of 30, but up to 120; re-inspected under 4× magnification	35; re-inspected under 2× magnification	Minimum of 30; re-inspect under magnification
Disinfection of equipment and supplies	Chlorine solution of 25 mg/L. Dessication for up to 4 d	Not specified	Chlorine solution of 25–250 mg/L. depending on type of material; dessication in warm dry air for 3–5 d
Monitoring			
Temperature, dissolved oxygen, and pH	Twice daily	Daily	At least daily
All other water quality variables	Daily to weekly	Daily to weekly	Daily to weekly
Disease and mortality	Not specified	Not specified	At least weekly

* All native mussels were rinsed with a high pressure hose before being placed into the quarantine facility.

facility modification and suitability, zebra mussel risk assessment and management procedures, and native mussel health and disease management procedures, that may have broad applicability to other situations and locations.

Facility-Specific Concerns and Procedures

The availability of aquatic facilities for long-term captive care of freshwater mussels is limited. Thus, most of the salvage and quarantine facilities have involved the short-term use of state and US Government owned fish hatchery ponds and raceways or similar research aquaculture facilities (Dunn & Layzer 1997. Pinder et al. 1999, Gatenby 2000, Newton et al. 2001). The main facility concerns have focused on the type of rearing or holding system (e.g., ponds, raceways, or above-ground tanks capable of housing hundreds to thousands of mussels), the facility's proximity to the source of relocated mussels (to reduce transportation time and handling stress), on-site water quality for maintenance of mussel health, and production of an algal-based food supply. The objectives of any given conservation project will likely dictate the type of facility or holding system used and any modifications that may be required. Nonetheless, whether used for short-term quarantine or for long-term captive care, all facilities should be able to provide space for isolation and quarantine, water quality characteristics to meet requirements for shell growth and metabolic processes, and food quantity and quality to support growth and reproduction (Table 1).

Specific isolation and containment modifications are probably necessary at most facilities to control and contain source water inflow and potentially contaminated outflow. For example, the outflow of water from quarantine units may need to be passed through filtration or disinfectant treatments to remove or kill potential zebra mussels before the water is discharged through normal routes. Containment procedures commonly used at facilities conducting zebra mussel research have included filtration of outflow water through small mesh bags (100 μm or smaller), chlorine treatment tanks (250 mg/L for 1 h), and sand filtration units (J. J. Rach, U.S. Geological Survey, Upper Midwest Environmental Sciences Center, La Crosse, WI, pers. com.). Additional facility precautions may include the capping of all exterior drains to prevent the release of potentially contaminated water from the affected

areas and the development of a flood risk assessment, if the facility is within a designated floodplain.

The type of facility selected, however, may influence the relative success of the conservation project. Success could depend on its use only as a short-term quarantine facility for subsequent relocation to a natural or artificial system, or its use for long-term captive care. For example, Newton et al. (2001) relocated five species of native mussels (1,392 mussels total) from the Upper Mississippi River to a fish hatchery pond after 35 d of quarantine in an artificial pond (81% of mussels survived during quarantine). Mussel survival in the hatchery pond averaged 80% after 1 y, but only 35% 3 y after relocation. Of the mussels in a handling-control treatment that were placed back into the Mississippi River after quarantine, survival was 80% after 1 y and 75% after 3.3 y. The authors attributed the differences in survival between the hatchery pond and riverine relocated mussels to inadequate nutritional resources in the pond. This study illustrates the potential utility of natural or managed refugia over artificial refugia for long-term conservation (Nichols et al. 2000, Cope et al. 2003). Gatenby (2000) observed similar decreases in survival of six large river species relocated to pond refugia after a 30-d quarantine in above-ground tanks. Mean survival of native mussels during quarantine was 97%. Mean survival after 1 y in the ponds ranged between 82 and 93%, depending on species. Despite an abundance of a suitable algal food supply and adequate water quality conditions in the ponds, however, the survival of relocated mussels decreased to 44% after 2 y and to 5% after 3 y. Gatenby (2000) attributed the mortality to high water temperatures in July and August during years 2 and 3 of that study. Large river species of mussels relocated (with no quarantine period) to fish hatchery raceways with flowing water and sediment also showed high survival (95%) after 1 y (Dunn & Layzer 1997), but their long-term (3–5 y) success in this type of system is unknown.

The relocation of native mussels after quarantine to natural refugia or raceway systems supplied by natural river water will likely have greater success for long-term preservation of the mussels than retention in artificial pond refugia for two key reasons: water temperature and food quality. These two components are critical to the livelihood of any aquatic organism. Rapid fluctuations in temperature, unnaturally high temperatures, and inadequate food supplies are known to cause stress in aquatic organ-

isms, and can lead to mortality (Bayne et al. 1973). Thus, temperature, food quality, and food quantity will also be key components to the success of native mussel captive care programs.

Zebra Mussel Risk Assessment and Management Procedures

Because the threat of zebra mussels to native mussels has been the primary causal factor for initiating most mussel conservation activities, special precautions have been necessarily incorporated into the collection and handling protocols where native mussels are relocated. These precautions taken during collection, transport, processing, and quarantine of native mussels are of utmost importance. Only the careful collection and handling of native mussels from zebra mussel-infested waters will ensure that hatchery fish, native mussels, and other aquatic species in the ecosystem are protected from the incidental introduction of zebra mussels.

In situations where there is uncertainty in the co-existence of zebra mussel populations in the watershed, the most prudent and conservative approach is to treat all native mussels as if they originated from zebra mussel-infested waters. A review of zebra mussel range distribution and population dynamics in the particular river basin is also warranted. Particular items of interest include, the nearest known reproducing population of zebra mussels to the native mussel collection site, the relative density and potential spawning periods of zebra mussels at that site, and the likelihood of an undetected presence at the native mussel collection site (e.g., lack of an active monitoring program).

The optimum time for collection of native mussels for a given conservation project is largely unknown. Conservation projects, however, should strive to select periods that reduce the stress associated with handling as much as possible. Potential criteria include choosing a period that coincides with the absence of zebra mussel larvae in the water column, minimizes the temperature differential between air and water, and does not interrupt the reproductive cycle for most of the species being relocated. Zebra mussel contamination can be minimized by collecting native mussels during early spring or late fall periods when zebra mussel larvae are likely not present in the water column (e.g., water temperatures <15°C, Mackie 1991) or when the settled juveniles are of a sufficient size to be easily seen (e.g., 2–5 mm in shell length), respectively. Freshwater mussels are categorized as either long-term (bradytictic) or short-term (tachytictic) brooders. Long-term brooders, like many species of lampsilines and anodontines, become gravid in late summer, retain the developing glochidia in the gill marsupia throughout winter, and spawn in early spring (McMahon & Bogan 2001). In contrast, short-term brooders, like many species of amblemines, become gravid in early spring and spawn in late summer (McMahon & Bogan 2001).

Newton et al. (2001) collected native mussels in early spring when water temperatures ranged between 11 and 14°C, a period before zebra mussel spawning, which generally occurs when water temperatures reach 15 to 17°C (between May and June), in northern temperate regions of the United States and Canada (Mackie 1991). The collection of native mussels in early spring also has an added potential benefit of reduced energetic stresses associated with handling because of the cooler water temperatures (Jokela 1996, Newton et al. 2001). For example, glycogen concentrations in *Amblema plicata* were highest between May and July and dropped precipitously thereafter—a pattern that closely paralleled reproduction in this short-term brooder (Monroe & Newton 2001).

Similarly, Jokela et al. (1993) observed that glycogen concentrations decreased substantially between July and October in *Anodonta piscinalis*, a long-term brooder. Furthermore, Jokela (1996) suggested that transplanting females before fertilization or during the early development of the brood had no detectable effect on reproductive output.

Data on energetic reserves in marine bivalves contradict the recently reported data in freshwater bivalves. In the marine environment, it has been suggested that mussels collected in fall may be able to better withstand handling stress because of their higher energy reserves and because their metabolism is slowed by the cooler water temperatures (Bayne et al. 1973). For example, by mid to late fall, the marine species *Mytilus edulis* and *M. trossulus* had accumulated abundant carbohydrate energy reserves (Hawkins & Bayne 1985, Kreeger 1993, Kreeger et al. 1995). The differences between marine and freshwater species may be caused by differing reproductive strategies. Results from a recent study with native freshwater mussels, however, suggest that some species of native mussels may build up their energy reserves in fall (Gatenby 2002). Obviously, this is an area where additional research is needed.

When native mussels are collected from multiple sites in a watershed with a known or suspected gradient in zebra mussel density, working from the least infested site to the most infested site will reduce potential zebra mussel contamination of boats and other equipment. Optimally, boats used to collect or deploy native mussels in zebra mussel infested areas should be cleaned (before and after) by a high-pressure hot-water wash and diver wet suits, supplies, and equipment (e.g., ropes, buckets, etc.) used in the study should be disinfected with a mild solution of chlorine bleach (25 mg/L) or air dried (3–5 d) before use (Gatenby et al. 2000).

If the quarantine or relocation facility is also an operational fish hatchery or aquaculture center, precautionary measures to protect endemic wild species and cultured fish species should be considered. Before entrance into the facility, a subsample of native mussels should be obtained from the collection site and submitted to a United States Fish and Wildlife Service, National Fish Health Center (Newton et al. 2001) or similar laboratory, to assess potential disease and pathogen presence (see section later on native mussel health and disease management procedures).

After screening for diseases and pathogens, collection of native mussels should proceed with procedures to minimize contamination from adult and larval zebra mussels. These include scrubbing individual native mussels with plastic bristled brushes, visual inspection of all exterior surfaces of the shell with magnifying lenses, and holding cleaned natives in zebra mussel-free water (Table 1). Care should be taken during scrubbing and inspection to avoid overlooking small zebra mussels that may be attached in crevices, in areas of shell erosion (native mussels with severely eroded or damaged valves should be discarded), or along the hinge line (Gatenby et al. 2000, Newton et al. 2001). Only personnel experienced in mussel biology should conduct the inspections to ensure accuracy and efficiency of these procedures.

During collection and processing of native mussels, emersion (exposure to air) and thermal stress should be kept to a minimum. Recent studies have shown that handling mussels over a range of emersion air temperatures (15–35°C) and emersion durations (15–60 min) did not acutely impair survival, behavior, or biochemical composition (Bartsch et al. 2000, Greseth et al. 2003). A minimal emersion time (<20 min), however, is generally recommended from recent efforts (Table 1). Moreover, water temperature and

dissolved oxygen concentrations in the holding vessels during collection should be measured frequently (at least once per hour) and maintained at or near (±2°C) the ambient stream conditions at the time of collection with non-chlorinated ice and external aeration, if possible (Gatenby et al. 2000).

Depending on the proximity of the native mussel collection site to the quarantine facility (a transport time generally <12 h), mussels should be transported in coolers covered with moist burlap and kept cool (within ±2°C of the water collection temperature, if possible) with ice in plastic bags without direct contact of ice bags and mussels (Gatenby et al. 2000, Newton et al. 2001, Cope et al. 2003). This method is advantageous over the use of water-filled, aerated tanks (Chen et al. 2001) because of the reduced need for costly and cumbersome trucks and equipment and of minimizing potential problems associated with maintaining stable dissolved oxygen concentrations in water during transport.

At the quarantine facility, native mussels have generally been held for a minimum of 30–35 d (Gatenby et al. 2000, Newton et al. 2001) to allow any small or previously undetected zebra mussels to become visually apparent on re-inspection. The 30–35 d quarantine period is based on reported zebra mussel growth rates of 0.06–0.15 mm/d (Mackie 1991, Martel 1995, Chase & Bailey 1999), which would allow a newly settled zebra mussel to reach a visible shell length of about 2–5 mm during quarantine. During this time, basic water quality measurements (e.g., temperature, dissolved oxygen, and pH) should be taken at least daily. Other water chemistry variables such as alkalinity, hardness, potassium, total ammonia nitrogen (TAN), and unionized ammonia should be measured at least weekly to ensure that water quality conditions for minimum life requirements are met (Table 1). In addition, mussels in quarantine should be monitored at least weekly for disease (see section below on native mussel health and disease management procedures) and mortality.

Isolation of native mussels from other aquatic species, their contact water, nets, or other equipment at the quarantine facility is necessary to protect organismal health and the physical facility. These concerns can largely be addressed by applying standard best practices for maintaining fish health. Disinfection of equipment and supplies for native mussel quarantine should be guided by National Fish Health Policy and Procedures, Part 713, sections FW1 and FW 3 (USFWS 1995); chlorine (200–250 mg/L for 1 h), sodium or potassium salts (saturated solutions) or other chemical treatments (e.g., benzalkonium chloride at 100 mg/L for 3 h) and desiccation (3–5 d) have been successfully used or recommended (Reid et al. 1993, Waller et al. 1996, Gatenby et al. 2000).

After the minimum quarantine period (30–35 d), individual mussels are thoroughly re-inspected by hand with magnifying lenses to evaluate the presence of zebra mussels. If zebra mussels are not found, the mussels are deemed zebra mussel-free and can be relocated elsewhere (e.g., to natural or artificial systems or to other facilities for long-term captive care). Because no zebra mussels were found after quarantine in the study of Newton et al. (2001), the mussels were subsequently relocated to fish hatchery ponds. In contrast, Gatenby et al. (2000) found zebra mussels on initial re-inspection and consequently held native mussels in quarantine for additional 30 d intervals each time zebra mussels were found, up to a total of 120 d. Because of declines in mussel health and condition over time during quarantine (Patterson et al. 1997, Newton et al. 2001), Gatenby et al. (2000) recommended re-inspection of mussels at 7 d intervals after the initial 30 d period when zebra mussels are found, and to hold them only for 30

additional days after the last zebra mussel is found, to shorten the overall quarantine time. However, the added stress of handling native mussels more frequently must be weighed against the probability of earlier detection of zebra mussels.

Additionally, native mussels could be treated with chemical disinfectants. Certainly, the benefit of this type of treatment must be weighed against the risk of added stress and reduced fitness in the native mussels, but a study by Waller and Fisher (1998) found that limited application of specific chemicals (e.g., 20,000 mg NaCl/L for 6 h) may be feasible for certain tolerant native species. They cautioned, however, that chemical disinfectants cannot guarantee the elimination of all zebra mussels from native mussel shells and stated that pre-treatment or multiple treatment (e.g., once per week) of native mussels and their holding tanks may be most valuable for reducing the time held in quarantine. Many fish hatchery and aquaculture facilities may already be using various chemical treatments (Waller et al. 1996, Edwards et al. 2000, Edwards et al. 2002) or hazard analysis protocols such as the Aquatic Nuisance Species-Hazard Analysis Critical Control Point (ANS-HACCP) approach (Gunderson & Kinnunen 2001) to prevent the spread of zebra mussels and other aquatic nuisance species during their activities, which may be adapted to the collection, transport, and quarantine of native mussels.

Native Mussel Health and Disease Management Procedures

Although little is known about the diseases of native freshwater mussels, recent studies have shown the potential for pathogen transmission among native mussels and fish (Starliper et al. 1998, Starliper & Morrison 2000). The primary concern for fish hatchery or aquaculture facilities that contain native mussels is the potential for transmission of disease and pathogens between host mussels and hatchery fish. Transmissions from hatchery fish to mussels and from mussel to mussel are also important vectors to control for maintaining mussel health. Therefore, a pathogen and disease monitoring plan for native mussels, similar to that commonly used for hatchery-reared fish, should be considered. Hatchery personnel are routinely trained in fish health protocols and record keeping; these procedures could easily be adapted for monitoring mussel health. The United States Government standards and protocols currently exist for a disease control and classification system for coldwater fish (salmonid) pathogens—similar guidelines for warmwater fish or native mussels do not exist (USFWS 1995). Revisions to the United States Fish and Wildlife Service, Fish Health Policies and Procedures are currently underway to include warmwater fish and other aquatic organisms (Richard Nelson, United States Fish and Wildlife Service, La Crosse Fish Health Center, Onalaska, WI, pers. com.). Until those changes are implemented, however, native mussels may only be screened in the near term for reportable coldwater pathogens and diseases. On a positive note, a recent study evaluating the effect of depuration on the transmission of the bacterial fish pathogen *Aeromonas salmonicida* (the causative agent of fish furunculosis) between the unionid *Amblema plicata* and two strains of Arctic char *Salvelinus alpinus* found that the minimum 30-d quarantine of native mussels recommended for preventing the spread of zebra mussels was sufficient for depuration of the fish pathogen and eliminating transmission of the disease (Starliper 2001). Therefore, when adequate safeguards and standard best practices for fish health are used in combination with a 30-d quarantine, disease and pathogen transmission risks should be minimal. Native mussels held in quarantine should be

screened before being placed in the quarantine facility and monitored monthly throughout the duration of their captive care to document disease and pathogen incidence and history. More research and policy development is needed in this area to ensure protection of fish and native mussels.

Maintaining the physiologic condition of native mussels during quarantine is difficult because diet and nutritional requirements are poorly understood. Although the specific time course for changes in biochemical indices of mussels caused by quarantine is unknown, recent studies have shown that substantial decreases in glycogen concentrations occur in as little as 7–35 d after quarantine. For example, Patterson et al. (1997) found that glycogen concentrations in mantle tissue in *Amblema plicata* and *Quadrula pustulosa* dropped significantly after 7 d in quarantine and by day 30, concentrations had declined to only 15–31% of that measured in wild-caught specimens. Likewise, glycogen concentrations in foot tissue of *A. plicata* decreased 44% from 279 ± 191 mg/g dry weight at day 0 to 178 ± 105 mg/g dry weight after 35 days in quarantine (Newton et al. 2001).

Based on the poor physiologic condition of native mussels after quarantine shown by previous studies, it is critical to provide the best source of nutrition during quarantine. Previous studies have relied on an algal-based diet, either produced *in situ* by stimulating algal growth with fertilizers in ponds or cultured indoors on site and added directly to mussel holding tanks (Gatenby et al. 1997, Patterson et al. 1997, 1999, Gatenby 2000, Gatenby et al. 2000, Newton et al. 2001). A number of algae have been tested as food for juvenile and adult mussels (Gatenby et al. 1997, Gatenby 2000, Beck 2001). Recent biochemical analysis of three algae (*Neochloris oleoabundans*, *Bracteacoccus grandis*, and *Phaeodactylum tricornutum*) indicate that these could be nutritionally suitable for maintaining freshwater mussels in captivity (Gatenby et al. 2002). If mussels are to be quarantined or relocated to ponds, the following should be kept in mind: (1) standard commercial pond fertilizers should not be used to stimulate growth of algae; (2) the potassium levels in commercial fertilizers are toxic to freshwater mussels (Imlay1973); (3) the nitrogen:phosphorous ratio (N:P) of the standard 10:10:10 nitrogen:phosphorous:potassium (N:P:K) fertilizer will not promote suitable algae for mussels that typically require an N:P ratio of 10:1 (McCombie 1953); and (4) an unsuitable, or indigestible filamentous blue-green algal bloom will result when 10:10:10 N:P:K is used. Therefore, we recommend using the fertilizers indicated in Table 1, following Gatenby et al. (2000). Although feeding requirements for native mussels will likely depend on the species involved, temperature conditions, and metabolic activity, Gatenby et al. (2000) recommended that native mussels be fed 1×10^5 cells/mL or 4.0 mg dry weight/L twice daily (Table 1). This was a conservatively high recommendation based on initial feeding studies and assimilation efficiencies. This concentration resulted in the greatest assimilation of organic carbon, but a significant amount of this ration went unused by the animals (Gatenby 2000). More recent data indicate that a diet ration of $2.0–5.0 \times 10^4$ cells/mL or 1.9 mg dry weight/L per feeding chamber should maintain mussel condition during summer growth periods (Gatenby 2002). Particle concentrations should be monitored and not allowed to drop below 60% of this recommended ration. Feeding frequency will depend on the species and total biomass being held in captivity (Gatenby 2002). Thus, monitoring the particle concentration on a daily basis is necessary. Initially, particle concentration may need to be monitored two to three times daily

until the manager is familiar with the particle depletion rate or clearance rate of the native mussels held in captivity.

CONCLUSIONS AND RECOMMENDATIONS

Native freshwater mussels should only be relocated from existing areas as a last resort (Cosgrove & Hastie 2001). Other options to relocation and salvage, such as periodic cleaning of zebra mussels from native mussels and replacement (Hallac & Marsden 2000, Hallac & Marsden 2001), and the use of natural or managed refugia (Nichols et al. 2000), should be considered as first alternatives where practical. For example, Hallac & Marsden (2000, 2001) suggested that periodic cleaning and replacement might be a viable option for conservation of native mussels, especially in areas where food is not limiting and where collection and cleaning are logistically feasible. If, however, freshwater mussel relocations are required to conserve localized populations from zebra mussels or other catastrophic events, the concerns and procedures described in this article should provide general guidance for developing plans to prevent the incidental introduction of zebra mussels during these activities and for maintaining the health of the native refugees while under captive care.

In addition, procedures for ensuring long-term viability of native mussel populations need to be considered throughout the planning and implementation process. For example, similarities in water quality, substratum characteristics, food, and necessary fish hosts among the systems are critical elements in a native mussel relocation strategy. Additional ecological and evolutionary concerns, such as retention of genetic diversity of the mussel populations, need to be carefully considered before relocating native mussels to natural refugia, especially if the mussels are to be relocated between river basins or between sub-basins of the same river system (Villella et al. 1998, Storfer 1999).

Because of costs and limited availability of facilities for quarantine and captive care of native mussels, the United States Fish and Wildlife Service and its resource conservation and management partners may wish to designate several facilities within regions of the United States that can accept, hold, and screen mussels for disease and pathogens. These facilities may include state or national fish hatcheries, research or aquaculture centers, and fish health centers.

To our knowledge, this synthesis represents the "state-of-the-science" for minimizing the incidental introduction of zebra mussels during native mussel conservation activities and for ensuring their short-term and long-term health and viability. Readers of this article should be cautioned that the information presented is only recommended guidelines and that future improvements to procedures will be made through research and policy development.

ACKNOWLEDGMENTS

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A COMPARISON OF THE PARASITE AND SYMBIONT FAUNA OF COHABITING NATIVE (*PROTOTHACA STAMINEA*) AND INTRODUCED (*VENERUPIS PHILIPPINARUM* AND *NUTTALIA OBSCURATA*) CLAMS IN BRITISH COLUMBIA

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ABSTRACT Native littleneck clams (*Protothaca staminea*), Manila clams (*Venerupis philippinarum*, inadvertently introduced in the 1930s), and varnish clams (*Nuttallia obscurata*, inadvertently introduced in the 1980s and 1990s) were collected from the same microsite at two different locations and examined for parasites and symbionts using histology and light microscopy. Varnish clams are currently being assessed for their long-term fisheries potential but there is little knowledge of their parasite and symbiont fauna. This study initiates the documentation of parasites and symbionts of varnish clams and adds to the continuing documentation of organisms found within native littleneck clams and Manila clams. Host exposure to potential parasites and symbionts that were prevalent in at least one of the clam species was assumed to be similar for all clams due to their close proximity. This close association in the natural environment allowed for the comparison of host specificity and response of the clams to multiple invasive species. All three of the clam species had a different assemblage of parasites and this pattern was mostly consistent for both sites. Host preferences of each type of parasite or symbiont were also consistent between sites and they were often restricted to a single host species. The most common parasites of varnish clams were *Nematopsis*-like spores, pea crabs (*Pinnixa faba*) and parasitic copepods (*Mytilicola* sp.) and less frequently a turbellarian inhabiting the kidney tubule. An undocumented eimeriorin-like kidney coccidian was found in 4% of Manila clams and two previously undescribed inclusions bodies were found in native littleneck clams at low frequencies.

KEY WORDS: bivalve, *Protothaca staminea*, *Venerupis philippinarum*, *Nuttallia obscurata*, parasites, symbionts

INTRODUCTION

In June of 2002 three species of clams (one native and two introduced) were chosen for a survey of parasites and symbionts. The native littleneck clam [*Protothaca staminea*; (Conrad 1837); = *Paphia staminea*, = *Venus staminea*] was the most important fresh-market clam until the advent of the Manila clam [*Venerupis philippinarum*; (Adams & Reeve 1850); = *Ruditapes philippinarum*, = *Tapes japonica*, = *Tapes philippinarum*, = *Tapes semidecussata*, = *Venerupis japonica*, = *Venerupis semidecussata*], another member of the family Veneridae with similar morphology to the native littleneck clam but with a longer market shelf-life. The Manila clam, also known as the Japanese littleneck clam, was first observed in British Columbia near Ladysmith Harbour in 1936 (Quayle 1964). Introduction presumably occurred during transplantation of Pacific oyster (*Crassostrea gigas*) seed from Japan, when young Manila clams of several millimeters in shell length may have been trapped in the oyster shells (Quayle 1964). The dispersal of Manila clams was rapid, and by 1941 they formed a significant proportion of the commercial catch and were the dominant lamellibranch of many beaches (Quayle 1964). They are now established along both coasts of Vancouver Island, although less abundant in the northern parts, and along similar latitudes on the mainland coast (Bourne 1982).

Varnish clams [*Nuttallia obscurata* (Reeve 1857); = *Soletellina obscurata*, = *Psammobia olivacea*, = *Soletellina japonica*], also known as purple mahogany or Savory clams, belong to the family Psammobiidae. Originally native to Korea and the Japanese Islands of Kyushu, Honshu, and Shikoku (Coan et al. 2000), they have been recently introduced to the Georgia Strait, probably via ballast water (Gillespie et al. 1999). They have since spread north into Johnstone Strait, along the west coast of Vancouver Island north to Checleset Bay, along the mainland coast, south into Puget

Sound and along the Oregon Coast to Port Townsend (Dinnel & Yates 2000, Gillespie et al. 2001). There have been some trial fisheries but the long-term potential of the fishery is currently under investigation (Gillespie et al. 1999, 2001).

The purpose of this study was to compare the parasites and symbionts found in each of the clam species at two different sites. Clams from each site were gathered at close proximity to each other and were assumed to have had similar exposure to the spectrum of parasites enzootic to that site. This sampling regimen helps minimize suspicions that observed differences could be the result of temporal or spatial variations, thereby increasing the interpretative value of negative results. This survey is the first to examine varnish clams for parasites and symbionts using histological methods and also contributes to the continuing documentation of parasites and symbionts found in Manila and native littleneck clams.

MATERIALS AND METHODS

On 10 June 2002, Manila clams, native littleneck clams, and varnish clams (*n* = 25) were collected from each of two locations within the Strait of Georgia on the coast of British Columbia for a total of 150 clams. The first 75 clams were collected from Crofton at a beach below a sewage outfall located between the ferry terminal and pulp mill, the others were gathered 2 h later from Boulder Point, Ladysmith. At each location clams between 40 and 57 mm in length were dug from a single site (2.0–2.5 m² in area, approximately 15 cm deep) within the mid-intertidal zone, away from evidence of eutrophication and fresh water runoff, where none of the target species were more than 1.5 times more abundant than another. All clams appeared healthy and were held in tanks (one tank per site) with flowing ambient seawater for 3–4 days. Each clam was then shucked, the shell length and wet weight of soft tissue recorded, superficially examined and pool fixed (5 per jar) in Davidson's solution. Pea crabs were collected, preserved in Davidson's solution and held for identification. After at least 24 h in the fixative two cross sections, one through the region of the

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stomach and digestive gland and the other through the kidney and heart were made. The labial palps, siphon and posterior adductor muscle were also sampled and processed with the cross-sections using routine histological techniques. Sections (5-μm thick) were cut and stained with Harris's modified hematoxylin and 0.5% alcoholic eosin. Additional sections from selected specimens were stained with Brown and Hopps Gram stain and also tested for the presence of DNA using the Feulgen stain reaction. All sections were examined under a compound microscope (100 to 1000×).

RESULTS

Average shell lengths of each clam species varied little between sites but clams collected from the Crofton site had lower wet weight to shell length ratios (Table 1). Native littleneck clams ranged in length between 41.6 to 50.1 mm from Boulder Point and 41.6 to 49.4 mm from Crofton; their wet weights were between 5.6 to 11.9 g from Boulder Point and 5.8 to 10.4 g from Crofton. Manila clams ranged between 41.4 to 56.4 mm from Boulder Point and 40.2 to 55.1 mm from Crofton; wet weights were between 6.1 to 14 g from Boulder Point and 4.7 to 12.9 g from Crofton. Manila clams showed the least difference in wet weight to shell length ratio (Table 1). Varnish clams ranged between 41.4 to 53.4 mm in length from Boulder Point and between 40.0 to 51.7 from Crofton, wet weights ranged between 4.6 to 11.6 g from Boulder Point and 3.9 to 7.8 g. from Crofton. The average wet weights to shell length ratio was much less in varnish clams collected from the Crofton site (Table 1).

Pea crabs (family Pinnotheridae) were collected from both Manila and varnish clams during the shucking process. Only one immature *Pinnixa faba* was found in the Manila clam sample, however 16–24% of varnish clams contained one pea crab (Table 2). These were also identified as *P. faba* and were either immature or male; the largest measured 13 mm across the carapace. The presence of pea crabs had no obvious pathological effects and did not affect the wet weight to shell length ratio. For example, the wet weight to shell length ratio of the six varnish clams from Boulder Point containing a pea crab was 0.18 g/mm whereas this ratio for the 19 varnish clams from the same location without pea crabs was 0.17 g/mm. All other organisms were found during histological examinations.

Colonies of intracellular prokaryotes (Rickettsiae or Chlamydiae) were observed within the epithelial cells of the gills and digestive gland tubules in both Manila and native littleneck clams (Fig. 1). Gill infections in Manila clams were less frequent (8–20%) and were considered to be of light intensity (<80 colonies) compared with native littleneck clams where there was a higher prevalence (≥88%) and many examples of moderate and high (≥200 colonies) intensities (Table 2). Infections within the digestive gland were also more prevalent in native littleneck clams than

in Manila clams (Table 2). The digestive gland was the most frequent site of infection in Manila clams whereas the gill infections greatly outnumbered digestive gland infections in native littleneck clams. Most digestive gland infections were light (<10) to moderate (10 to 24) in both species except for two cases of heavy infection in native littleneck clams from the Crofton site where as many as 55 colonies were counted. The identity of the intracellular prokaryotes is unknown and may be representative of more than one species. The colonies within the digestive gland tubules appeared to be denser than those found within the gill tissue where it was often possible to see the individuals within the colony. Between hosts, the colony morphologies were consistent and appear to be the same agents as those described by Bower et al. (1992). No associated host response was observed, however the infected cells (especially gill epithelium) were often swollen beyond their normal size (Fig. 1). In many cases, host cells of gill infections were ruptured and the prokaryotes were leaking out into the water channel.

Colonies of large intracellular rod shaped bacteria (Fig. 1) were observed at low intensities within gill epithelial cells of 4–52% of native littleneck clams. The maximum size of these bacteria was 6.3 μm long by 1.4 μm wide but there were also smaller variants. Staining characteristics ranged from strongly to very weakly basophilic and were predominantly gram positive, however, there were also Gram-negative representatives throughout the entire size range. Colonies were often 28 μm in diameter but did not appear to incite any hemocytic response or otherwise show any indication of pathology. There was a weak correlation between intensity of Rickettsia or *Chalmydia*-like infections and the number of colonies of rod shaped bacteria observed, clams containing colonies of rod shaped bacteria were usually infected with moderate to high numbers of Rickettsia or *Chalmydia*-like colonies.

Another inclusion body, also unique to native littleneck clams, was found in low intensities with 12% prevalence at both sites (Table 2). These bodies were large, with an average diameter of 65 μm, and bound by hemocytes that appeared to have flattened against the infected cell forming a thick eosinophilic membrane (Fig. 2). The material within was basophilic, Feulgen positive and Gram negative, it was of a very fine matrix and denser near the edges of the colony. The infection was found in nearly every tissue (heart, kidney, gonad, gill, and palps) and appeared to be the result of an infected, extremely hypertrophied hemocyte.

Apicomplexan spores resembling *Nematopsis* sp. were observed at least once in all three species, however, mainly in Manila and varnish clams collected from the Crofton site (Table 2). The prevalence in native littleneck clams was very low (4% and 12%) and there were never more than two spores within an infected clam. One spore was in the gill epithelium and the others were found within the gill connective tissue, those found within the

TABLE 1.

Average shell length and wet weight to shell length ratios of native littleneck clams (*Protothaca staminea*), Manila clams (*Venerupis philippinarum*), and varnish clams (*Nuttallia obscurata*) examined from two locations in British Columbia, Canada (*n* = 25 for each species at each location).

	Native Littleneck Clams Boulder Pt./Crofton	Manila Clams Boulder Pt./Crofton	Varnish Clams Boulder Pt./Crofton
Average Shell length (mm)	43.2 / 44.9	45.7 / 48.5	47.7 / 44.7
Wet weight to shell length ratio (g/mm)	0.20 / 0.17	0.19 / 0.18	0.17 / 0.12

TABLE 2.

Prevalence* and intensity† of parasites and symbionts in native littleneck clams (*Protothaca staminea*), Manila clams (*Venerupis philippinarum*), and varnish clams (*Nuttallia obscurata*) from two localities in British Columbia, Canada.

Parasite/Symbiont	Native Littleneck Clam Boulder Pt. / Crofton	Manila Clam Boulder Pt. / Crofton	Varnish Clam Boulder Pt. / Crofton
Rickettsia or Chlamydia in gill	88%: 19L, 3M (1–130) / 100%: 6L, 7M, 12H (12–600)	8%: L (1–2) / 20%: L (1–30)	0% / 0%
Rickettsia or Chlamydia in digestive gland	48%: 9L, 3M (1–24) / 52%: 7L, 4M, 2H (1–55)	36%: 7L, 2M (1–24) / 32%: 5L, 2M (1–20)	0% / 0%
Large intracellular rod shaped bacteria	4%: L (1) / 52%: L (1–16)	0% / 0%	0% / 0%
Fine-matrix inclusion bodies	12%: L (1–9) / 12%: L (3–5)	0% / 0%	0% / 0%
Apicomplexan spores *Nematopsis*-like	4%: L (1) / 12%: L (1–2)	0%: / 80%: 17L, 2M, 1H (3–200)	4%: L (1) / 100%: 16L, 6M, 3H (3–230)
Trichodina spp.	0% / 0%	20%: L (1–3) / 56%: L (1–11)	0% / 0%
Order Rhynchodida	0% / 0%	20%: L (1–5) / 44%: L (1–15)	0% / 0%
Eimeriorin-like coccidian (Apicomplexa)	0% / 0%	4%: L (4) / 4%: L (3)	0% / 0%
Copepods, (*Mytilicola*-like)	4%: L (1) / 8%: L (1)	4%: L (1) / 0%	64%: L (1–4) / 60%: L (1–4)
Other copepods	4%: L (1) / 0%	0% / 0%	4%: L (1) / 0%
Trematode metacercariae	0% / 0%	8%: L (1) / 0%	0% / 0%
Turbellarians	0% / 0%	0% / 4%: L (1)	8%: L(1) / 0%
Pinnotheridae	0% / 0%	4%: L (1) / 0%	24%: L (1) / 16%: L (1)

* Recorded as the percentage of each clam species infected with a given organism at each location.

† Recorded as the number of clams with heavy (H), moderate (M), or light (L) infections (as defined in text), followed by the range of colonies or individuals of each parasite/symbiont observed in parenthesis.

connective tissue were accompanied by a mild hemocytic response. Manila clams were only infected at the Crofton site; the majority of these infections were light (<60 spores per histological section) with only a few cases of moderate or high (>150) intensity. Gill connective tissue was the primary focus of infection but was accompanied by a light infection (one to five spores) of the palps in 28% of the clams. There was also one instance where a single spore was found in the gonadal tissue. The spores appeared opaque, with no visible internal structures or nuclei, and were usually accompanied by a focal hemocytic response, identical to those described by Bower et al. (1992). Spores found in varnish clams also occurred predominately within the gill connective tissue but there were also a few spores in the palps (three clams) and kidney (two clams). A little over half of the spores were of the same morphology typical to the Manila clams (Fig. 3) and usually showed a hemocytic response. The remaining spores (Fig. 4) often contained a nucleus and were clustered within clumps of hemocytes, making them difficult to discern and accurately count. These variations appeared to be part of the host immune response since there was no evidence to suggest that the spores were alive and capable of progenesis. There also appeared to be a size difference between the two spore morphologies with one having an average length of 9.26 ± 1.33 μm ($n = 30$) and the other an average length of 7.91 ± 1.31 μm ($n = 30$). However the size differences were not statistically significant.

The remaining protistan parasites detected were an eimeriorin-like coccidia (Apicomplexa) and two ciliates, a *Sphenophyra*-like ciliate of the order Rhynchodida and a *Trichodina* spp., and all were found exclusively in the Manila clams. The coccidian was observed within the kidney tissue in one Manila clam from each location (Table 2) but only the macrogamont stage was observed (Fig. 5). The macrogamonts were spherical, with a granular cyto-

plasm and a large central nucleus. These macrogamonts have not been previously observed in Manila clams but ones with similar morphology have been observed in native littleneck clams (Desser and Bower 1997a). Although the large size of the macrogamonts (32–33 μm in diameter) was sufficient to stretch the kidney tubules there appeared to be very little impact on the host due to the low intensity and lack of other life stages. The Rhynchodyda-like ciliate was attached by a stalk between the cilia of the gill epithelium in 20% and 44% of Manila clams (Table 2). They had a large prominent nucleus and appeared to be the same as those described by Bower et al. (1992). There was no evidence of a hemocytic response and the intensity of infection appeared too light to have a significant pathological effect. *Trichodina* spp. (similar to those described by Bower et al. 1992) were found attached to or closely associated with the foot, inner surface of the siphon and in one case the mantle. The prevalence of these organisms was 20% and 56% and the intensity was light (Table 2). There was no evidence of tissue disruption or hemocytic response indicative of a pathological impact.

Copepods resembling *Mytilicola* spp. (commonly called red worms; Fig. 6) were observed at least once in all three clam species (Table 2), although predominately in varnish clams (60% and 64% infected) and rarely in the other species (4% to 8%). They were usually found within the lumen of the stomach or intestine but one was found in the digestive gland duct of a native littleneck clam (Fig. 7). Intensity was recorded as the number of cross sections and therefore the same organism may be represented more than once. In cases where there was more than one cross section in one part of gut, the lumen was somewhat distended (Fig. 6), otherwise there was no indication of serious pathology. These copepods have been observed previously in Manila clams and native littleneck clams as well as other bivalves (Bower et al. 1994).
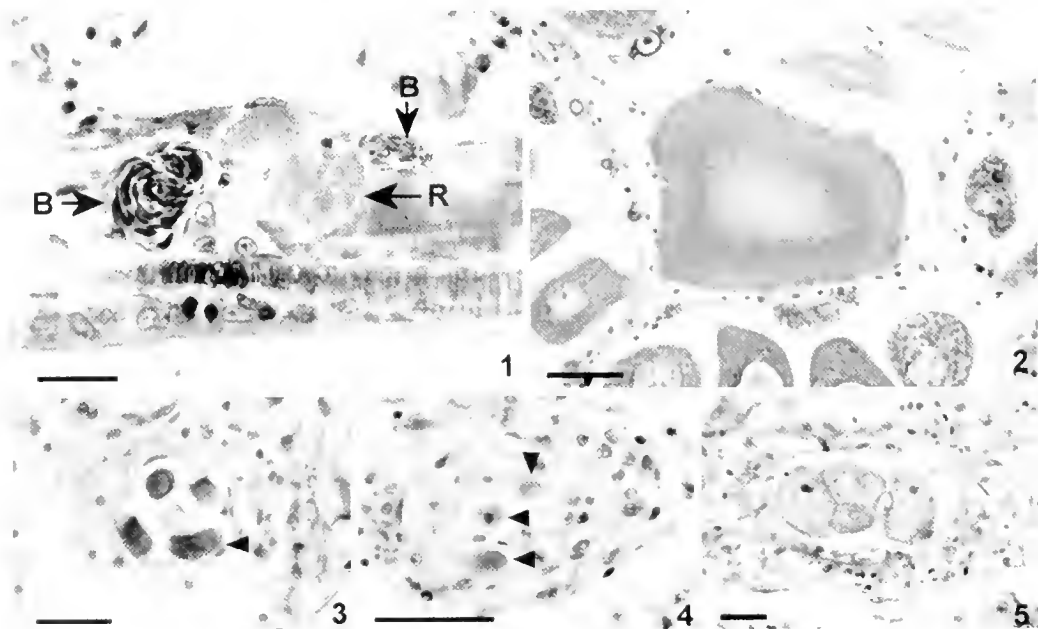
Figures 1–5. Inclusion bodies and protozoa observed in histological sections of clams from British Columbia, Canada (hematoxylin and eosin stain, scale bars are 20 μm).

Figure 1. Two strongly basophilic rod-shaped bacteria colonies (B) next to a Rickettsia or *Chalmydia*-like inclusion (R) in the gills of a native littleneck clam (*Protothaca staminea*). Note the size difference in individuals in each type of colony. Both types of inclusions cause considerable distortion of the host cell.

Figure 2. Large inclusion bound by hemocytes within the gonad of a native littleneck clam (*P. staminea*). The thick membrane surrounding the inclusion appears to be the result of layers of flattened hemocytes.

Figure 3. Four *Nematopsis*-like spores (arrowhead) surrounded by hemocytes within the water channel of a varnish clam (*Nuttallia obscurata*).

Figure 4. Three *Nematopsis*-like spores (arrowheads) in the water channel of a varnish clam (*N. obscurata*) gill. Note the smaller spore size and greater number of responding hemocytes compared with Figure 3.

Figure 5. Three macrogamonts of an eimeriorin-like coccidia in the kidney tubule of a Manila clam (*Venerupis philippinarum*). The tubule is greatly distended as a result of the large size of the macrogamonts.

All other metazoans observed were copepods, turbellarians, or trematode metacercariae and all occurred at low frequencies (Table 2). Two different copepods were found, one in the gill of a native littleneck clam and the other in the gonad of a varnish clam (Table 2). The gill copepod (Fig. 8) was large, nearly 750 μm long in the tissue section, and was observed within the water channel of the gill. It did not appear to be attached and despite its size there was no significant tissue disruption. Two metacercariae were found in Manila clams, one in the digestive gland (Fig. 9) and another unencysted one within the pericardial space. The metacercaria within the digestive gland was surrounded by a thick layer of hemocytes that caused some local tissue disruption. One turbellarian was found within the intestine of a Manila clam (Fig. 10) and two turbellarians were found in the kidney tubules of varnish clams (Fig. 11). The turbellarians found in the varnish clams both appeared to be of the same species and were quite large, one was over 200 μm in diameter, and therefore caused considerable swelling of the tubule, otherwise no pathological effects were observed.

DISCUSSION

Comparisons of parasite and symbiont prevalences between Manila, native littleneck, and varnish clams provide strong evidence that there are host preferences. Each parasite/symbiont had the same order of host preference at both locations except in the case of *Nematopsis*-like spores. *Nematopsis*-like spores were rarely observed at the Boulder Point site but were common in clams from Crofton. Because *Nematopsis* spores do not reproduce once they are inside the molluscan host (Sprague and Orr 1955) the clams from Crofton had a significantly higher rate of invasion. This may be related to the fact that known species of *Nematopsis* require a decapod host to complete their life cycle (Lauckner 1983); possibly the Crofton site was more suitable for the alternate host(s). Another possibility may be related to differences in exposure, the Crofton beach was in a bay and had more protection from waves and current due to the nearby ferry dock and marina. The infectious agents may have been washed away from the Boulder Point site before they reached the filtration field of their potential bivalve host. These data are limited by time of year and are representative of a small geographic area. Whether these patterns of host specificity are constant throughout seasonal fluctuations and at different locations is unknown. The assumption that clams of similar sizes dug from the same micro-site have similar exposures to potential parasites and symbionts does not work as well for parasites that are accumulated at low intensities over a long period of time. Because size is not an accurate measurement of age, clams of similar sizes cannot be assumed to have the same exposure times, also clams found in the same micro-site one year may have been more widely separated in previous years.
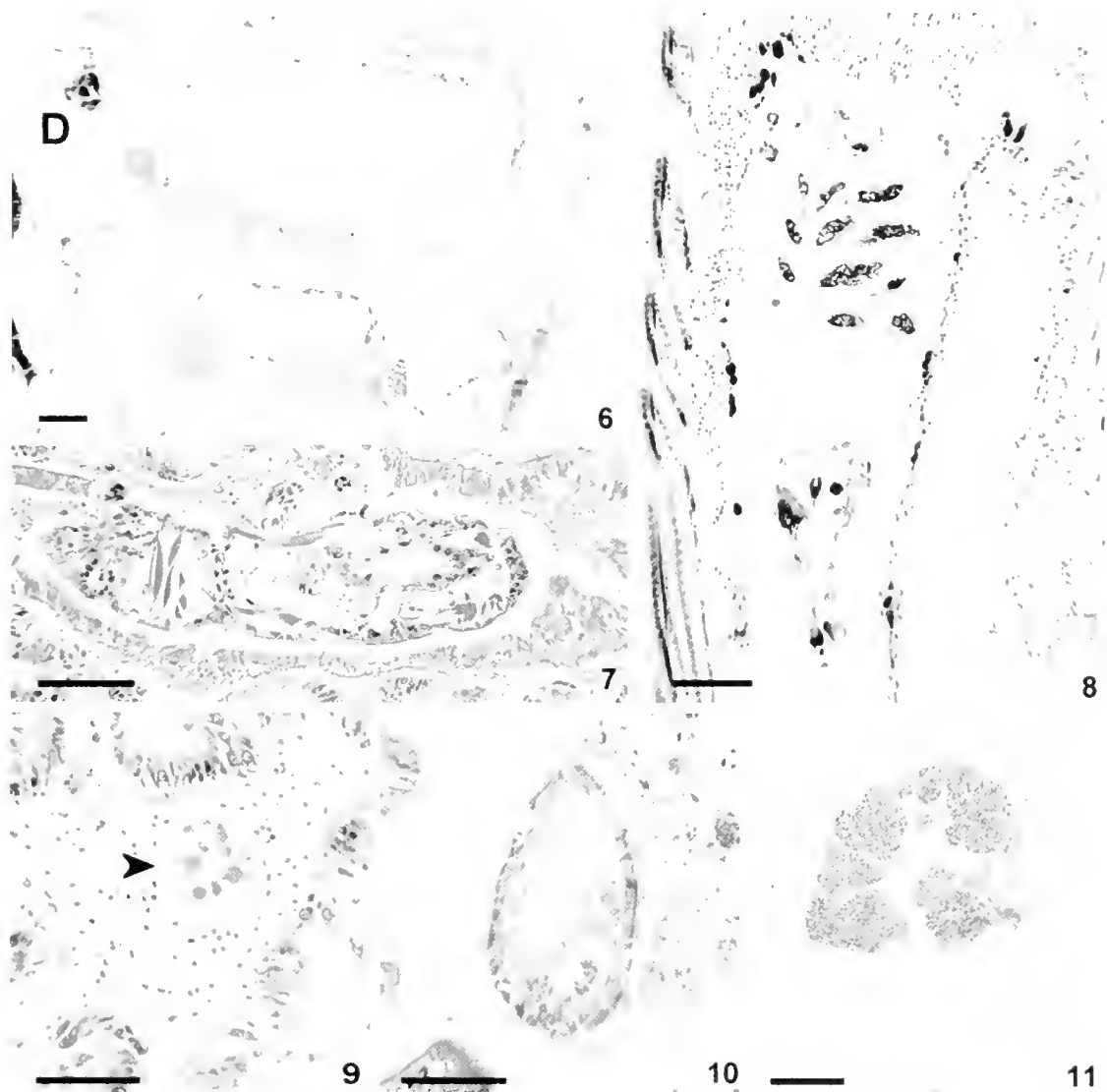
Figures 6–11. Metazoa in clams from British Columbia Canada (hematoxylin and eosin stain).

Figures 6–8. Copepods found during histological examination (scale bars are 100 μm).

Figure 6. *Mytilicola* spp. in intestine of varnish clam (*Nuttallia obscurata*). Multiple sections may represent the same organism folding back on itself. Damage to intestine wall (D) appears to be a sectioning artifact.

Figure 7. *Mytilicola* spp. in a duct of the digestive gland of a native littleneck clam (*Protothaca staminea*). Note damage to intestinal wall in upper left of photo between appendages of the copepod.

Figure 8. Section shown is through the appendages and abdomen of a copepod within the water channel of a native littleneck clam (*P. staminea*) gill.

Figure 9–11. Metacercaria and turbellarians (scale bars are 50 μm).

Figure 9. Metacercaria (arrow) within the digestive gland of a Manila clam (*Venerupis philippinarum*) surrounded by a focal hemocytic response.

Figure 10. Turbellarian in the intestine of a Manila clam (*V. philippinarum*).

Figure 11. Turbellarian within a kidney tubule of a varnish clam (*N. obscurata*). The kidney tubule is grossly distended to accommodate the large size of the turbellarian.

Nematopsis-like spores are able to gain entry into many species of bivalves (Sprague & Orr 1955, Bower et al. 1994) but do not always remain viable (Bower et al. 1992). None of the *Nematopsis*-like spores observed in these clams appeared to be alive and were probably within the wrong host. Viable interactions between bivalve host and *Nematopsis* spp. are likely to be highly specific

(Sprague & Orr 1955). There also appears to be some inhibition of infection because native littleneck clams were not infected to the same degree as varnish or Manila clams. Native littleneck clams have been known to contain *Nematopsis*-like spores (Bower et al. 1994) but these may represent a different species than those encountered in this study. The few spores observed in native little-

neck clams here were slightly smaller and may have represented a different species that was less abundant. It is uncertain whether the spores of two different sizes found in the varnish clams were the same species. However, both spore types were found in the same tissues and were proportional in abundance so could represent different stages of host response.

There were many instances where a parasite or symbiont was unique to only one host, for example, *Trichodina* spp., Rhynchodida-like and eimeriorin-like protozoa were only found in Manila clams. *Trichodina* spp. and Rhynchodida-like ciliates have been observed on other bivalve species (Bower et al. 1994) and have a worldwide distribution. Both of these ciliates can be found in association with Manila clams throughout their range (Bower et al. 1992); the particular species found on Manila clams may be enzootic and introduced to British Columbia along with their host. Both are believed to be benign, large numbers of Rhynchodida-like ciliates have been reported with no obvious host response or mortalities (Bower et al. 1994).

The presence of eimeriorin coccidia in Manila clams and not in native littleneck clams was unexpected. An eimeriid coccidian parasite from the kidney of the native littleneck clam has been described in Washington State, USA (Morado et al. 1984). A similar, presumably the same, parasite was described and named (*Margolisiella kabatai*) by Desser and Bower (1997a) in a low percentage of native littleneck clams from Southern Vancouver Island. The macrogamonts observed in the Manila clam appeared similar to those described in native littleneck clams, however *M. kabatai* shares many ultrastructural similarities to coccidian macrogamonts found in California abalone (*Haliotis* spp.; Friedman et al. 1995). Because macrogamonts were the only stage observed it is impossible to determine whether this is a different species or if *M. kabatai* is also able to invade Manila clams. More than one host species is not unknown in eimeriorin coccidia (Léger 1897, Léger & Duboscq 1915); however, a survey of 994 Manila clams (Bower et al. 1992) came across no evidence of this parasite. A possible explanation may be related to geographic distribution of the parasite. The Manila clam survey performed by Bower et al. (1992) only sampled 80 clams south of Nanaimo and those were sampled in early spring. All records of the kidney coccidia lie further south than the boundaries of the Manila clam survey, it is possible that *M. kabatai* may only be infecting Manila clams from more southern populations. Although heavy infections of kidney coccidia in native littleneck clams can damage the architectural integrity of the kidney due to lethal hypertrophy of parasitized cells containing maturing macrogamonts (Morado et al. 1984), the intensity of infection observed in this study probably had minimal effect on the host. No link between clam behavior and coccidian infection has been established in British Columbia, unlike those reported in Washington by Morado et al. (1984). Possibly this parasite has a greater impact at lower latitudes.

Native littleneck clams were the only clams infected with fine matrix inclusion bodies and colonies of large rod shaped bacteria. Both of these infections are previously undocumented and may be unique to native littleneck clams. Native littleneck clams have not been surveyed as intensively as introduced and farmed species of shellfish so these infectious agents may have escaped detection until now. Those native littlenecks that have been surveyed were collected at different locations (Bower et al. 1992), so range or annual fluctuations may be an explanation. The fine matrix inclusions have potential to be harmful to the host due to their extreme size if they multiplied or accumulated in vast numbers.

The rod shaped bacteria were at first reminiscent of Rickettsia or *Chalmydia*-like prokaryotes but these individuals were larger than others described from those groups (see review in Elston and Peacock 1984). Most of the colonies were much more basophilic and were usually Gram positive, unlike the paler Gram negative colonies of what were more typical of Rickettsia-like prokaryotes. The variations in Gram staining may be related to stages in development; there was a tendency for the larger individuals to be Gram positive but this was not always the case. The correlation between the intensities of infection of colonies of typical Rickettsia-like prokaryotes and rod shaped bacteria may be a function of clam filtering activity or maybe some individuals are more susceptible to gill infections than others. Unfortunately it was impossible to compare clam size to infection intensity because the clams had been pool-fixed. Although this paper separates these bacteria from the more typical Rickettsia-like colonies it is not unusual to find variations in the sizes of individual prokaryotes in bivalve inclusions (Elston & Peacock 1984). However, the differences are not usually as great as those observed here. The taxonomy of intracellular prokaryotes from bivalves is very poorly understood and is based on morphological observations as opposed to biochemical, infective or taxonomic relationships with similarly named organisms in higher animals.

Parasitic or commensal crustaceans are common within most bivalve species; however, those encountered in this survey were predominantly in varnish clams. Manila clams can be host to more than one species of pea crab (Bower et al. 1992) but all accounts to date have found only one species (*P. faba*) in varnish clams (Gillespie et al. 2001). Immature *P. faba* are found in many species of clams in British Columbia but mature pairs are most often found in the horse clam, *Tresus capax* (Hart 1982). Pea crabs are usually harmless to their host however one study of Manila clams in Japan found that the presence of pea crabs was related to a decrease in the ratio of wet weight to shell length compared with unexposed clams (Sugiura et al. 1960). This relationship has not been observed in any bivalves examined as such in British Columbia. The prevalence of pea crabs found in the varnish clams is consistent with a more extensive count by Gillespie et al. (1999) but the reason varnish clams have so many is unknown.

None of the clams in the present survey were examined fresh; thus, the specific identity of the *Mytilicola*-like copepod was not determined. However the most common *Mytilicola* spp. encountered in British Columbia is *Mytilicola orientalis*, which was introduced via Pacific oyster seed (Bernard 1969). It is improbable that these copepods are enzootic to varnish clams and introduced at the same time since varnish clams are presumed to have arrived here in a larval form within ballast water. Rates of infestation of *Mytilicola intestinalis* between individuals of the same bivalve species is passively determined by the host's field of filtration (Gee & Davey 1986) and are often found in greater abundance in larger sized hosts (Goater and Weber 1996). This does not explain their predominance in varnish clams since they are less dependent on filter feeding and were not significantly larger. Either more larvae are entering varnish clams or the survival rate is lower in Manila and native littleneck clams. Varnish clams are deposit and pedal feeders in addition to filtering (Gillespie et al. 1999), this action may stir up the sediment more, re-suspending larvae and increasing the incidence of infection. Some experiments using *M. intestinalis* in Europe have been linked to poor growth, tissue damage and gut metaplasia in oysters and mussels (Koringa 1952,

Odlaug 1946, Sparks 1962) however no pathology has been reported in British Columbia as a result of *M. orientalis* (Chew et al. 1965, Bernard 1969).

Both gill and digestive gland Rickettsia or *Chlamydia*-like infections showed the same order of host preference with a complete absence from varnish clams. Although there was no correlation between numbers of gill colonies compared with number of digestive gland colonies in infected individuals this trend in host specificity may indicate a close relationship between these two types of infections. Possibly they are the same species and only appear different because they are found in different host cells. The similarity in appearance between species supports this theory and suggests that one agent may be responsible for these infections. However, detailed ultrastructural observations, serological or genetic analysis is necessary to make these distinctions. A greater dependence on filter feeding does not completely explain why native littleneck and Manila clams have these colonies while varnish clams do not as the prokaryotes are not picked up indiscriminately by passive filtration. Gulka and Chang (1984) tried infecting other bivalves with a rickettsia isolated from a scallop (*Placopecten magellanicus*) but were unsuccessful. This suggests that these organisms are fairly host specific and those found here were not able to infect varnish clams. It is possible that these intracellular prokaryotes are a natural parasite/symbionts of native littleneck clams and are able to successfully colonize Manila clams at a lower rate due to certain similarities between the hosts. The prevalence found in Manila clams from this study is similar to that found by Bower et al. (1992), in comparison the prevalence and intensity found in native littleneck clams was very high. Infections of this degree have been observed in farmed scallops without any indication of pathology, in this case the intensity decreased after

the scallops were moved from contained aquaculture ponds to the open environment (S. Bower & G. Meyer, personal communication). This was another case in which location had a pronounced effect on frequency and intensity of infection, possibly related to the differences in wave and current exposure between the two locations. In general these types of prokaryotic infections are not linked to a pathological response but it has been suggested that heavy infections may reduce the metabolic efficiency and reduce the nutritional status of the host (Otto et al. 1979, Elston 1986). There are a few cases linking intensity of Rickettsia or *Chlamydia*-like infections to mortality (Gulka & Chang 1983, Le Gall et al. 1988, Leibovitz 1989) but no detrimental effects have been reported in British Columbia.

The low prevalence or absence of some organisms is also worth noting. Native littleneck clams collected by Bower et al. (1992) in 1986 and 1990 and by Desser and Bower (1997b) in 1995 were infected with the elongate sporozoites of a Coccidia-like Apicomplexan (37% to 100% prevalence), these organisms were also found in Manila clams near the Northern end of their distribution. Some of these samples were taken at the same time of year as the samples in this study, so seasonal fluctuations are probably not the cause. These parasites may have been in low abundance in 2002 or possibly the unknown alternate host does not occur in the Georgia Strait. There were also fewer turbellarians observed than expected, this is may be due to an annual fluctuation since they are usually common in both Manila and native littleneck clams.

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POPULATION DYNAMICS OF THE ASIATIC CLAM, *CORBICULA FLUMINEA* (MÜLLER) IN THE LOWER CONNECTICUT RIVER: ESTABLISHING A FOOTHOLD IN NEW ENGLAND

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ABSTRACT The founding population of *Corbicula fluminea* in the Lower Connecticut River, discovered in 1990, was studied for ten years (1991–2000). Seasonal abundance of six size classes was monitored near three electric power plants. *Corbicula* abundance varied seasonally as well as annually, but peaked in 1992. Winter survival of clams was positively correlated with the average winter water temperature and negatively correlated with frequency of daily mean water temperatures $\leq 1°C$ and with frequency of daily mean April spring freshet flows ≥ 1700 m³/s. Higher winter survival at Middletown Station sites during most years, when compared with survival near Connecticut Yankee, was attributed to the influence of the Middletown Station thermal discharge. Thermal discharge did not support a permanent population at Connecticut Yankee because of temperature extremes during power plant operation in summer. Clam growth under ambient river temperatures began in May when water temperatures exceeded 10°C and ceased in December when temperatures fell below this threshold. Cooling water discharges altered this seasonal growth pattern; growth began in November, as temperatures fell below 35°C, and ceased in the summer, when discharge temperatures exceeded this upper thermal threshold. Reproduction occurred in the river when water temperatures were between 17°C and 28°C, typically from June to October. Peak spawning occurred in August. Discharge temperatures shifted clam reproduction back to spring (March to May). The key to *Corbicula*'s success in establishing a population in the Connecticut River is its ability to colonize refugia from winter temperature and spring freshet flow extremes that often cause high clam mortality.

KEY WORDS: Asiatic clams, *Corbicula fluminea*, thermal discharges, electric power plants, winter survival, thermal tolerance, reproduction, growth, invasive species

INTRODUCTION

The Asiatic clam (*Corbicula fluminea*) is a freshwater bivalve, native to southeast Asia, that is now common in Europe, Africa, the Pacific Islands, and North and South America. Early evidence of *Corbicula* in North America was empty shells collected in 1924 at a British Columbia site (Counts 1981) and at a Columbia River site in Washington, United States in 1938 (Burch 1944). Today, *Corbicula* is reported in 37 US states including, most recently, New York and Connecticut (McMahon 1983; Foehrenbach & Raeihle 1984; Morgan et al. 1992). The rapid spread and persistence of *Corbicula* throughout North America is related to its rapid growth rate, early onset of maturity, high fecundity, and its ability to tolerate a wide range of environmental conditions (Mattice & Dye 1976, Aldridge & McMahon 1978, Graney et al. 1980, McMahon & Williams 1986a, McMahon & Williams 1986b, McMahon 2002).

While *Corbicula* is considered an economically important food species in its native range (Chen 1990), it is recognized as a nuisance in North America (Ingram 1959, Sinclair 1964, Prokopovich 1969, McMahon 1977, McMahon 1983, Isom 1986). Its ability to clog water systems makes *Corbicula* a serious and costly problem for the electric generating industry (Goss & Cain 1975, Mattice 1979, Page et al. 1986). Thus, the discovery of Asiatic clams in water systems at Connecticut Yankee Nuclear Power Station (CY) on the Connecticut River in May 1990 (Morgan et al. 1992) received considerable attention. The range extension of *Corbicula* to the Connecticut River, the northernmost population in the eastern United States, was not expected because river temperatures frequently drop below 2°C, the minimum temperature tolerated by this clam (Mattice & Dye 1976). This study was initiated in 1991 as a condition of a Connecticut Department of Environmental Protection (CTDEP) permit to allow CY to continuously chlorinate its service water system to prevent *Corbicula* biofouling. Monitoring was later expanded upriver to the Middletown and South Meadow power plant sites. This study examines the abundance, growth, and reproductive phenology of *Corbicula* under ambient Connecticut

River conditions and under thermal discharge conditions at the Connecticut Yankee and Middletown power plant sites.

SITE DESCRIPTION

The Connecticut River originates in northern New Hampshire near the Canadian border and flows south for 660 km, dropping 800 m in elevation by the time it reaches the mouth at Long Island Sound (LIS) (Merriman & Thorpe 1976 and Fig. 1). Annual average water flow, measured at Thompsonville CT (102 km from LIS), during the period 1991 to 2000 ranged from a low of 410 m³/s in 1995 to a high of 735 m³/s in 1996 (USGS 2002). Daily maximal rates usually occur in April, often exceeding 1700 m³/s.

The focus of this study is the lower Connecticut River extending downstream from Hartford, Connecticut to a point 30 km above the mouth of the river (Fig. 1). The survey area extends over a 51 km section of river and encompasses three electrical power plant sites: South Meadow Station (SM), a 68.5 megawatt, solid waste-to-energy plant; Middletown Station (MS), an 856 megawatt oil fired power plant; and Connecticut Yankee (CY), a 582 megawatt nuclear power plant (Fig. 1). River width varies between ~400 m and 600 m over the study area. Depths at sampling sites were 1–6 m below mean low water. Semidiurnal tides affect river flow, bringing on average 425 m³/s of additional flow to the lower Connecticut River in the vicinity of CY (Merriman & Thorpe 1976), causing periodic fluctuations in river height of ~1 m (NSI 1995, Rozsa 2001). The tidal influences are large in relation to natural flow during periods of low river discharge, and absent or nearly absent during freshet conditions (Boyd 1976, Rozsa 2001). The study areas at CY and farther north at MS and SM are above any seawater incursion. Daily average ambient water temperatures were similar for all three power plants and ranged between −1.7 and 30.6°C during the 10-year study period (Fig. 2). The river frequently freezes over during the winter in our study area, but the duration of ice cover varies from year to year. Discharge water temperatures at CY during plant operation were 8 to 12°C above ambient river temperatures at a maximum flow rate of 25 m³/s.
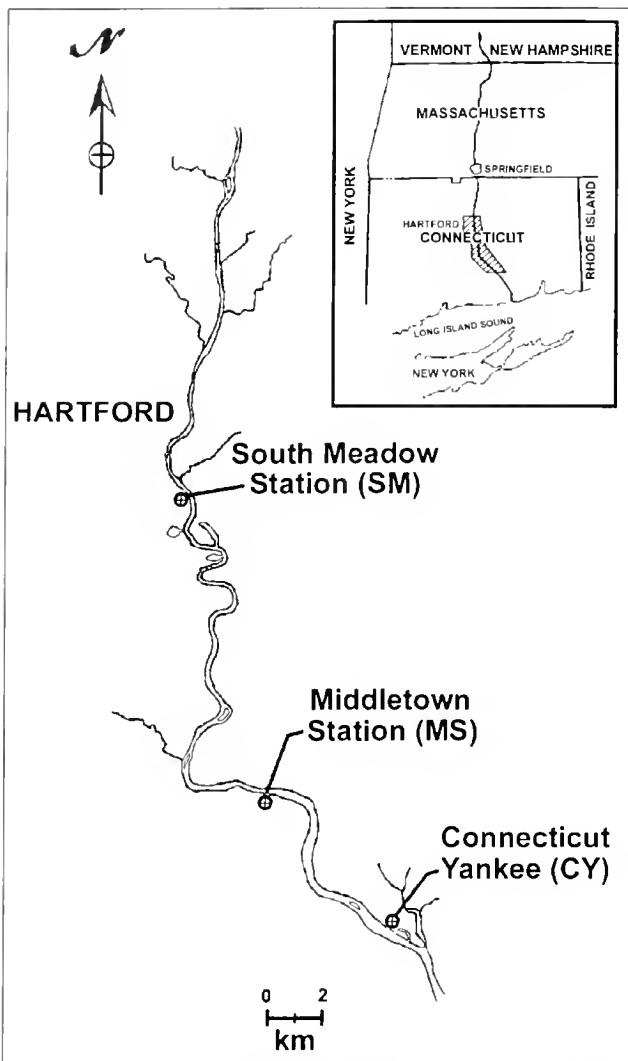
Figure 1. Location of Asiatic clam study area and sampling sites on the Connecticut River, showing the three electric power station sites (SM, MS, and CY).

The CY cooling water discharge flows through a man-made canal 1 km long before mixing with ambient Connecticut River waters. Connecticut Yankee ceased operation on July 22, 1996. At MS, the average sustained discharge temperatures from 1992–1994 ranged between 7 and 10°C above ambient river conditions with an average discharge of 3.6 m³/s. At MS and SM, the cooling water is discharged directly to the river.

MATERIALS AND METHODS

This study was conducted between August 1991 and November 2000. Data at CY were collected during the entire study at four sampling sites located in the river near the power plant and one site in the discharge canal (CY discharge). The four CY river sites were similar in *Corbicula* abundance and the data from each were combined for data analysis (CY). Sampling was extended to three sites at MS in May 1992 and continued through November 1994; two sites were grouped for data analysis as river sites (MS), and the third, adjacent to the cooling water discharge (MS discharge), was analyzed separately. At SM, a single river site downstream of the cooling water discharge (minimal thermal influence) was sampled between August 1993 and November 1994.

In the first year of the study (1991), field sampling was conducted in August and November. For the remainder of the study period (1992–2000), field sampling was conducted three times each year, in May, August, and November. To collect *Corbicula*, five 0.1 m² bottom sediment samples were obtained at each sampling site using a weighted Peterson grab (Wildlife Supply Company, Buffalo, NY). Sample processing techniques were similar to those of Gardner et al. (1976). Grab samples were sieved in the field by passing the sample through a series of three screens (6.3, 2.0, and 1.0 mm mesh size). Clams and sediment retained on the 1-mm screen were subsampled in the field by placing a well-mixed 1-L sample in an elutriator (Magdych 1981) for 3 min at a water flow of 20–30 L/min. The overflow from the elutriator was collected on a 1-mm mesh sieve and sorted in the laboratory under a dissecting microscope (10×). Sediment and clams retained on the 6.3 and 2.0 mm screens were taken to the laboratory and washed through a series of five US Standard Testing Sieves (19.0, 12.5, 6.3, 3.4, and 2.0-mm mesh sizes). Size classes were determined based on the mesh size on which clams were retained. Clams
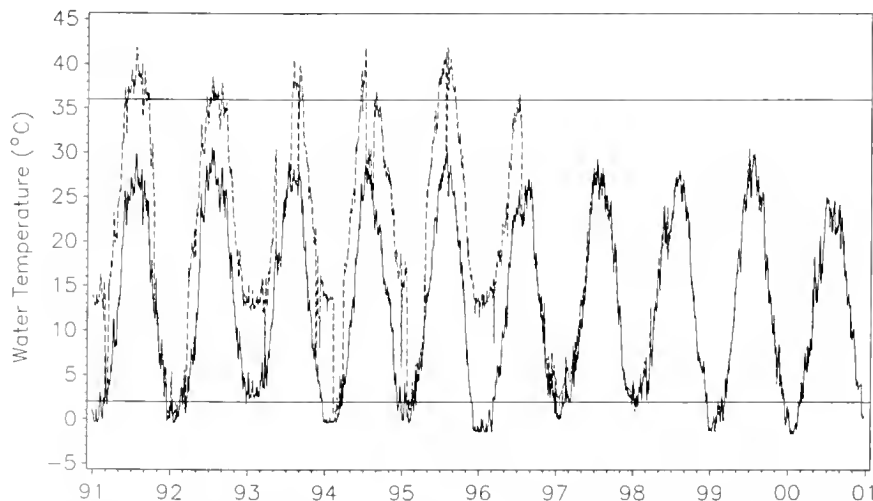
Figure 2. Intake (—) and discharge (- - - -) water temperatures at CY from January 1, 1991 to January 1, 2000. Horizontal reference lines represent upper and lower lethal temperature limits for *Corbicula fluminea*.

Figure 3. Average abundance (# clams/m²) of *Corbicula fluminea* by size class (graph) and total (table, ±95% CI) at CY river sites.
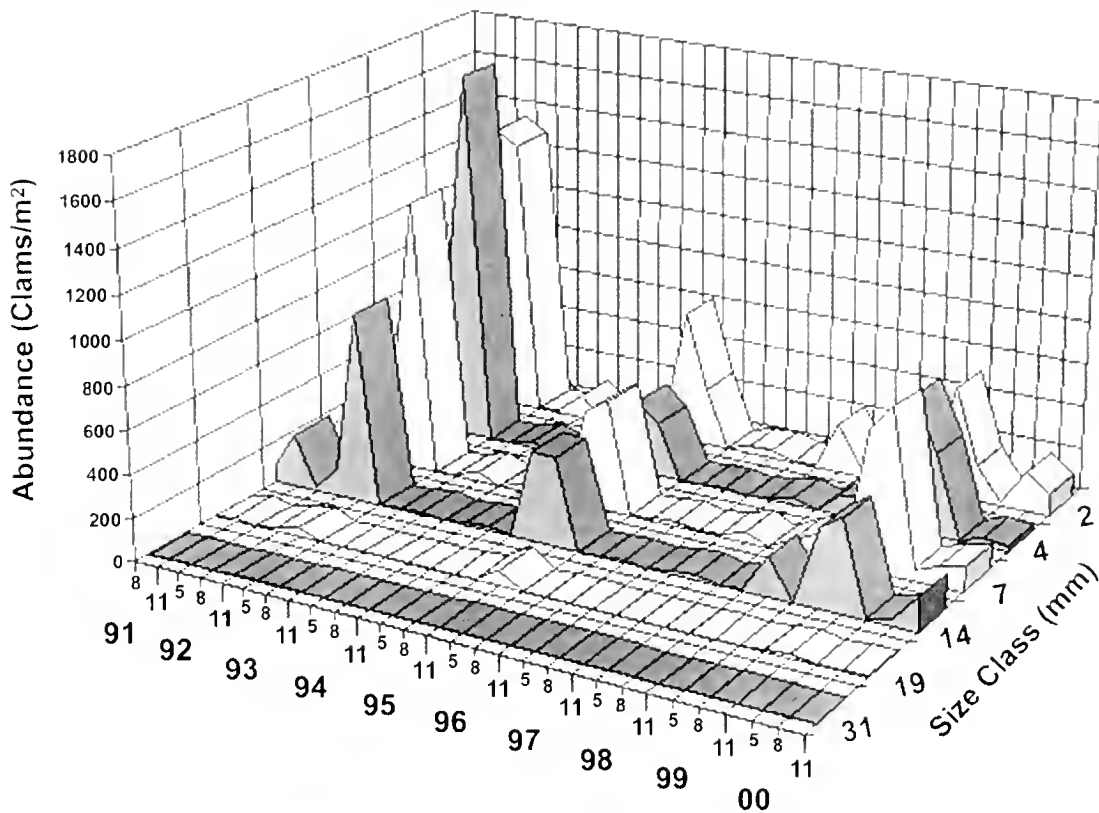
Month	Year									
	1991	1992	1993	1994	1995	1996	1997	1998	1999	2000
5	-	55 ±40	4.0 ±8.5	0	124 ±56	0	8.0 ±7.5	38 ±33	94 ±35	78 ±58
8	399 ±131	2,568 ±1,538	35 ±24	68 ±22	1,828 ±622	56 ±22	57 ±28	291 ±130	2,148 ±617	366 ±138
11	807 ±387	5,209 ±2,630	206 ±91	225 ±118	1,522 ±565	80 ±38	350 ±136	649 ±272	1,758 ±635	412 ±189
ANNUAL	-	2,610 ±1,136	89 ±40	98 ±45	1,158 ±328	45 ±16	138 ±59	326 ±116	1,334 ±362	286 ±85

retained on the 1.0-mm sieve averaged 2.0 mm in shell length; on the 2.0-mm sieve, 4.1 mm; on the 3.4-mm sieve, 6.7 mm; on the 6.3-mm sieve, 14.1 mm; on the 12.5-mm sieve, 19.3 mm; and on the 19-mm sieve, 31.1 mm.

Individual clam growth was monitored monthly in 1993 and 1994 using shell length measurements to the nearest 0.1 mm. In the river near the CY plant intakes, marked clams maintained in lantern nets were used for monitoring growth. In the CY discharge canal, 12 clams collected randomly from lantern nets were measured monthly to assess growth.

Clam fecundity was determined monthly using techniques of Aldridge and McMahon (1978). Several hundred adult clams (>8.0 mm in shell length) were collected from the river in May/June of 1991 through 1994 and held in lantern nets placed at two locations, one in the river near CY plant intakes, the other in the CY discharge canal. Clams were collected monthly from river nets until winter, when no live clams remained in lantern nets. In the CY discharge canal, all clams were dead by June (when water temperatures at this site exceeded 37°C). In this study, data for fecundity in the discharge canal were collected from November 1992 to July 1993, and June and July fecundity data were at ambient river temperature due to a power plant shut down. Twelve clams were subsampled monthly from each net. In the laboratory, each clam was held under static conditions at 20°C for 24 h in a 100-ml
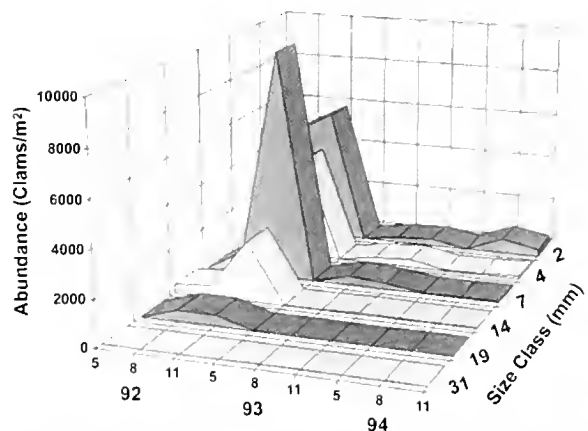
Month	Year		
	1992	1993	1994
5	758 ±688	43 ±36	138 ±68
8	10,413 ±7,233	878 ±451	786 ±577
11	23,275 ±5,494	928 ±339	533 ±295
ANNUAL	11,482 ±4,416	616 ±227	555 ±253

Figure 4. Average abundance (# clams/m²) of *Corbicula fluminea* by size class (graph) and total (table, ±95% CI) at MS river sites.

beaker filled with filtered Connecticut River water. The number of juveniles released during this period, determined with a 10× dissecting microscope, was recorded as an index of spawning activity. Additional fecundity assessments were made by dissecting these clams and noting the presence of brood. Maturity of gametes was assessed by removing egg and sperm cells from the gonadal tissues and examining the cells under a compound microscope (400×).

Statistical analyses were performed using SAS version 8 software (SAS Inc., Cary, NC). Abundance data in figures are presented using arithmetic means and non-transformed data. Statistical comparisons of abundance data were always carried out after log transformation. The relationships between winter clam survival (defined as the ratio of May clam abundance to November clam abundance from the previous year, expressed as a percentage) and temperature or river flow indices were assessed using the rank-order Spearman correlation. Growth and reproduction data were not transformed prior to statistical testing.

RESULTS

Abundance

Corbicula abundance exhibited high intra- and inter-annual variability. Year to year abundance fluctuations were considerable at all ambient temperature river sites (Figs. 3, 4, 5; note different vertical scales). At CY, mean annual clam abundance in 1992, 1995, and 1999 (range 1,158–2,610 clams/m^2) was significantly higher ($P < 0.05$) than in all other years (range 45–326; Fig. 3). At MS, mean annual abundance in 1992 (11,482 clams/m^2) was significantly higher ($P < 0.05$) than in 1993 or 1994 (616 and 555 clams/m^2, respectively; Fig. 4). At SM, mean annual abundance was low, with 82 clams/m^2 in 1993 and 67 clams/m^2 in 1994 (Fig. 5).

Of ambient temperature river sites, seasonal abundance at CY
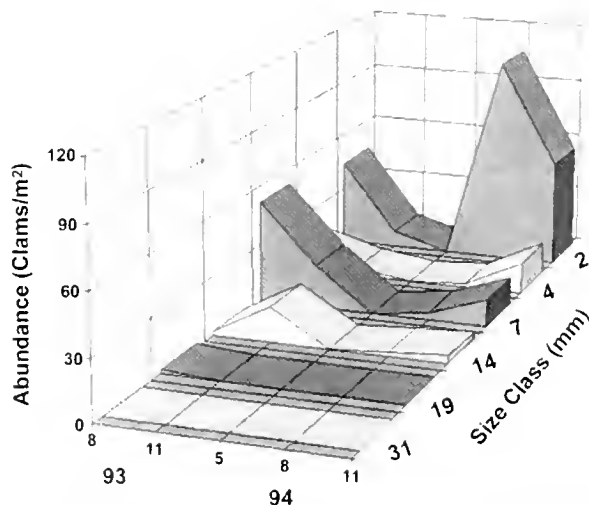
over a 10-year period was significantly higher ($P < 0.05$) in November than in May or August. November abundance at CY ranged from 80 clams/m^2 in 1996 to 5,209 clams/m^2 in 1992. By contrast, over the 3 years surveyed at MS (1992–1994) and 2 years surveyed at SM (1993 and 1994), abundance was not significantly different ($P > 0.05$) between August and November samples. November abundance at MS in 1992 (23,275 clams/m^2) was the highest observed during the study. Lowest November abundance occurred at SM in 1993 (52 clams/m^2). At all sites, clam abundance in May was significantly ($P < 0.05$) lower than that in either August or November.

Of thermally influenced sites, seasonal clam abundance in the CY discharge canal had significant differences ($P < 0.05$) among the three sampling periods (Fig. 6). May abundance ranged from 0–92 clams/m^2. August abundance ranged from 0–12,174 clams/m^2. November abundance ranged from 24 to 880 clams/m^2. At the MS discharge, August and November abundance estimates were not significantly different ($P > 0.05$), ranging from a low of 322 clams/m^2 in November 1993 to a high of 7,100 clams/m^2 in November 1992 (Fig. 7). As with river sites, May abundance at both CY and MS discharge sites was significantly lower ($P < 0.05$) than that in August and November.

Annual abundance was variable at the CY discharge site. A pooled *t*-test of total abundance during operational (1991–1996) vs. post-operational years (1997–2000) indicated that clam abundance increased significantly ($P = 0.007$) during post-operational years. This increase was the result of higher abundance of larger size class clams (7–14 mm and 19–31 mm) following power plant shutdown. At the MS discharge site, total clam abundance was significantly higher ($P < 0.05$) in 1992 (3,322 clams/m^2) than in 1993 and 1994 (496 and 549 clams/m^2, respectively; Fig. 7). Clam abundance was not significantly different ($P > 0.05$) between the river and discharge sites at MS, except for the largest clams (31 mm size-class), which were most abundant at the MS discharge site. In fact, the largest clam measured during the entire study (37.6 mm) was collected at MS in August 1992.

Winter Survival

Declines in clam abundance from November of one year to May of the next were used to determine winter survival; values at CY ranged from 0% in 1994 and 1996 to 55% in 1995 (Fig. 3). The effects of winter water temperatures and peak river flows on clam winter survival were examined using Spearman-ranked correlation (Table 1). The severity of winter water temperatures, as indicated by the number of days with average water temperature ≤2°C, was not significantly correlated ($r_s = -0.65$, $P = 0.081$) with clam winter survival. The number of days, however, ≤1°C was negatively correlated ($r_s = -0.73$, $P = 0.040$) with winter survival, and average December through April water temperature was positively correlated ($r_s = +0.87$, $P = 0.004$). Highest average monthly flow in the Connecticut River typically occurs in April (Fig. 8). Accordingly, the number of days each year exceeding 1,700 m^3/s in April was used as an index of spring freshet severity. This index was negatively correlated with winter clam survival ($r_s = -0.91$, $P = 0.002$). Data from 1993 were omitted from this analysis because a single storm in March caused total mortality of clams at our sampling sites.

Growth

Corbicula growth rates under ambient river conditions exhibited seasonal cycles, and growth of marked clams was size-

Month	Year	
	1993	1994
5	-	0
8	112 ±30	114 ±103
11	52 ±21	88 ±75
ANNUAL	82 ±27	67 ±43

Figure 5. Average abundance (# clams/m^2) of *Corbicula fluminea* by size class (graph) and total (table, ±95% CI) at SM.

Figure 6. Average abundance (# clams/m²) of *Corbicula fluminea* by size class (graph) and total (table, ±95% CI) at CY discharge.
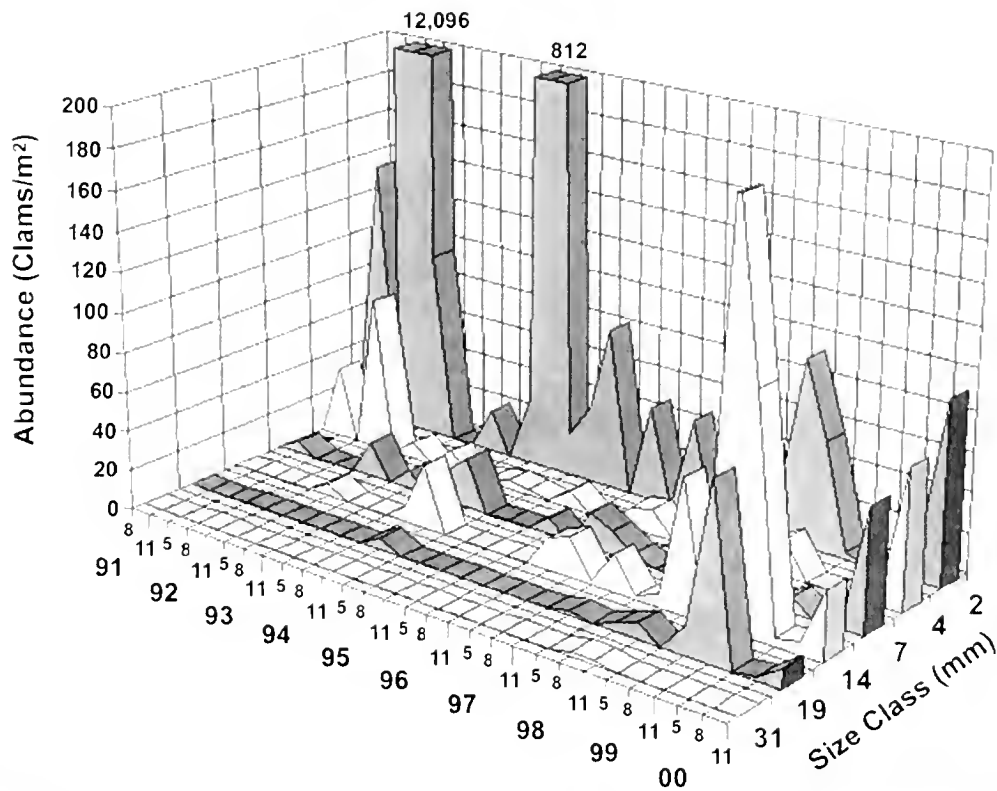
Month	Year									
	1991	1992	1993	1994	1995	1996	1997	1998	1999	2000
5	-	2 ±5.0	32 ±22	0	24 ±29	8.0 ±5.1	92 ±62	20 ±22	76 ±37	4 ±10
8	34 ±58	12,174 ±30,269	0	60 ±54	38 ±50	48 ±75	6 ±15	62 ±83	243 ±172	30 ±35
11	178 ±181	96 ±121	42 ±26	880 ±1254	90 ±187	44 ±70	24 ±6.3	212 ±227	210 ±73	268 ±181
ANNUAL	-	4,091 ±8,454	25 ±14	313 ±397	51 ±53	33 ±28	41 ±27	98 ±78	173 ±63	101 ±83

dependent (Figs. 9 and 10). In 1993, clams with an initial shell length of ~14.5 mm had a higher growth rate (0.54 mm/wk) from June to October than those starting at ~17.5 mm (0.41 mm/wk), and ~21.7 mm (0.35 mm/wk). A similar size-dependent relationship was also observed in the 1994 study; clams with an initial length of ~12 mm grew fastest from June to October (0.51 mm/wk), followed by ~20 mm (0.32 mm/wk) and ~30 mm (0.14 mm/wk) clams. Growth rates were significantly different ($P < 0.05$) among the three size classes through August. In September through December, however, mean monthly growth rates for all size classes were generally low and not significantly different from each other.

Clam growth rates in the CY discharge canal from November 1992 to February 1993 were ≤0.18 mm/wk, when water temperatures were 13–19°C, 10–12°C above ambient river temperatures (Table 2). As these clams were not marked, negative growth rates could occur as a result of mortality of large individuals. Growth rates were as high as 0.27 mm/wk from March to May when water temperatures ranged from 13–27°C. Maximum growth rates at this site occurred during June (0.38 mm/wk) and July (0.33 mm/wk), when canal temperatures were similar to those at ambient river conditions because of a power plant outage. All clams died after the power plant restarted and discharge water temperatures exceeded 37°C (July).

Reproduction

Microscopic examination of gametic tissues of clams held under ambient river and CY discharge conditions show that eggs and sperm were continually present as long as clams were alive (Fig. 11). For clams held at ambient river temperatures, the presence of embryos and veligers in the demibranchs (brooding) and the active release of juveniles occurred primarily over a 4-month period (June to September). By October, only one clam out of 48 examined was still spawning. The maximum number of juveniles released per clam per day typically occurred in August across all 4 years in which reproduction was monitored (2,862 juveniles/clam/day; Fig. 12). This pattern of juvenile release allowed maximum recruitment to occur just after the period of maximum river water temperature (July, with a 4-year average of 27.5°C). The number of juveniles released per adult in August was positively correlated with the size of the clam ($r_s = 0.77$; $P < 0.01$; Fig. 13).

The reproductive cycle of *Corbicula* in the CY discharge canal was seasonally shifted (Fig. 11). Brooding and releasing of juveniles first occurred in November 1992 when discharge temperatures averaged 18.3°C, and ceased from December through February when temperatures averaged <14°C. Spawning began again in March and increased through April when discharge temperatures averaged 17°C. The sharp decrease in May was the result of
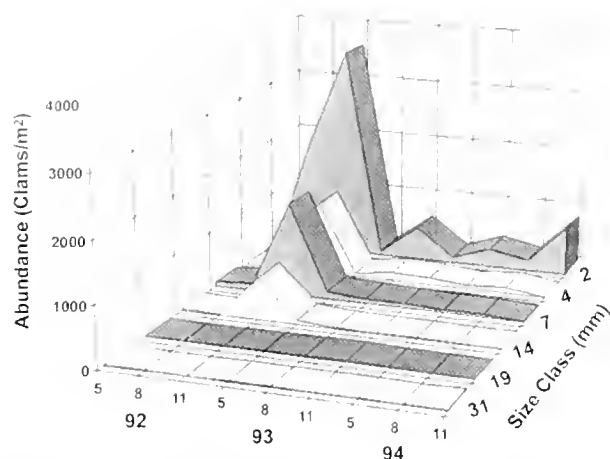
Month		Year	
	1992	1993	1994
5	200 ±122	326 ±141	448 ±277
8	2,666 ±836	840 ±450	340 ±116
11	7,100 ±1,896	322 ±69	860 ±931
ANNUAL	3,322 ±1,721	496 ±186	549 ±283

Figure 7. Average abundance (# clams/m²) of *Corbicula fluminea* by size class (graph) and total (table, ±95% CI) at MS discharge.

a power plant outage beginning on May 15, which dropped cooling water temperatures from 30°C to 18°C in a single day (Fig. 14). Spawning activity recovered and peaked in June and July as the plant outage continued, similar to the pattern observed at ambient river temperatures (17–27°C). On July 21, 1993 the power plant restarted and temperatures increased to >35°C in 4 days. By August 18, 1993 all clams held in the CY discharge were dead.

DISCUSSION

Corbicula fluminea was first documented in the Connecticut River in May 1990 (Morgan et al. 1992), the first report of this nonindigenous clam in New England waters. Before this discovery, *Corbicula* was not expected to colonize the Connecticut River because water temperatures routinely fall below 2°C for prolonged periods. It is commonly accepted among researchers that the lower lethal temperature limit for *Corbicula* is ~2°C (Horning & Keup 1964, Bickel 1966, Mattice & Dye 1976, Rodgers et al. 1979, Cherry et al. 1980).

Corbicula abundance varied seasonally as well as annually, but
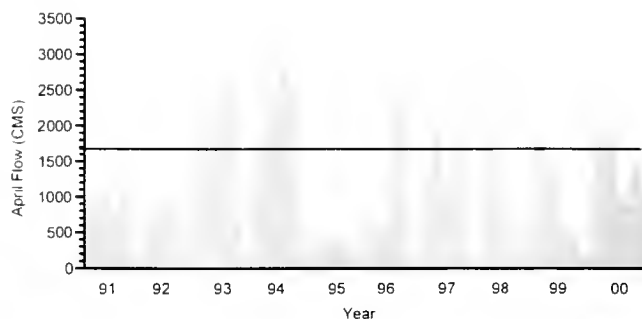
Figure 8. Connecticut River daily flow rates (m³/s) at the Thompson-ville, CT gaging station in April from 1991 to 2000.

clearly peaked in 1992. Survival of clams from one year to the next is positively correlated with the average December to April water temperatures and negatively correlated with the number of days the river water temperature was below 1°C and the number of days that river flows exceeded 1700 m³/s in April. For example, no clams were observed in May at our Connecticut Yankee sampling sites following the two coldest winters (1993–1994 and 1995–1996), when river water temperatures dropped below 2°C for 12–15 weeks and the highest winter survival occurred in 1995 when daily average river flow in April never exceeded 1700 m³/s.

Low survival at Connecticut Yankee and Middletown Station during the winter of 1992–1993, when water temperature did not drop below 2°C, was attributed to winter storm Joshua (March 13, 1993). This storm produced low water levels (1–2' below normal) and left shoal areas, specifically our sampling areas, exposed to air temperatures as low as −8°C, freezing sediment and clams (NUSCO 1994).

Higher winter survival at Middletown Station sites, when compared with those around Connecticut Yankee, was attributed to the influence of the Middletown Station thermal discharge. River water temperatures seldom dropped below 2°C in the Middletown Station discharge mixing zone (NUSCO 1994). Other over-wintering populations likely exist in the river in refugia provided by other industrial thermal discharges or in areas of the river receiving regular influxes of groundwater that maintains a temperature of 9.0 ± 2°C (R. Lewis, State of Connecticut Geologist; pers. comm.). Graney et al. (1980) and Kreiser and Mitton (1995) suggest that warm water refugia such as these were assisting the Asiatic clam in expanding its geographical range northward.

Clam densities in the Connecticut Yankee discharge canal were

TABLE 1.

Spearman correlations coefficient (r$_s$) for percentage winter survival of *Corbicula fluminea* at CY versus indices of winter temperatures and Connecticut River flow.

Variable	r$_s$	Prob >|r|	n[a]	Mean	Std Error	Min.	Max.
Percentage Survival[b]	—	—	8	12.7%	6.23%	0%	54.9%
Ave. Winter Temp.[c]	+0.87	0.004	8	2.93	0.37	1.32	4.86
No. Days ≤1°C	−0.73	0.040	8	54.9	8.75	17	93
No. Days ≤2°C	−0.65	0.081	8	70.6	8.12	28	103
Flow ≥1700 m³/s[d]	−0.91	0.002	8	6.4	1.54	0	13

[a] 1993 data were omitted because of the mortality caused by the March storm Joshua (see text).

[b] % Survival = (May abundance/prior November abundance) × 100.

[c] Average Winter Temperature = the annual December to April mean daily Connecticut River temperature at CY.

[d] Number of days in April when the Connecticut River flow equaled or exceeded 1700 m³/s.
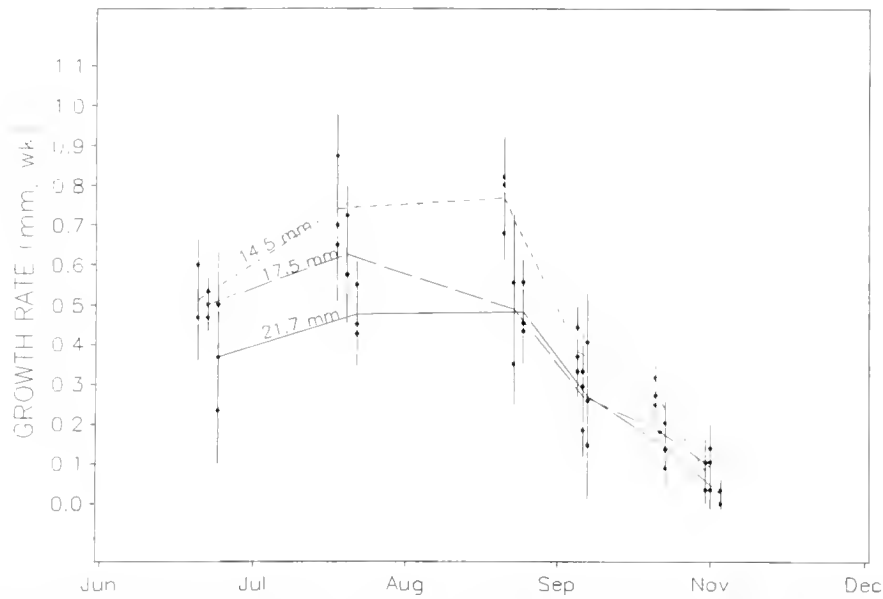
Figure 9. *Corbicula fluminea* growth rates (mm/wk) in 1993 for marked clams within initial size classes based on shell length. Vertical bars represent two standard deviations around the mean growth rates for three individuals in each size class.

most variable. Large numbers of small (2 mm) clams that apparently survived passage through the power plant cooling water system characterized transient populations in the canal. A permanent population, however, was not established during power plant operation because summer water temperatures often exceeded 37°C, the upper lethal temperature limit for *Corbicula* in our study. Mc-Mahon and Williams (1986b) reported similar findings for *Corbicula* living in the thermal discharge of the Handley Power Station in Texas. Following Connecticut Yankee closing in 1996, size range of clams collected in the discharge canal has increased with shell lengths now ranging from 2–19 mm. These results indicate not only that clams are successfully over-wintering in the canal under ambient river temperatures, but also surviving for >1 year.

The canal essentially has become a cove where circulation is dependent on semidiurnal tidal exchange, and not vulnerable to high spring freshet water flows.

Clam abundance in the Middletown Station discharge area also fluctuated, but was consistently higher than abundance at CY discharge during the same period. Similar to the CY discharge, the population near the MS discharge was dominated by clams 2 mm in size. In contrast, however, to the CY discharge, clams of all size classes, including those in the 31 mm class, were regularly collected at the MS discharge. The presence of larger size clams suggests that this area provided a more stable refugium. The 37-mm clam collected at this site in 1992, along with growth rates observed during our study, suggests that *Corbicula* has been
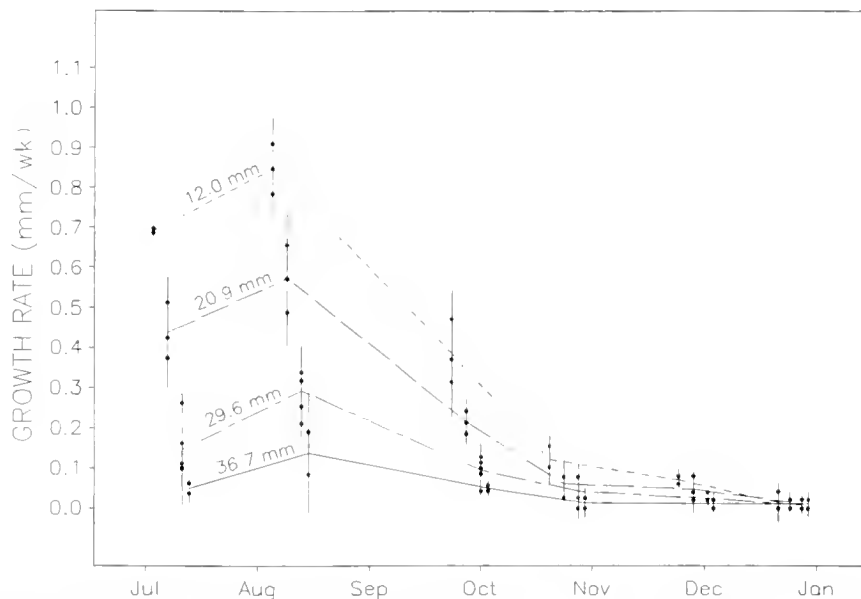
Figure 10. *Corbicula fluminea* growth rates (mm/wk) in 1994 for marked clams within initial size classes based on shell length. Vertical bars represent two standard deviations around the mean growth rates for two to five individuals in each size class.

TABLE 2.

Corbicula fluminea growth in the CY discharge canal from November 1992 to July 1993.

Date	Growth Week	Average Length (mm)	n	SE	Minimum Length (mm)	Maximum Length (mm)	Growth Rate (mm/wk)
11/10/92	0	20.20	12	0.69	15.3	24.0	
12/22/92	6	20.04	12	0.38	18.1	21.5	−0.026
01/26/93	11	20.97	12	0.59	17.7	26.5	0.185
02/23/93	15	20.89	12	0.48	18.6	23.8	−0.019
03/23/93	19	21.96	12	0.43	19.2	24.4	0.267
04/29/93	24	23.04	12	0.42	20.6	24.9	0.205
05/18/93	27	22.57	12	0.31	20.4	24.1	−0.172
06/24/93	32	24.57	11	0.40	21.5	26.1	0.378
07/22/93	36	25.89	12	0.39	24.2	27.6	0.330
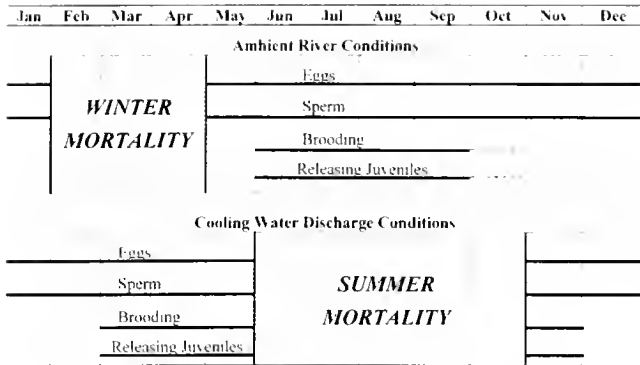
Figure 11. Summarization of the 1991 to 1994 annual reproductive cycle of *Corbicula fluminea* under ambient Connecticut River conditions and the thermally elevated conditions of the CY cooling water discharge.

present in the river since 1988. Winter water temperatures were moderated by the Middletown Station thermal discharge, and summer thermal stress was reduced because of rapid dilution of discharge waters with ambient river water. In addition, the MS thermal discharge flow was only ~15% that of CY.

Corbicula growth in the Connecticut River under ambient water temperatures is consistent with reports by other researchers in North American (Morton 1977, Britton et al. 1979, Eng 1979, Mattice 1979, McMahon 1983, Welch & Joy 1984, Joy 1985, Mattice & Wright 1986, McMahon & Williams 1986b, Doherty et al. 1990, French & Schloesser 1991), and was primarily influenced by water temperature. Growth began in May when water temperatures rose above 10°C and continued until December when water temperatures dropped below this threshold. Other researchers reported 9–15°C to be the lower temperature threshold for growth of *Corbicula fluminea* in their studies (Hall 1984, Mattice & Wright 1986, McMahon & Williams 1986a, French & Schloesser 1991).
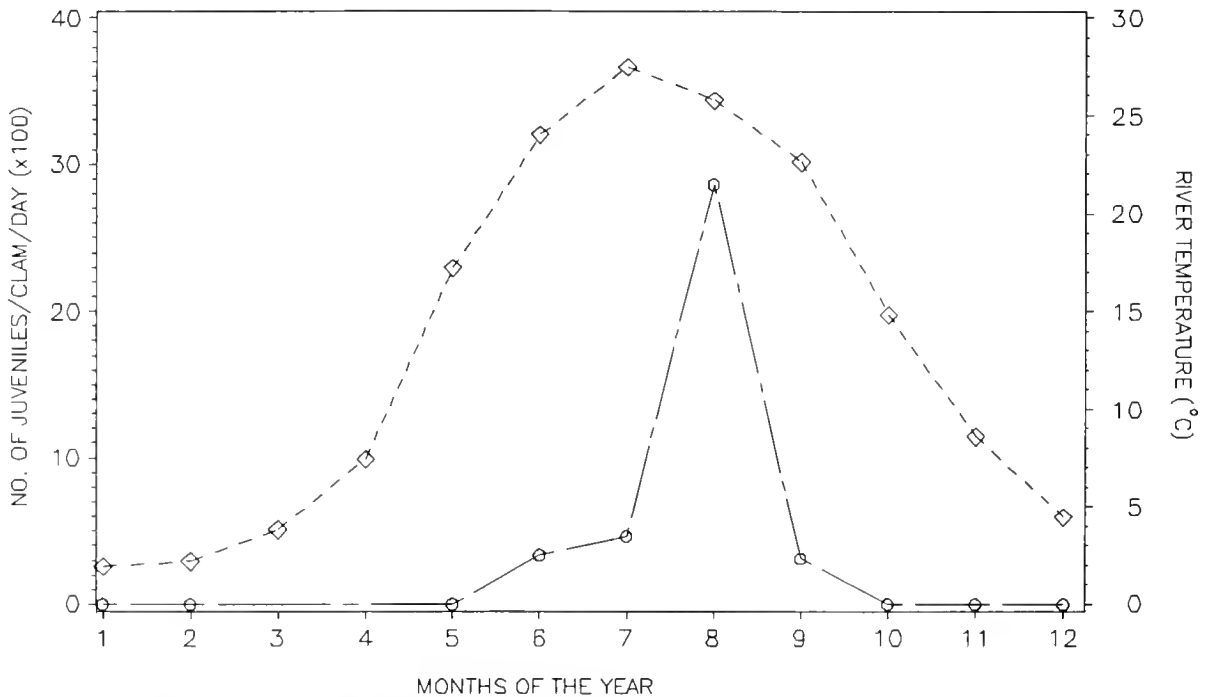
Figure 12. *Corbicula fluminea* fecundity (−○−) and water temperature (- - ◊ - -) for clams held in ambient temperature Connecticut River water from 1991–1994.
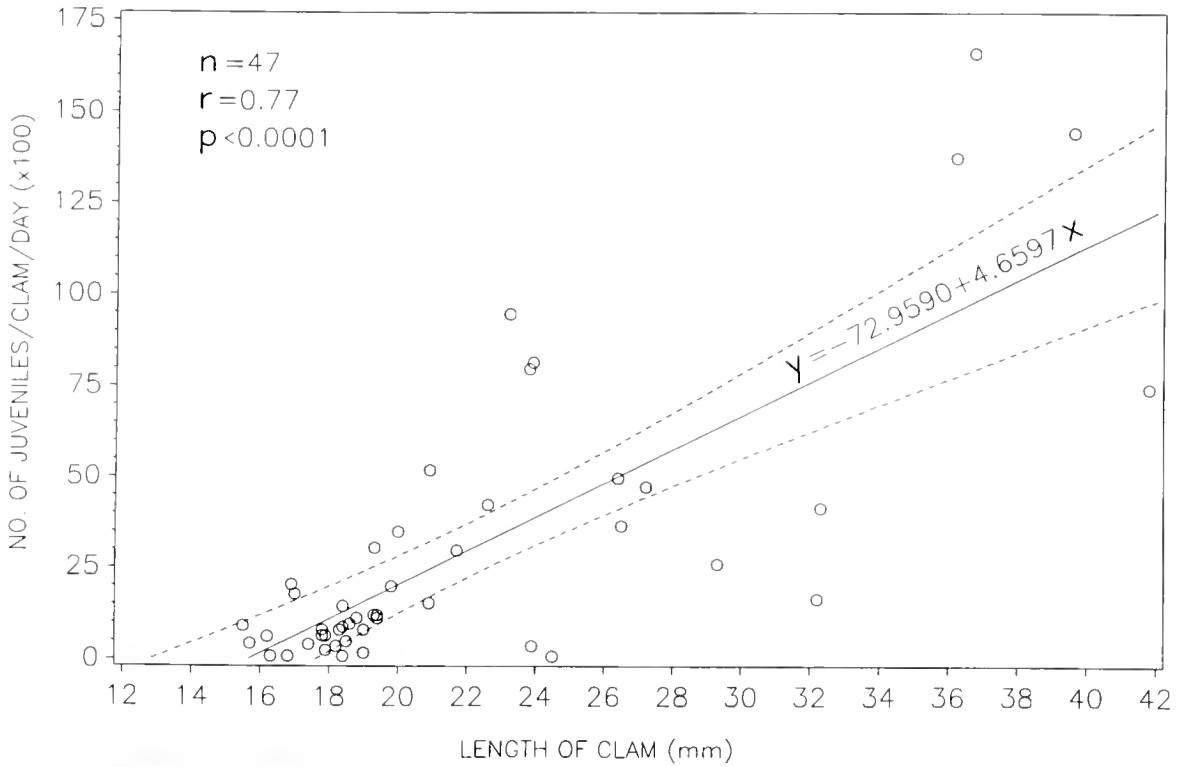
Figure 13. Linear regression with 95% CI on mean predicted values for the number of juveniles released per day in relation to shell length (mm) of the spawning *Corbicula fluminea* during the peak spawning month of August in the mainstem Connecticut River.
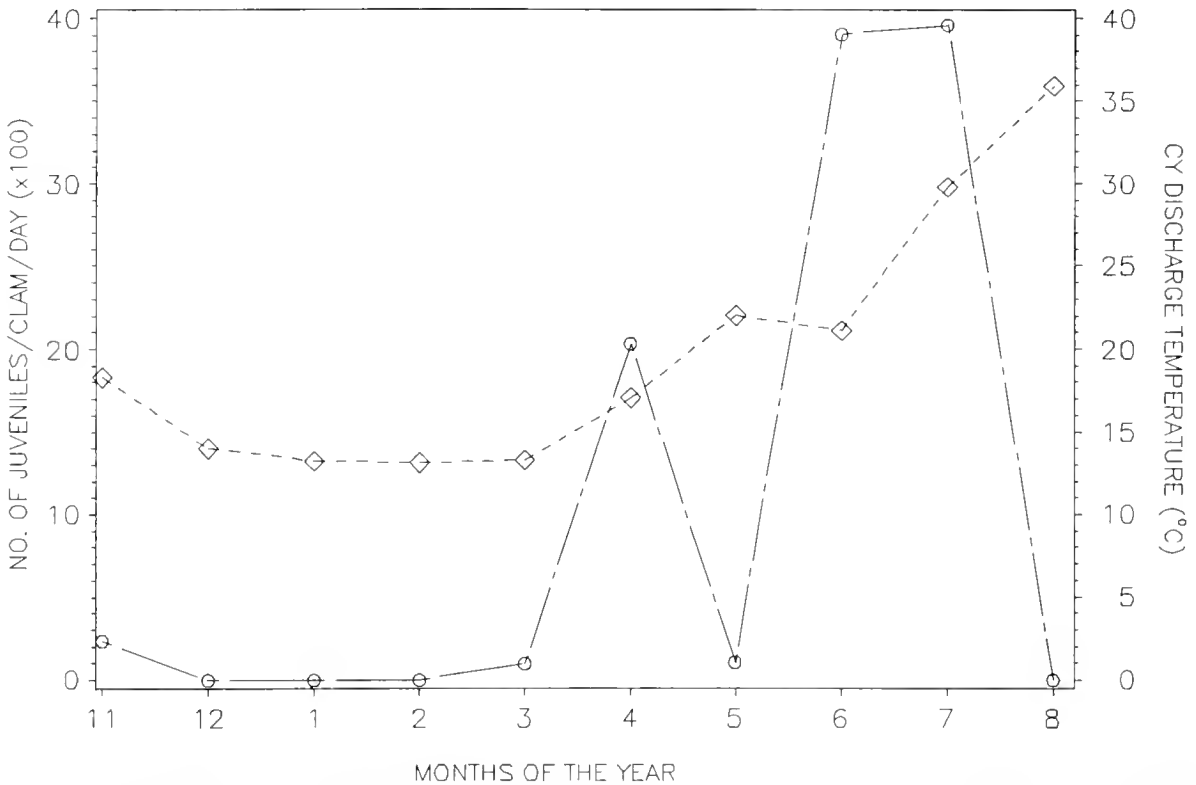
Figure 14. *Corbicula fluminea* fecundity (–○–) and water temperature (- - ◇ - -) for clams held in the discharge canal at CY from November 1992 to August 1993.

Highest growth rates occurred in July and August, when river water temperatures peaked (25–30°C), and growth rates were significantly higher for the smaller clam sizes.

The upper temperature tolerance of *Corbicula* determined in this study is within ranges reported by other researchers in laboratory and field experiments (Mattice & Dye 1976, Dreier 1977, Mattice 1979, Cairns & Cherry 1983, McMahon & Williams 1986a). *Corbicula* growth in the CY thermal discharge canal was initiated in November 1992 when water temperatures dropped to <35°C. Growth continued until August 1993, when water temperatures were >37°C and clams died.

Seasonal water temperatures also control reproductive cycles of the Connecticut River *Corbicula* population. The presence of eggs and sperm was continuous in the Connecticut River population of this species as long as water temperatures supported its survival. Brooding and releasing of juveniles occurred when water temperatures were between 17–28°C, typically from June to October. Spawning temperatures of 14–27°C were reported by other researchers in North America (Eng 1979, Mattice 1979, Hall 1984, Cherry et al. 1986, Foe & Knight 1986; McMahon & Williams 1986a; Doherty et al. 1987; Rajagopal et al. 2000).

A single annual spawning peak for the *Corbicula* population in the Connecticut River occurred in August. Others reported two *Corbicula* spawning peaks, one in spring and one in fall (Heinsohn 1958, Aldrige & McMahon 1978, Eng 1979, McMahon 1983, Foe & Knight 1986, McMahon & Williams 1986a). Several others have reported a single spawning peak (Bickel 1966, Hornback 1992, Mouthon 2001). The presence of a single reproductive peak in the Connecticut River population may be related to longer periods of cold-water conditions, more severe spring flooding, and the quantity and quality of available food.

The altered thermal regimen within the CY discharge canal shifted the period of reproduction from the ambient river period of June through September to November and March through May when water temperatures in the canal ranged between 16–30°C. Spawning during July and August 1993 occurred because the power plant was off-line and the discharge water temperatures were not elevated. These results demonstrate that thermal discharges can alter the reproduction cycle of *Corbicula*. Aldridge and McMahon (1978) and Dreier and Tranquilli (1981) reported that *Corbicula fluminea* spawning activities stopped at temperatures of 30–34°C, most likely due to thermal stress. Graney et al. (1980) speculated that elevated temperatures in thermal discharges may extend the spawning season into the winter.

In conclusion, this study showed that the Connecticut River has supported a fluctuating *Corbicula* population for at least 10 years. Cold water temperatures (<2°C) for several weeks, and high water flow in the spring caused high mortality of clams in the river during the winter and early spring. Growth and reproduction for *Corbicula* in the Connecticut River peaked in July and August when river temperatures ranged between 24–30°C and only one spawning peak occurred each year. The key to *Corbicula*'s unexpected success in establishing a population in the Connecticut River is its ability to colonize refugia from cold winter water temperatures and spring freshet flows that cause high clam mortality. Following the closing of the CY power plant, *Corbicula* continued to populate the CY river sites establishing a more mature population in the discharge canal. Based on our observations of *Corbicula* in the Connecticut River, we expect that this species will continue to successfully colonize other rivers and lakes in New England, where similar winter refugia exist.

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QPX, A PATHOGEN OF QUAHOGS (HARD CLAMS), EMPLOYS MUCOID SECRETIONS TO RESIST HOST ANTIMICROBIAL AGENTS

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ABSTRACT The thraustochytrid protist quahog parasite unknown (QPX) has caused mass mortalities of hard clams (*Mercenaria mercenaria*) in Atlantic Canada and Massachusetts. It typically secretes copious mucus *in vivo* and *in vitro*. *M. mercenaria* plasma contains naturally-occurring agents that modulate growth of QPX cultures. This activity was shown by exposing washed, mucus-free QPX (wQPX) to filter-sterilized *M. mercenaria* plasma. Low plasma protein concentrations (<10 μg/ml) in the medium tended to stimulate QPX growth; higher concentrations (10–50 μg/ml) produced dose-dependent inhibition. If wQPX were incubated for various times before exposure to an inhibitory concentration of *M. mercenaria* plasma, a time-dependent protection from the plasma was observed; total protection was seen after ~24 h preincubation. This effect was probably a result of the re-establishment of the mucoid coats around the wQPX during preincubation. These data suggest that the mucoid secretion of QPX may represent an important virulence factor.

KEY WORDS: quahog parasite unknown (QPX), *Mercenaria mercenaria*, virulence factors, clam diseases

INTRODUCTION

Whyte et al. (1994) described a protistan parasite that caused high mortalities in a hard clam (*Mercenaria mercenaria*) hatchery on Prince Edward Island, Canada; the causative agent was named quahog parasite unknown (QPX). This organism was similar or identical to the clam pathogen first observed by Drinnan and Henderson (1963) in New Brunswick, Canada. Subsequently, QPX has been cited as the cause of mass mortalities of *M. mercenaria* in Massachusetts (Smolowitz et al. 1998) and has been reported in several Virginia coastal embayments (Ragone Calvo et al. 1998). Molecular phylogeny studies based on sequencing of 18S ribosomal RNA suggest that QPX is a member of the phylum Labyrinthulomycota (Maas et al. 1999, Ragan et al. 2000), in the thraustochytrid phylogenetic group (Stokes et al. 2002).

A medium developed by Kleinschuster et al. (1998) has permitted *in vitro* cultivation of QPX. In culture, thalli were shown to grow and mature into sporangia containing numerous vegetative endospores. The endospores were released on rupture of the sporangia and in turn matured to form thalli, and the stages of the vegetative life cycle were repeated. Whyte et al. (1994) and Kleinschuster et al. (1998) reported conversion of endospores to motile zoospores in sterile seawater. Later studies (Brothers et al. 2000), however, were unable to replicate these findings. The vegetative life stages of QPX have been observed in the tissues of infected *M. mercenaria*. In many instances, the QPX cells were seen in histologic sections to be enclosed by a translucent space; this was initially attributed to lysis of host tissue by enzymes secreted by the parasite (Whyte et al. 1994). Subsequently, Smolowitz et al. (1998) determined that in live animals, the space is occupied by a muco-fibrillar substance produced by the parasites; and that this substance is removed by histologic processing. It was suggested in that study that phagocytosis of the parasite in the clams' tissues is inhibited by the mucofibrillar secretions of the parasite.

The disease caused by the Canadian strain (CA QPX) as described by Whyte et al. (1994) is similar to that described for the Massachusetts strain (MA QPX) by Smolowitz et al. (1998). MA QPX, however, primarily infected the mantle and gill and sometimes produced nodules; CA QPX infections were more commonly seen in the connective tissue of the foot and were rarely associated with nodules. Areas of infection by CA QPX and MA QPX triggered inflammatory responses involving extensive infiltration of adjacent host tissues by hemocytes, with some evidence of phagocytosis and/or encapsulation of the parasites. Inflammatory foci caused by MA QPX sometimes contained phagocytic multinucleated giant cells similar to those produced *in vitro* by Anderson (1987). Apparently QPX infection elicits a vigorous cellular response, but this activity is insufficient to control the disease. Humoral QPX modulatory agents in *M. mercenaria* plasma are described for the first time in this article, and the role of QPX mucoid secretions in protection from them.

MATERIALS AND METHODS

QPX

These studies were carried out using MA QPX obtained from Dr. R. Smolowitz, Marine Biologic Laboratory, Woods Hole, MA. They were propagated in the medium of Kleinschuster et al. (1998). The initial seeding density was 10^4/ml and the cultures were maintained at 23°C and were harvested at 7 d (168 h) while still in exponential growth phase. The QPX cells were enveloped by a heavy mass of mucoid secretion, which was routinely washed off the cells by dilution with a saline solution, IO (25 ppt, Instant Ocean®, Aquarium Systems Inc.; Mentor, OH), followed by repeated centrifugations (300 × g, 10 min, 21°C, ×3). Washed QPX (wQPX) were >90% viable by the trypan blue exclusion assay (Hanks & Wallace 1958) and almost immediately resumed mucus secretion. The numbers of QPX cells in particular cultures and cell numbers required for subsequent experiments were quantified spectrophotometrically using a standard curve of the numbers of

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wQPX (as determined in a hemacytometer) as a function of their absorbance at 560 nm.

C9G

Another thraustochytrid, C9G, closely related to QPX (Anderson et al., in press) was isolated from gill tissues of Canadian *M. mercenaria* and provided by Mr. G. S. MacCallum and Dr. S. McGladdery, Gulf Fisheries Center, Moncton, Canada. Like QPX, C9G was maintained in the medium of Kleinschuster et al. (1998) at 25°C and subcultured at 7 d.

M. mercenaria *Plasma*

M. mercenaria, collected from the Ware River, VA by a commercial supplier, were maintained with recirculating water (25 ppt, IO, 11°C). Hemolymph samples were withdrawn by syringe from an adductor muscle hemolymph sinus and held on ice in polypropylene tubes. The hemocytes were centrifuged out of suspension (300 × g, 10 min, 4°C). The pooled supernatant (plasma) was sterilized by filtration (0.2 μm pore size), and assayed for protein content (BCA kit, Pierce Co., Rockville, IL). Individual plasma samples from three to four hard clams were pooled and were frozen (~20°C) in aliquots. The frozen samples were used soon because the QPX-modulatory activity declined after ~2 mo in storage. In one series of experiments, plasma was heat-treated by exposure to 65°C for 10 min, the plasma was cooled to room temperature (~25°C) before use.

Immediate Exposure of Thraustochytrids to Plasma

QPX cells from 7d cultures were washed, as described above, and resuspended (2.5 × 10⁵/ml) in 25 ppt IO. Plasma protein concentration was standardized (usually to 0.2 mg/ml) by dilution with IO and serial dilutions prepared. Replicate culture flasks for each protein concentration tested were prepared with experimental (1.9 ml Kleinschuster's minimal essential medium (KMEM), 0.1 ml QPX suspension, and 0.5 ml plasma dilution), control (1.9 ml KMEM, 0.1 ml QPX suspension, and 0.5 ml IO), and the necessary blanks. After 7 d incubation at 24°C, the contents of each flask were removed, and the QPX washed thoroughly and quantified, as described previously. In related experiments, QPX or C9G were incubated for 2 h in IO containing plasma, washed, and resuspended in KMEM. Percent inhibition was determined using the following formula:

$$\% \text{ inhibition} = 1 - \frac{\text{experimental value}}{\text{control value}} \times 100$$

Delayed Exposure to Plasma

In the delayed exposure experiments, wQPX were permitted to incubate in KMEM for various time intervals ≤24 h before exposure to 40 μg/ml *M. mercenaria* plasma proteins. The QPX cells resumed typical secretory activities during these pre-exposure periods, as seen by microscopic examination. This plasma protein concentration was selected because it had been shown in previous immediate exposure experiments to inhibit ~95% of the growth of QPX cultures.

Viability Assays

QPX viability tests were carried out using viability/cytotoxicity kit #1 (Molecular Probes, Eugene, OR). The test is based on the differential permeability of live and dead cells to a pair of fluo-

rescent stains. Cell populations exposed simultaneously to both dyes become differentially stained: live cells are stained green and dead cells appear red. This assay was used to check wQPX viability after exposure to IO or *M. mercenaria* plasma.

RESULTS

Effects of M. mercenaria *Plasma on Washed QPX*

At the lower plasma concentrations tested, inhibition was low and variable, with some pools actually stimulating growth (Fig. 1). However, at plasma protein concentrations ≥10–50 μg/ml, a dose-dependent inhibition was consistently recorded (~100% inhibition was seen at >50 μg/ml). The inhibitory EC_{50} was calculated to be ~19 μg/ml. When this procedure was carried out with heat-treated (65°C, 10 min) plasma, the stimulatory effects of the lower concentrations were not evident (Fig. 2). The inhibitory EC_{50} for heated plasma was ~32 μg/ml; therefore, this heat treatment only partially inactivated (~40%) the growth inhibitory factor(s).

The inhibitory effects of *M. mercenaria* plasma were exerted in a short period. When wQPX were exposed to 40 μg/ml plasma for 2 h, washed free of plasma and cultured for 7 d in plasma-free medium, the resultant QPX cell numbers were 80.7 ± 13.3% (n = 3) reduced as compared with untreated controls. A similar degree of inhibition (94.3 ± 5.1%, *n* = 4) was seen when 40 μg/ml plasma was left in the medium for the entire duration of the assay. No significant difference was found between these means by way of a 2-tailed, unpaired *t*-test. The inhibition produced by 2-h exposure of wQPX to 40 μg/ml plasma protein did not result from QPX-cidal activity. Plasma-treated and untreated wQPX were similar (treated: 94.0 ± 1.7%, *n* = 3; and untreated: 94.0 ± 3.0%, *n* = 3 viable). A degree of specificity for *M. mercenaria* plasma is also indicted because exposure of wQPX to 40 μg/ml produced >90% inhibition, whereas under the same conditions, C9G was minimally inhibited (Fig. 3).

Reactions of M. mercenaria *plasma with mucus-enveloped QPX*

The typical response obtained by exposing wQPX immediately to *M. mercenaria* plasma (Fig. 1) was not seen after comparable
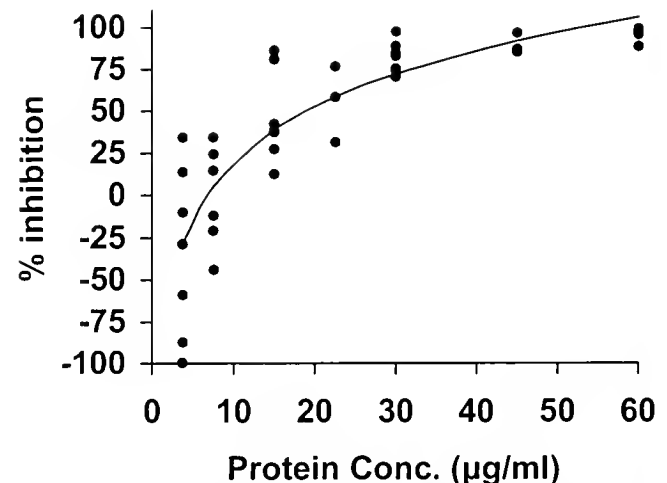
Figure 1. QPX-modulatory activity of *M. mercenaria* plasma expressed as percent inhibition of cultures after 7 d incubation. Final plasma protein concentration in the medium is indicated. Linear regression (y = 111.3[log x] – 92.31; r² = 0.7497) of log-transformed concentrations was used to calculate the inhibitory EC_{50} = 18.99 μg/ml.
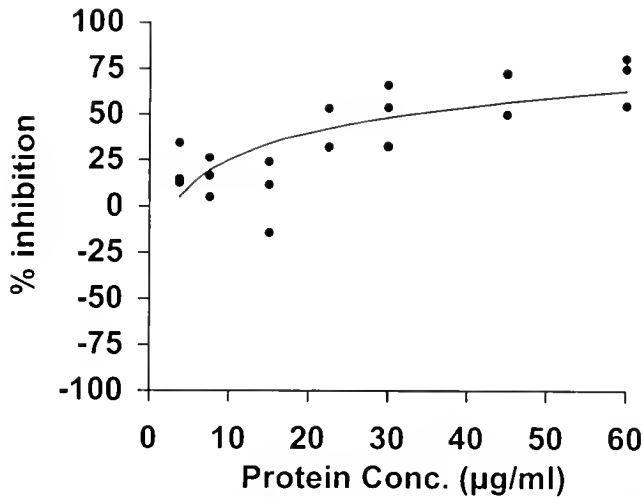
Figure 2. QPX-modulatory activity of heat-treated (65°C, 10 min) $M.$ $mercenaria$ plasma expressed as in Figure 1. Linear regression (y = 48.22[log x] – 22.71; r^2 = 0.6715) of log-transformed concentrations was used to calculate the inhibitory EC_{50} = 32.20 μg/ml.

Figure 4. Growth of wQPX preincubated for 24 h in plasma-free medium before exposure to 40 μg/ml $M.$ $mercenaria$ plasma. The QPX cells continuously secreted mucoid material during the preincubation period.

exposure of wQPX that was incubated for 24 h before the addition of plasma (Fig. 4). The lowest dose tested (3.75 μg/ml) apparently produced some inhibition, whereas all other doses (≤60 μg/ml) seemed to stimulate the QPX cultures. The apparent inhibition produced by the lowest concentration tested was not significantly different from zero (P > 0.05, one sample t-test, 2-tailed). The higher concentrations tested were all stimulatory. (P > 0.05, one sample t-test, 2-tailed). wQPX cells were either immediately exposed to a highly inhibitory plasma concentration (40 μg/ml) or allowed to incubate in plasma-free medium for 2–24 h before exposure; in these delayed exposure experiments, a time-dependent linear decrease in growth inhibition was observed (Fig. 5). Unlike QPX, C9G cells in culture secreted no mucoid material visible in preparations examined under the microscope. Preincubation of washed C9G cells for 24 h before exposure to 40 μg/ml plasma had no significant protective effect as compared with cells immediately exposed.

DISCUSSION

When wQPX cells were introduced into media containing various concentrations of $M.$ $mercenaria$ plasma, their subsequent growth was altered according to plasma concentration. This may be seen in Figure 1 where 7 d QPX culture growth was often stimulated in the presence of low plasma levels but consistently suppressed at >10 μg/ml. These effects could be explained by the presence of two QPX-modulatory agents in the plasma. Stimulation at low protein levels might be caused by a factor with high QPX-affinity and low to moderate activity. The effect of this stimulator would be lost at higher protein levels if a low QPX-affinity, higher activity inhibitor were present. The presence of two growth modulators was also suggested by the differences in thermal sensitivity (Fig. 2). Heat treatment of 65°C for 10 min seemed to eliminate all stimulatory activity; however, the inhibitory effects persisted with somewhat reduced activity. The growth modulating activity of $M.$ $mercenaria$ plasma takes place rapidly after interaction with wQPX. If wQPX was exposed to an inhibitory concentration of plasma (40 μg/ml) for 2 h, and then washed free of plasma proteins before growing the culture in plasma-free medium, culture growth was inhibited to about the same extent because it would have been if the cells had been continuously ex-

Figure 3. The effects of 40 μg/ml $M.$ $mercenaria$ plasma proteins on growth of 7 d cultures of QPX and C9G, a closely related thraustochytrid also isolated from hard clams. The protists were exposed to the plasma for 2 h, washed, and cultivated (7 d) in KMEM. Mean percent inhibition and standard deviations are indicated.
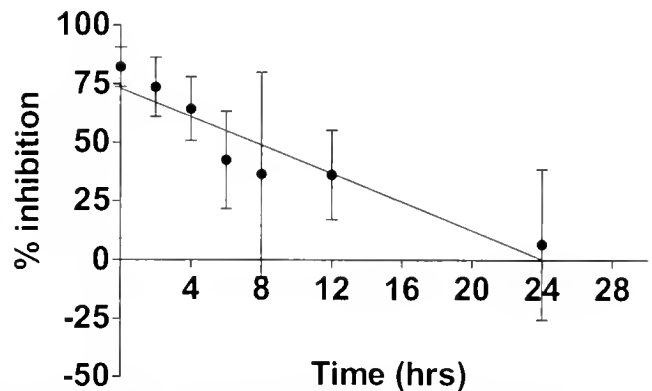
Figure 5. Effects of length of wQPX preincubation before exposure to 40 μg/ml $M.$ $mercenaria$ plasma. Mean percent inhibition and standard deviations are indicated.

posed to 40 μg/ml plasma. These experiments could not establish whether the inhibitory effects produced by *M. mercenaria* plasma on the cell density of 7 d QPX cultures were caused by growth inhibition or by cidal activity. Direct killing was ruled out by the fact that 40 μg/ml exposed, (potentially highly inhibited) wQPX and unexposed wQPX were ~95% viable.

Figure 3 presents evidence that the QPX-inhibitory plasma factor shows target specificity; C9G growth was hardly affected by 40 μg/ml. Sequence analysis of C9G placed it in the thraustochytrid phylogenetic group as a sister taxon to *Thraustochytrium pachydermum*, and these sequences were grouped with QPX with a parsimony jackknife support value of 100 (Anderson et al. in press). Clearly, QPX sensitivity to low (≤40 μg/ml) plasma concentrations exceeds that of C9G; however, C9G growth was inhibited (~60%) by exposure to ≥180 μg/ml plasma (Anderson et al. in press). Because the pathogenicity of C9G for *M. mercenaria* has yet to be established, it is not known whether inhibition differences caused by clam plasma between QPX and C9G reflect differences in pathogenicity.

Incubation of wQPX in plasma-free medium allowed the cells to resume mucus secretion. The cells underwent minimal division for the first 48 h in culture, then proceeded to grow with a doubling time of ~3 d (QPX growth curve not shown). The wQPX cells were suspended in a loose gelatinous mass by 24 h. This mucoid secretion often infiltrated the entire culture medium by 7 d in culture. When the cells were permitted to develop their mucoid covering for 24 h before the addition of plasma (Fig. 4), concentration of ~7–60 μg/ml failed to inhibit QPX growth in 7 d cultures. Unexpectedly, the lowest concentration tested (3.75 μg/ml) seemed to have inhibitory activity, but the mean of these experimental values were not significantly different from zero. These data suggested that the mucus material might protect QPX from *M. mercenaria* humoral defense mechanisms such as antimicrobial factors. This hypothesis was supported by the results of the delayed exposure experiments, where protection from growth inhibition was dependent on the time of incubation before exposure to 40 μg/ml plasma protein (Fig. 5). Because QPX cells in clam tissues are typically enveloped by mucus, a role of this secretion as a virulence factor seems likely. This is supported by a recent report that clams injected with wQPX did not develop infections or disease (Smolowitz et al. 2001).

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A PORTABLE AND PRACTICAL METHOD TO MONITOR BIVALVE FEEDING ACTIVITY IN THE FIELD USING TIME-LAPSE VIDEO TECHNOLOGY

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ABSTRACT We developed a simple method to measure feeding activity of *Mytilus edulis* using a camcorder placed inside an underwater housing, a plastic frame for holding mussels and time lapse videography. Exhalant siphon area, indicative of feeding activity, was monitored in laboratory mussels exposed to filtered seawater and various concentrations of microalgae, including *Pavlova lutheri* or *Tetraselmis suecica*. Exhalant siphon area increased as algal concentration increased from zero to ~25–30 × 10³ cells ml⁻¹, but declined again at higher concentrations. Advantages of this method include portability and relatively low cost, high resolution of data over short and long temporal scales, potentially large sample sizes, and minimum logistics required for deployment in a variety of different environments. Once relationships between exhalant siphon area and other indicators of feeding such as filtration rate have been established, this method could greatly improve our understanding of bivalve feeding *in situ* and how they respond in dynamic natural conditions.

KEY WORDS: *Mytilus edulis*, bivalve feeding, time-lapse recording, exhalant siphon area, particle concentration

INTRODUCTION

There have been numerous studies on measuring feeding activity in a variety of suspension-feeding bivalves over the last several decades. There has recently been much discussion and debate on whether or not bivalves have the capability of physiological regulation or are pumping at full capacity all the time (Jorgensen 1996, Bayne 1998, Hawkins et al. 2001). This includes numerous comments on the proper interpretation of the published literature and diverse opinions on the reliability of some of the methods used (Cranford 2001, Riisgård 2001, Widdows 2001).

One such method considered to have good potential for assessing feeding activity remotely with little interference by the observer and minimal disturbance to the bivalve is the estimation of valve gape and siphon area in mussels (Newell et al. 2001). Positive relationships have been reported between pumping rates of mussels, valve gape and the exhalant siphon area (Jørgensen 1960, Riisgård & Randløv 1981, Famme et al. 1986, Jørgensen et al. 1988, Jorgensen 1990) and between exhalant siphon area and mussel filtration rates (Newell et al. 2001).

Filtration rates of mussels have been shown to be linked to particle concentration with low levels observed for filtered water but increasing with natural levels of seston before decreasing again at higher seston loads (Foster–Smith 1975, Winter 1973, Bayne 1993). Riisgård and Randlov (1981) found comparable reductions in filtration rates and valve gape of blue mussels at densities of *Phaeodactylum tricornutum* lower than 1,500 cells ml⁻¹ and higher than 30,000 cells ml⁻¹. Newell et al. (2001) found a similar apparent threshold for the filtration response to particle concentration to occur at 2,000–6,000 particles ml⁻¹ in a flume environment. Dolmer (2000a, 2000b) observed that high algal concentrations may lead to decreases in valve gape as well as estimates of filtration in the field.

There is ample evidence to suggest that exhalant siphon area is a useful indicator of feeding activity in mussels and it is responsive to variations in the concentration of suspended particles. The purpose of this study was to develop a portable and reliable method to remotely estimate exhalant siphon area for numerous undisturbed mussels simultaneously. It would be particularly advantageous if the method could be deployed to the field where mussel response could be continuously evaluated while natural seston and flow conditions are monitored. The combination of time lapse capabilities and high resolution image of a digital camcorder, a portable underwater housing, a plastic frame for holding mussels, and readily available image analysis software provides an effective tool for studying mussel feeding activity. Exhalant siphon area was monitored in this study in mussels exposed to various concentrations of cultured microalgae in the laboratory environment.

MATERIALS AND METHODS

Mussels (*Mytilus edulis* Linnaeus 1758) were collected from an inlet in the Pasamoquoddy Bay, New Brunswick and transported to University of New Brunswick in Saint John, New Brunswick, Canada. Mussels were acclimated to laboratory conditions for a minimum of 2 d and a maximum of 7 d. Experiments were performed in a 530 l (244 cm long, 66 cm wide, and 33 cm deep) tank with well mixed recirculating seawater, flowing approximately 5–10 cm s⁻¹. Experiments were performed in full room light and temperature and salinity were maintained at 12°C and 35–36‰, respectively. Water was pre-filtered in the tank with three inline filters of 20, 5, and 1 μm. Mussels were exposed to filtered seawater and cultured microalgae ranging in initial concentration from 5,000–85,000 cells ml⁻¹ while siphon area was monitored over periods of hours using time-lapse videography. Mussels were exposed to experimental conditions for 30–60 min prior to measurements to ensure feeding activity had resumed. With a few exceptions experiments for each series of mussels typically ran for 2–4 h to ensure a good time series of measurements and a detectable change in particle concentration. Algal concentration was measured using an electronic particle counter (Coulter Multisizer II) with a 100 μm tube orifice diameter. Algal diets provided in experiments were one of *Pavlova lutheri* (Provasoli–Guillard CCMP1325) or *Tetraselmis suecica* (Provasoli–Guillard CCMP904) or a mussel spat formula of *Nanochloropsis oculata*, *Chaetoceros*-B, and *Phaeodactylum tricornutum* (Innovative Aquaculture Products Ltd.).

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At least one day prior to the experiments Velcro was attached to the mussel shell using cyanoacrylate cement and, after drying, mussels were attached to individual plastic posts also covered in velcro. The posts containing the mussels were secured to a plastic plate and attached to a frame connected near the lens of a video recording device (Fig. 1A). The number of mussels observed (usually 9–12 adults) in the video frame depended on the size of the mussels and the efficiency of arranging mussels to adequately view the external siphon. A Sony Mini DV (model DCR-TRV900) three ccd camcorder was enclosed in an Amphibico 900 underwater housing and set to an interval recording mode of 2 s every 30 s over the entire period of each experiment to capture siphon activity.

Multiple images from the mini DV tapes were collected using the photo feature of the camcorder and stored on memory cards before being transferred to a personal computer (Fig. 1B). Variation in siphon area was estimated for individual mussels using the program Image J (NIH public domain Java image processing program—URL: http://rsb.info.nih.gov/ij). Siphon area was calibrated using a 1 cm mark on the mussel posts. The inherent variation in measuring exhalant siphon area was 2.4–3.8%. To standardize individual responses for different sizes of mussels to different algal concentrations, exhalant siphon area data were converted to percent of maximum values observed for each mussel.
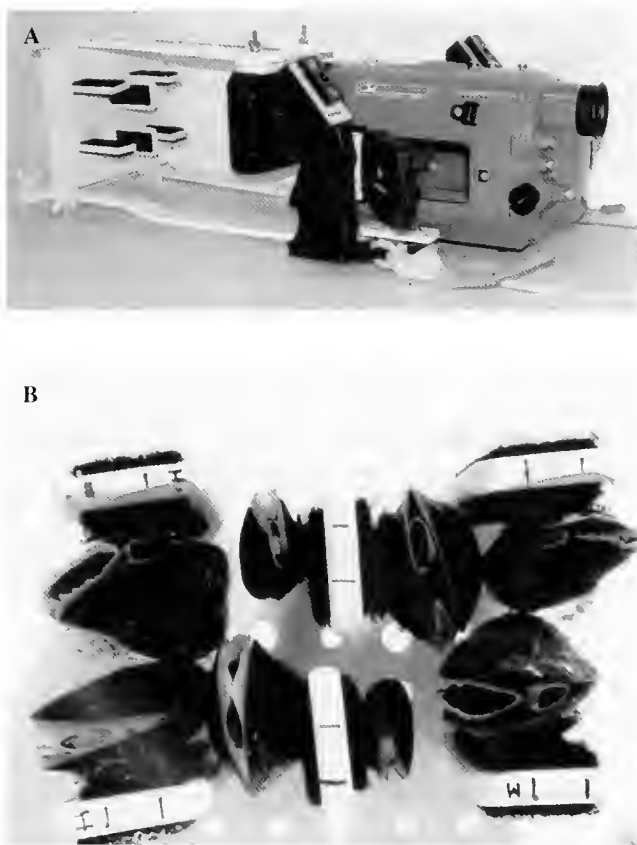
Figure 1. (A). An adjustable plastic frame attached to the front of an underwater video housing containing a high resolution camcorder with time-lapse capabilities. Mussels are secured with Velcro to moveable posts inserted into a plate positioned in front of the video lens. (B) A typical black and white photo made from a video frame captured from the mini DV tape. Exhalant siphons are clearly visible for several mussels simultaneously.

RESULTS

There was a consistent decline in algae over time in all the experiments, indicating removal of microalgae by the mussels in the course of the experiments (Fig. 2). Exhalant siphons were opened, confirming feeding activity by the mussels. The fitted lines for the uptake rates of algae typically had r^2 values exceeding 0.90–0.95 in all examples.

The percent maximum exhalant siphon area in individual mussels exposed to filtered seawater (no algae) was consistently lower than the siphon areas reported for the same mussels exposed to microalgae (Fig. 3A). A similar trend of greater exhalant siphon area was also observed for groups of mussels exposed to different concentrations of microalgae compared to those held in filtered seawater (Fig. 3B). Note that mussel exhalant siphon area was still approximately 20–30% of the maximum when exposed to filtered seawater.

The percent maximum exhalant siphon area in mussels increased with increasing particle concentrations to a maximum of near 90–95% at concentrations approaching 25–30,000 cells ml^{-1} (Fig. 4) Further exposure to concentrations above 30,000 cells ml^{-1} resulted in a decline in percent maximum exhalant siphon area.

DISCUSSION

By modifying an underwater housing and combining it with a high resolution camcorder capable of time-lapse videography we have developed a simple and relatively inexpensive method to remotely study bivalve feeding behavior. There have been other devices developed to remotely monitor bivalve activity but, for various reasons, they have not been readily adopted by scientists working on bivalves. This includes The Musselmonitor® developed as a biological early warning system containing sensors to record shell opening and closing while mussels are exposed to various pollutants (Baldwin & Kramer 1994). Manuel and Lobsiger (1999) developed the MarineCanary™ as a biomonitoring tool using an underwater camera and a time-lapse system to assess the marine environment through changes in bivalves' valve gape and mantle activity.

Using this new method we have established a positive relationship between exhalant siphon area and the concentration of cultured microalgae, also observed by Newell et al. (2001) in their study. Feeding activity is this study was confirmed by the continuous decline in the concentration of microalgae in the experi-
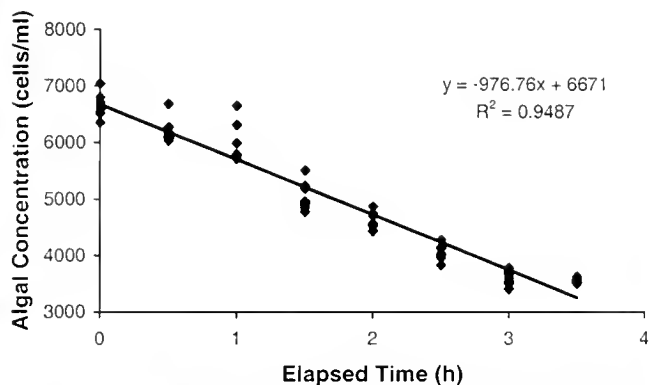
Figure 2. An example of variation in declining algal concentration, attributable to mussel feeding, during a typical medium—low concentration experiment.
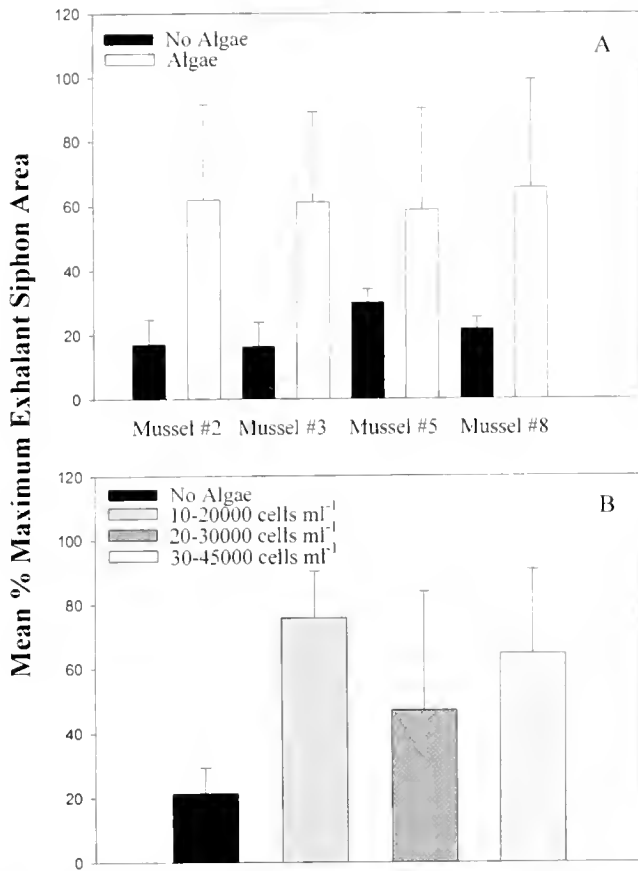
Figure 3. (A) Variation in individual mean percent maximum exhalant siphon area of representative mussels held in filtered seawater and exposed to microalgae in different algal concentrations (5–45,000 cells ml⁻¹). (B) Mean response for groups of mussels exposed to filtered seawater and three different experimental concentrations of microalgae (5–45,000 cells ml⁻¹). Values are means ± 1 SE.

Algal concentration (cells ml⁻¹)

Figure 4. Variation in percent maximum exhalant siphon area of mussels exposed to different concentrations of microalgae. The closed diamond represents an experiment where 8 mussels were subjected to algal concentrations from no algae to 45,000 cells ml⁻¹; the open circle, 13 mussels subjected to algal concentrations of 0–85,000 cells ml⁻¹; the open triangle, 8 mussels subjected to algal concentrations of 0–17,000 cells ml⁻¹. Values are means ± 1 SE.

mental tanks (Fig. 2). The shape of the line when fitted to semi-log transformed data (i.e., rate of clearance) was comparable to the reduction observed by Riisgård (1991) when *Mytilus edulis* was grazing on *Rhodomonas baltica*. The positive relationship between particle concentration and exhalant siphon area was apparent until concentrations reached 25,000–30,000 cells ml⁻¹ and exhalant siphon area appeared to decrease with further increases in concentration (Fig. 3B). Clausen and Riisgård (1996) also observed that mussels partly closed their valves and reduced the opening of the exhalant siphon at high algal concentrations but they found this reduction to occur at around 13–24,000 cells ml⁻¹. Note that we did observe some moderately high values for siphon area for mussels at the highest algal concentrations. This may have been an artefact of the experimental design where a group of starved mussels were exposed initially to very high concentrations of microalgae.

There are several advantages to the time-lapse videography method for the observation of feeding activity in bivalves. This includes its size, cost, portability and readily available components including public domain software. A variety of underwater housings are available today for most commercial camcorders capable of using time-lapse technology. Because of the small size of the housing, they can be placed unattended in a wide variety of habitats for extended periods of time—up to 10–15 hours with the new

generation of long-life batteries. Short-term bivalve feeding responses will be estimated more accurately *in situ* by monitoring their activity continuously and uninterrupted rather than relying on measurements at regular intervals or convenient points in time. It is not necessary, as with more traditional methods to measure feeding activity, to confine the bivalve in any kind of experiment chamber, which may facilitate measuring the change in particle concentration over time but exposes the bivalve to unrealistic flow conditions. Barrington et al. (2002) have successfully used this method to compare feeding activity in mussels held near salmon cages to mussels held in adjacent reference sites. We have observed between 8 and 12 mussels simultaneously, an obvious advantage for sampling rate and statistical power over methods that observe a single bivalve at a time. However, there exists a trade-off between the number of mussels that can be observed and the resolution of the siphon area for individuals obtained from the video tape.

Filtration rate by mussels is a function of pumping rate, particle concentration and filtration efficiency, such that control over pumping rate is viewed as a major factor contributing to energy acquisition by bivalves. As any one of these factors changes, there may be an uncoupling between exhalant siphon area and filtration rate. In order for this method, or any other method that measures exhalant siphon area, to be used to estimate a rate of feeding the variation in relationships between exhalant siphon area and filtration or pumping rate must be established in future studies. We are proposing that this method, using a camcorder in an underwater housing, a plastic frame for holding mussels and time lapse videography, is a practical and potentially useful tool to address many questions on how bivalves respond, in real time, to changes in a naturally dynamic environment.

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PARALYTIC SHELLFISH TOXINS IN PUGET SOUND, WASHINGTON STATE

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ABSTRACT The first illnesses and only deaths in Washington State resulting from paralytic shellfish poisoning were documented in the 1940s, resulting in the establishment of one of the longest monitoring programs for paralytic shellfish toxins in commercial and recreational shellfish in the United States. An analysis of the Washington Department of Health's monitoring data for the Puget Sound area has allowed us to examine temporal changes in shellfish toxin levels and geographical distribution of shellfish harvesting closures. The values of toxins in shellfish were normalized to control for variable levels of toxin accumulation in different shellfish species by dividing individual values by the yearly average for a given species. These normalized values increased significantly over the past five decades, indicating that the observed increase in paralytic shellfish toxin levels in Puget Sound shellfish was not caused by the shift in species monitored. A geospatial map of the first shellfish closures or paralytic shellfish-poisoning event in each Puget Sound basin suggests that over time, toxigenic *Alexandrium* cells have been transported from northern to southern Puget Sound. Shallow sills that restrict the exchange of water between adjacent basins have hindered the transport of toxic dinoflagellates, especially because these cells generally do not prosper in mixing conditions that are characteristically found at sills. Large-scale events, such as the bloom that occurred in the Whidbey and Central basins in 1978, may have been induced by global climate changes or shifts, such as the Pacific Decadal Oscillation. Although greater numbers of closures have been observed over time in basins of Puget Sound, closures as a percentage of total samples analyzed have decreased or remained constant in all basins, indicating that the Washington Department of Health has established an effective monitoring program to protect public health while allowing for maximum harvest potential.

KEY WORDS: paralytic shellfish poisoning, saxitoxin, Puget Sound

INTRODUCTION

Background

Paralytic shellfish poisoning (PSP) is an acute illness in humans caused by eating bivalve shellfish (e.g., mussels and clams) that have ingested dinoflagellates that produce neurotoxic compounds. The dinoflagellate, *Alexandrium catenella* (Whedon and Kofoid) Balech, previously described as belonging to the genus *Gonyaulax* Whedon and Kofoid or *Protogonyaulax* Taylor, has been identified as the primary causative organism on the west coast of North America, but recent evidence indicates that at least five known species of *Alexandrium* can produce PSP toxins (PSTs) in Northwest waters (Horner et al. 1997). These dinoflagellates occur either as single cells or as chains of cells. Their two flagella enable them to vertically migrate to the surface during the day and to depth at night, giving them advantages over nonflagellated phytoplankton. Generally, dinoflagellates thrive in stratified water because of their motility and ability to move to nutrient-rich areas within the water column. When conditions for growth become less favorable, *A. catenella* cells form resting cysts that settle to the sediments, where they await the return of favorable growth conditions (Anderson 1980).

Historically, PSP has been known in the Pacific Northwest and Alaska for centuries. Records of PSP events date back as early as June 15, 1793 (Vancouver 1798), when a member of Captain George Vancouver's exploration team died after eating contaminated mussels harvested in the uncharted coastline of what is now known as British Columbia. In 1799, 100 Russian hunters died after consuming toxic mussels near Sitka, Alaska (Halstead 1965). The first recorded outbreak of PSP on the eastern shore of Vancouver Island, Canada, in October 1957 caused serious illness in a number of people (Waldichuk 1958) and resulted in a mandatory monitoring program for PSTs in Washington State.

The PSTs include saxitoxin and at least 12 structurally related chemical compounds (see, for example, Baden 1983). The record level of PSTs in shellfish ever measured along the Pacific coast of North America was 31,000 μg saxitoxin equivalents (STXeq)/100 g shellfish in October 1989 in the inside passage of British Columbia, just north of the US and Canadian border (Bricelj & Shumway 1998).

PSP in Washington State

The Washington State Department of Health (WDOH) initiated a shellfish toxicity surveillance program in the early 1930s (Lilja 1978) as a joint effort between WDOH and the George Williams Hooper Foundation for Medical Research in San Francisco. This initial monitoring by WDOH focused on commercial shellfish and was expanded to include recreational shellfish in the early 1990s when the Puget Sound Water Quality Authority gave WDOH the authority to monitor recreational species. Washington State's only three fatalities due to PSP were recorded in 1942 (Quayle 1969) near the entrance to the Strait of Juan de Fuca (Fig. 1). Since then, the Washington Department of Fisheries has imposed annual harvesting closures for all shellfish except razor clams from April 1 to October 31 in the area west of Dungeness Spit (near Port Angeles, WA; Fig. 1) along the Strait of Juan de Fuca and southward along the coast to the Columbia River (Nishitani & Chew 1988). In general, razor clams do not retain high levels of PSTs but are now known to accumulate domoic acid (Wekell et al. 1994). The shellfish surveillance program in Washington State was terminated in 1946 when it was believed that this seasonal closure was effectively protecting public health. In June 1957, PST monitoring was reestablished to include all species of commercial shellfish in areas of north Puget Sound and the outer coast after WDOH was advised

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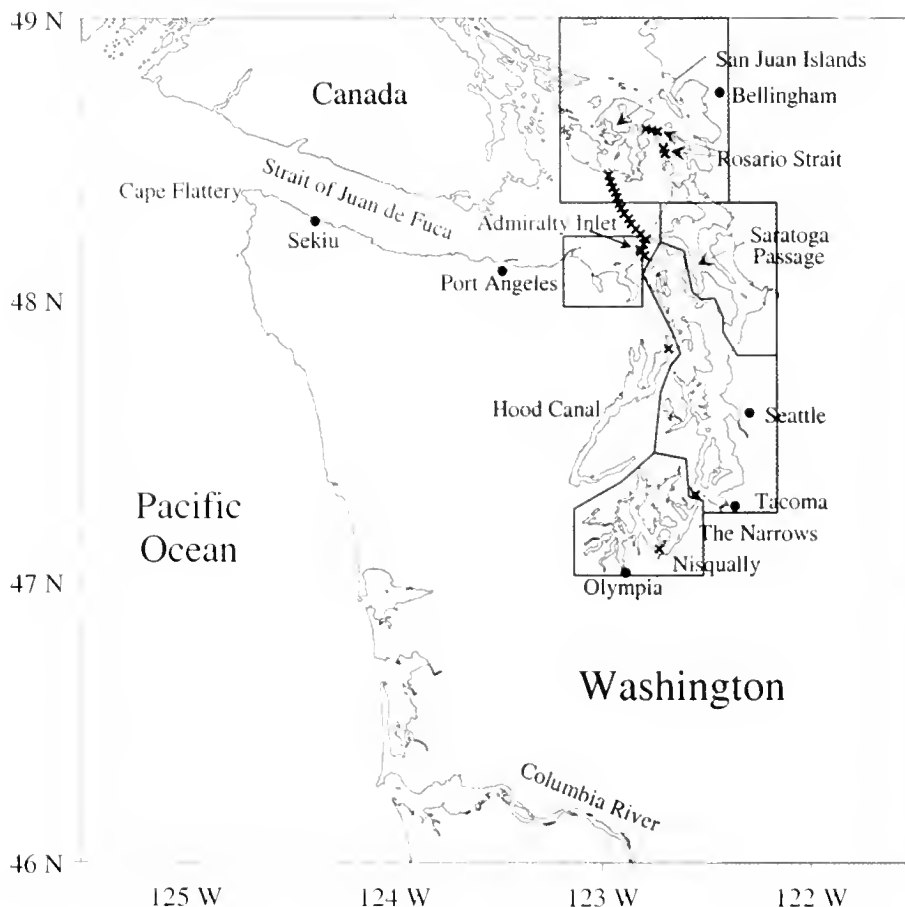
Figure 1. Map of western Washington. Puget Sound basins described in the text are outlined. Locations of sills less than 70 m deep are noted by Xs.

of the prevalence of PSTs in British Columbia shellfish. The monitoring of recreation and sport harvesting on the outer coast and in Puget Sound was sporadic until the early 1970s, when closures caused by PSTs in shellfish above the FDA regulatory limit of 80 μg STXeq/100 g shellfish occurred in the Bellingham area (Fig. 1) for the first time.

Physical Oceanography

Puget Sound is a complex fjord made of several distinct environments that are each influenced by different forces and conditions, including river runoff controlled by dams, free flowing rivers that undergo flooding due to snow-melt or heavy mountain rain and tidal flushing (Strickland 1983). Because of these distinguishing environmental factors, Puget Sound can be partitioned into a series of basins or environments using the descriptions and chart developed by Strickland (1983). The North basin extends from the Canadian border and includes the Strait of Georgia, San Juan Islands and Samish bay. In the North basin, the San Juan Islands are partially bounded from the Northwest basin by a sill at their southern edge (Fig. 1). In addition, the waters in Bellingham Bay are partially separated from the San Juan Islands by sills in the Rosario Strait. The Northwest basin is comprised of two semi-enclosed bays, Sequim and Discovery bays, with oceanic influence from the Strait of Juan de Fuca. This basin has the longest recorded history of PSTs in the Puget Sound with frequent blooms of varied intensity and duration. The Central, Whidbey, and South basins

are partially bounded from the Strait of Juan de Fuca by a sill at Admiralty Inlet to the north and west (Fig. 1). The Whidbey basin is relatively shallow and strongly influenced by high volumes of fresh water from the Skagit River, controlled by a series of hydroelectric dams on its upper reaches. The Central basin fronts the high population center of Seattle and contains the deepest waters of Puget Sound. While the Central basin receives fresh water inputs from a number of rivers to the north and south, the volume of its salt water mass is enormous compared with the other basins. Its circulation is influenced by sills at both the northern (Admiralty Inlet) and southern (Tacoma Narrows) ends. The sill at Tacoma Narrows also borders the South basin that extends to the southernmost reach of Puget Sound as a series of small, finger-like shallow fjords. The eastern and western finger inlets of south Puget Sound are believed to be two dynamically distinct water bodies with separate circulation (Ebbesmeyer et al. 1998). The primary freshwater influence in the South basin is the Nisqually River, fed by melting snows from Mt. Rainier and the surrounding mountain ranges. Currents in the South basin are strongly influenced by tides, due largely to the shallowness of this area. Finally, Hood Canal is partially isolated by a sill near its entrance that limits the transport of deep marine waters in and out of the canal (Burns 1985). Currents in Hood Canal are slow, perhaps because the basin is a closed-ended fjord without large volume rivers. It is the most poorly flushed of all inlets in Puget Sound (Strickland 1983), but the strongest currents tend to occur near the entrance at the north.

In summary, all Puget Sound basins are strongly influenced by fresh water input, resulting in density-dependent stratification, especially in the summer months.

The spread of PSTs into previously unaffected areas, such as south Puget Sound (Nishitani & Chew 1988) has raised an awareness of the significant and expanding threat to human health and economics of some of the most productive recreational and commercial shellfish regions on the US west coast. An analysis of PST data for the Puget Sound areas collected over the past five decades has allowed us to examine changes in PST levels and geographical distribution over the past five decades. This assessment will allow us to evaluate whether modifications of the current monitoring program or additional preventive measures are needed to effectively protect seafood consumers as well as assist aquaculturists.

METHODS

WDOH Database

Shellfish toxin data were provided by the WDOH Office of Food Safety and Shellfish Programs that routinely monitors PSTs throughout the state in both commercial and recreational shellfish. The data have been collected over a period of more than 40 y from samples submitted by commercial growers and local health agencies as required by federal and state regulations. In some cases, local health agencies have collected samples directly from beaches in their jurisdictions but have also relied on samples submitted by volunteers.

In the last 20 y, mussels have been selected as a sentinel species for PSTs because they bioaccumulate the toxins at a faster rate than other shellfish. However, in the early years of monitoring (1960–1980), Pacific oysters (*Crassostrea gigas*) and butter clams (*Saxidomus giganteus*) constituted the major species sampled for PSTs (Table 1). Since 1989, WDOH established a sentinel mussel-monitoring program (Nishitani 1990) in which the blue mussel, *Mytilus edulis*, generally was sampled; however, *M. galloprovincialis* and *M. californianus* were collected at a few Puget Sound sites (Determan 2000). At most sites, mussels were sampled every 2 wk during the year from wire mesh cages suspended about one meter deep below floats and docks. These cages were periodically restocked with mussels. About 100 mussels provided the 100 g of

tissue needed for toxin analysis. Mussels were collected, packed with frozen gel packs, and shipped to WDOH for analysis.

WDOH performed all testing for PSTs using the standardized mouse bioassay. The procedure has been modified since its inception in the 1920s by the inclusion of a saxitoxin standard provided by the US Food and Drug Administration (FDA), and expression of results in saxitoxin equivalents, STXeq (AOAC 1990). Early data from the 1950s and 1960s expressed as "mouse units," were converted to the newer designation by multiplying the mouse units (MU) by the factor 0.2. Thus 400 MU/100 g shellfish tissue is equivalent to 80 μg STXeq/100 g, the current "action level" specified by the FDA (AOAC 1990).

Data collected over the years by WDOH were not intended for establishing trends but rather were collected solely to protect the health of shellfish consumers. In other words, there was increased sampling during a toxic event, to characterize the extent and severity of the event, resulting in a greater proportion of tests that are positive for toxin. For the purpose of this study, we included all data for shellfish collected from 1957 through 1999. Blue mussels, butter clams, littleneck clams (*Protothaca staminea*), and Pacific oysters make up the largest number of samples analyzed (Table 1). During the 20-y period from 1957 to 1977, sampling by WDOH was relatively constant, averaging about 145 samples per year. After the record-breaking PST level measured in 1978 (30,360 μg STXeq/100 g), the agency increased its sampling.

Data Analysis

A shellfish toxin database was constructed from individual PST test data sets from the WDOH for each year from 1957 through 2000. These data sets were formatted and imported into a data table in Microsoft Access (Microsoft Corp, Bellevue, WA). The sample numbers that were assigned by WDOH were used as unique identifiers for each record. A table containing latitude and longitude coordinates along with sampling site descriptions was linked to the PST data table through a field containing a code that uniquely identified each sampling site. Similar methods were used to link tables containing common names for the samples and the names of the counties in which the sampling sites were located. Queries were constructed that allowed fields in any of the tables in the database to be searched.

RESULTS

Data Reduction for Trend Analysis

Sampling intensity throughout Puget Sound has been variable over the past 40 y, primarily because of budgetary constraints of the WDOH monitoring program. A variety of edible shellfish species with different toxin accumulation and retention capabilities was selected for monitoring purposes primarily because of availability. The major species used for monitoring in each basin since 1957 were oysters (North basin), littleneck clams (Northwest basin), blue mussels (Whidbey basin), littleneck clams (Central basin), and blue mussels (South basin; Table 2). These shellfish have different rates of accumulation and depuration of PSTs. For example, butter clams are known to retain high levels of toxin for months, whereas mussels are known to depurate toxins over a period of days (Bricelj & Shumway 1998). Additional variability in the data is caused by a disproportionate increase in sample size over time in certain basins relative to other basins. During recent decades, more reports of PSP illness, especially in south Puget

TABLE 1.

Number of shellfish samples collected by the Washington State Department of Health during each decade.

Decade	*Mytilus edulis*	*Saxidomus giganteus*	*Protothaca staminea*	*Crassostrea gigas*	Other*
1050s	4	127	20	169	69
1960s	0	208	146	362	157
1970s	649	1248	471	684	422
1980s	2361	3977	2712	2327	1773
1990s	9246	2498	4237	7078	779

*Other species (not all shellfish) include: *Cancer magister, Chione* sp., *Chlamys rubida, Clinocardium nuttalli, Crassadoma gigantea, Crassostrea sikamea, Ensis americanus, Fusitriton oregonensis, Haliotis kamtschatkana, Macoma nasuta, Macoma secta, Modiolus modiolus, Mya arenaria, Mytilus californianus, Mytilus galloprovincialis, Ostrea edulis, Ostrea lurida, Panopea abrupta, Parastichopus californicus, Patinopecten caurinus, Polinices lewisii, Tapes philippinarum, Tresus nuttallii.*

TABLE 2.

Shellfish collected by the Washington State Department of Health (1957–1999).

Basin	Total	*Mytilus edulis* (%)	*Saxidomus giganteus* (%)	*Protothaca staminea* (%)	*Crassostrea gigas* (%)	Other* (%)
North	10,175	18	19	11	31	21
Northwest	5,961	12	22	38	13	15
Whidbey	3,696	55	29	7	1	8
Central	13,673	25	25	26	8	16
South	5,644	43	4	4	33	16

* Other species (not all shellfish) include: *Cancer magister, Chione* sp., *Chlamys rubida, Clinocardium nuttallii, Crassadoma gigantea, Crassostrea sikamea, Ensis americanus, Fusitriton oregonensis, Haliotis kamtschatkana, Macoma nasuta, Macoma secta, Modiolus modiolus, Mya arenaria, Mytilus californianus, Mytilus galloprovincialis, Ostrea edulis, Ostrea lurida, Panopea abrupta, Parastichopus californicus, Patinopecten caurinus, Polinices lewisii, Tapes philippinarum, Tresus nuttallii.*

Sound, have required an increase in PST testing. The different sampling intensity as well as the shift in shellfish species collected over time has necessitated data reduction for the purpose of trend analysis. Because we examined the data for trends in PST activity, only samples having quantifiable levels ($\geq$32 µg STXeq/100 g) of PST by mouse bioassay were included. All the quantifiable PST data for San Juan Island shellfish are shown in Figure 2A. San Juan Island was chosen because one of the longest historical records in Puget Sound is available from this site. Data were simplified by showing only the highest annual level of PST (Fig. 2B). Averages per decade (Fig. 2C) of those maximum annual levels were calculated in all shellfish from the San Juan area from the 1950s to the 1990s. Finally, data were normalized to control for different rates of uptake and depuration of PSTs in all shellfish tested by dividing individual PST values by the average for that species. The maximum normalized PST values were determined for each year then averaged for the decade (Fig. 2D). When the normalized maxima per decade for the 1950s through 1970s were compared with the past two decades (1980s and 1990s), the more recent two decades were significantly higher (*t*-test, *P* < 0.001). The rise in PST values over the past several decades is clearly seen in Figures 2C and D.

PST in Basins of Puget Sound

A series of environmental factors such as the presence of bounding sills, river input, and unique bathymetry were used to divide Puget Sound into distinct basins (Strickland 1983; Fig. 3). Sites that show typical PST levels within a given basin were selected for this study upon recommendation by WDOH. Because central and south Hood Canal shellfish have remained essentially free of PSTs, this arm of water west of the Central basin was not included as part of this analysis. A summary of averages by decade of maximum PSTs in all defined basins in Puget Sound showed increasing magnitude of toxins in all shellfish monitored at all sites with the exception of Whidbey and Central basins (Fig. 3). In the North basin, Samish Bay had relatively low levels of PSTs during the past three decades, whereas San Juan Island and Georgia Strait had more intense toxic events with the average by decade of annual maximum levels increasing from the 1970s to the 1990s. In the Northwest basin we observed obvious increases in levels of PSTs in both Sequim and Discovery bays over several decades. In the Whidbey basin, PST levels remained relatively low, except for an anomalously high level of toxin (30,360 µg STXeq/100 g) in 1978 at Holmes Harbor. Levels of this magnitude had never before (and have not yet again) been observed in Washington State. Rec-

ord levels of shellfish toxin measured from Whidbey Island south to central Puget Sound in 1978 were responsible for the anomalous peaks seen in Holmes Harbor and Agate Pass in the 1970s (Fig. 3). In the Central basin, Quartermaster and Kilisut harbors, as well as
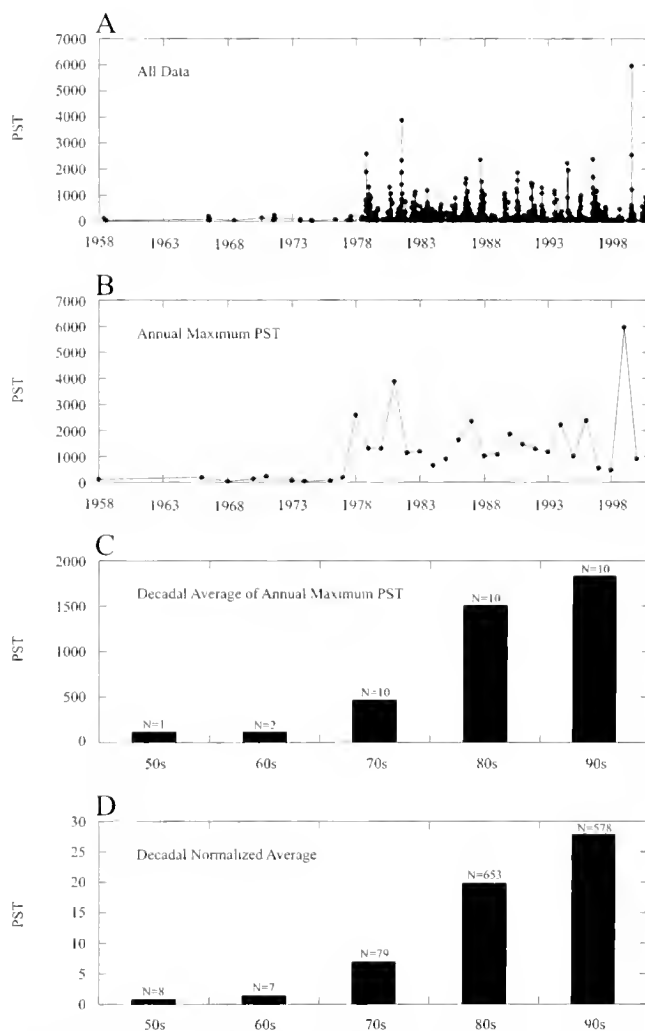
Figure 2. PST levels (µg STXeq/100 g) in all shellfish from San Juan Island, collected from Jan 1958 to Nov 1999. All data (A), maximum annual PST levels (B), and average per decade of annual maximum PST levels (C), and normalized average per decade of annual maximum PSTs (D) are shown.
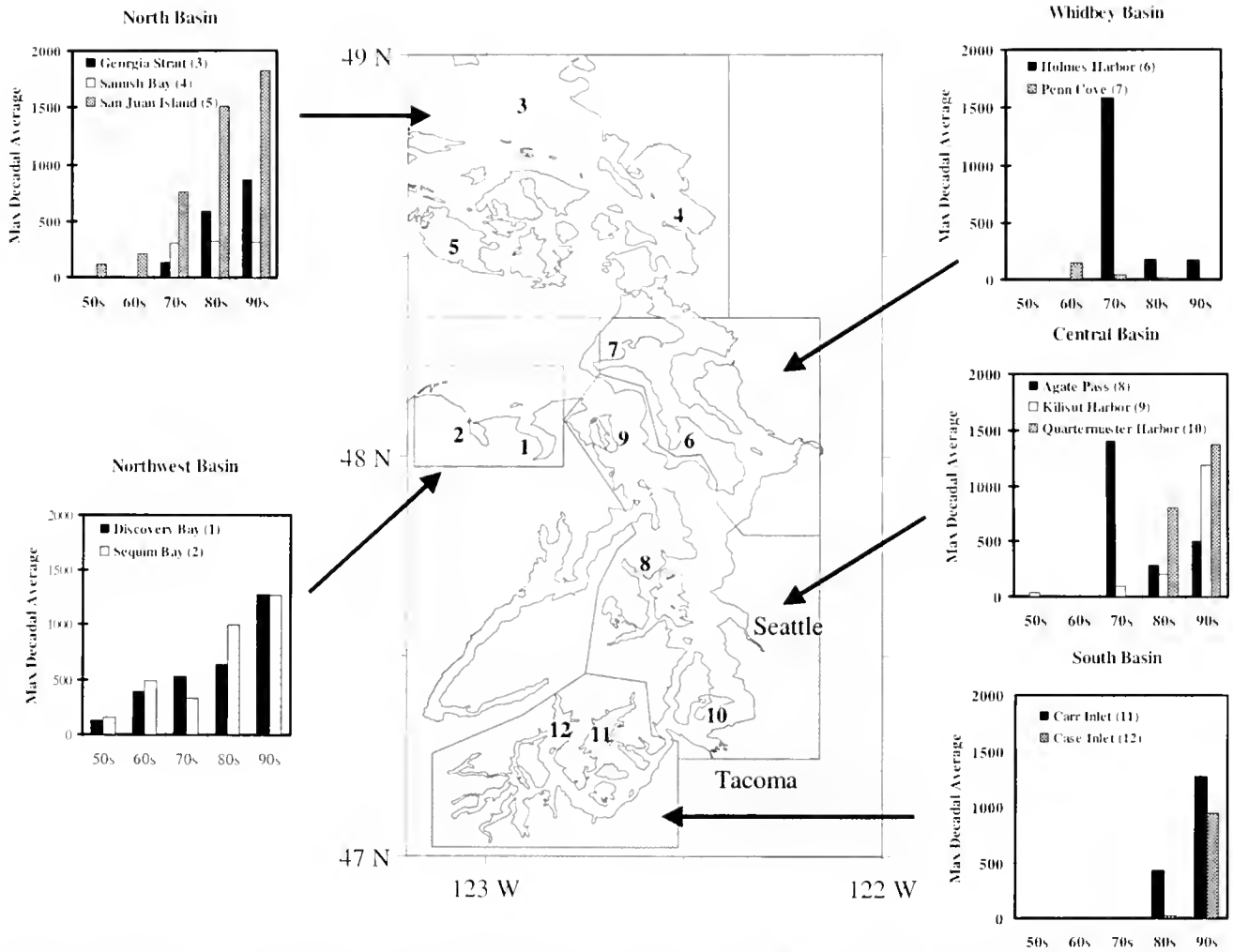
Figure 3. Averages per decade of maximum PSTs (µg STXeq/100 g) in each Puget Sound basin. The locations of representative sites in each basin are numbered.

Agate Pass, showed clear increases in average of annual maximum levels over the past two decades. In the South basin, PST levels have recently reached record highs. Carr Inlet had its first shellfish harvesting closures in 1988, although monitoring had been done at this site since 1957. Before 1988, PSTs had only occasionally been measured in the South basin but at levels below regulatory limit. Nearby Case Inlet had its first closure in 1991. Since the 1991 event, this area has experienced more frequent toxic events and

higher levels of PSTs, reaching a maximum of 13,769 µg STXeq/100 g in blue mussels in 2000.

Frequency of PST Closures

The frequency of PST closures over time in each Puget Sound basin is shown in Table 3. Although the number of samples collected over time has increased, closures as a percentage of total

TABLE 3.

Number of closures in Puget Sound basins, also as a percentage of total samples analyzed during each decade.

Decade	Northwest		North		Whidbey		Central		South	
	Closures	%	Closures	%	Closures	%	Closures	%	Closures	%
1950s	32	25	1	2	0	0	0	0	0	0
1960s	195	45	2	1	0	0	0	0	ND*	ND
1970s	227	27	260	20	165	39	109	18	0	0
1980s	610	34	827	22	119	7	912	23	238	22
1990s	387	14	486	10	31	2	1088	12	998	22

* ND = No data

samples analyzed in each basin were variable. However, in general a decrease in percentage of closures in each basin during the 1990s relative to previous decades was evident, except in the South basin, where 22% of the samples analyzed resulted in closures in both the 1980s and 1990s.

Seasonal Duration of Closures

The greatest number of closures during each decade occurred from July through November with 81% of all closures occurring during these months in the 1950s, 69% in the 1960s, 63% in the 1970s, 65% in the 1980, and 73% in the 1990s (Table 4).

Spread of PSTs

The historical record of PSP events causing illness and death in humans and initial shellfish closures in the different regions of Puget Sound is shown in Figure 4. The death of three people and illness of two others after their consumption of mussels and butter clams from the beach in Sekiu in 1942 was the first evidence of high levels of PSTs in Washington State. The death of three members of the Ucluelet Tribe after eating mussels containing PSTs on the west coast of Vancouver Island, British Columbia, Canada, was recorded three days prior to the mortalities in Sekiu (L. Hanson, pers. comm.), indicating that this event was probably widespread in the Pacific Northwest. From 1942 to 1957, Washington State monitoring was sporadic and was actually temporarily stopped in 1946 because of blanket closures that were in effect at this time (Lilja 1978). Monitoring for PSTs in Washington became formalized in 1957 after a large outbreak of PSTs occurred in British Columbia, Canada (Waldichuk 1958). During this year, the first shellfish closure occurred in Sequim Bay when a level of 162 μg STXeq/100 g was measured in butter clams. The first shellfish closure in the San Juan Islands occurred in 1958 when a level of 122 μg STXeq/100 g was measured in butter clams. In the early 1970s, when WDOH monitoring efforts increased, shellfish containing PSTs were found further east in Lummi Bay (Fig. 4) when 465 μg STXeq/100 g was measured in Pacific oyster in 1973. In 1978, anomalously high PST levels (up to 30,360 μg STXeq/100 g) caused the first shellfish closures in both Whidbey and Central Puget Sound basins. Over a period of several weeks, the contamination spread southward in Puget Sound to an area between Seattle and Tacoma in south-central Puget Sound. In 1987, levels of PSTs
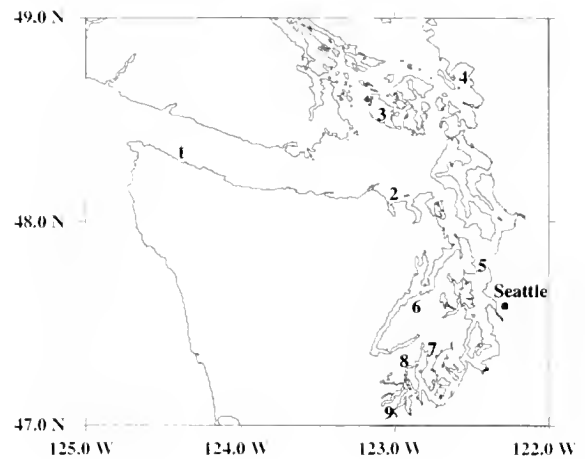
First Shellfish Harvesting Closures and PSP Event in Each Region

Record	Year	Region	Location of first closure and/or PSP event
1	1942	NW	Five cases of PSP in Sekiu, three deaths
2	1957	NW	Sequim Bay/Discovery Bay
3	1958	N	San Juan Islands
4	1973	N	Lummi Bay
5	1978	C	Whidbey Basin/Central Basin, 9 cases of PSP
6	1987	C	Northern Hood Canal
7	1988	S	Carr Inlet, 1 case of PSP
8	1991	S	Case Inlet
9	1997	SW	Totten and Eld Inlets

Figure 4. First recorded PSP events and shellfish harvesting closures in each Puget Sound basin. Locations of each event are numbered on the map of Puget Sound.

in northern Hood Canal were measured above the closure limit for the first time since WDOH sampling began (234 μg STXeq/100 g in Pacific oyster). The first closures of shellfish harvesting in south Puget Sound in 1988 were due to PST levels up to 10,982 μg STXeq/100 g in Carr Inlet. One person was hospitalized after ingesting oysters from Minter Bay, Carr Inlet in September 1988 (F. Cox, pers. comm.). In 1991, the first incidence of shellfish

TABLE 4.

Number of monthly closures, also as a percentage of total closures during each decade.

Month	1950s		1960s		1970s		1980s		1990s	
	Closures	%	Closures	%	Closures	%	Closures	%	Closures	%
January	1	2	10	5	40	5	80	3	82	4
February	0	0	11	5	30	4	49	2	66	3
March	0	0	8	4	35	5	97	4	53	2
April	4	9	14	7	65	8	173	7	60	3
May	2	4	1	0	33	4	155	6	70	3
June	2	4	2	1	42	5	238	9	147	7
July	3	7	24	12	101	13	442	17	353	16
August	13	28	20	10	109	14	449	18	337	15
September	5	11	27	13	98	13	408	16	321	14
October	6	13	41	20	115	15	260	10	393	18
November	10	22	29	14	65	8	111	4	204	9
December	0	0	15	7	37	5	94	4	150	7

closures occurred in Case Inlet, with levels of 779 µg STXeq/100 g in blue mussels. In the fall of 1997, PST levels up to 6799 µg STXeq/100 g were measured in Eld and Totten inlets, causing the first shellfish closures in these small southwestern finger inlets of south Puget Sound. Previous routine monitoring, necessitated by the presence of commercial shellfish operations at these sites, detected only low levels of PSTs that were below the regulatory limit of 80 µg STXeq/100 g (Saunders et al. 1982, Determan 2000). For example, the first measurement of PST in Carr Inlet was in 1981 at a level of 57 µg STXeq/100 g in blue mussels.

When the highest annual PST levels exceeded 80 µg/100 g even once at a particular monitoring site during a given decade, that site was shown to have a closure during that decade (Fig. 5). Although samples were tested in several areas throughout Puget Sound, in the 1950s and 1960s the only areas with shellfish clo-

sures were in the Northwest and North basins. In the 1970s, the number of sampling sites increased substantially, and closures were seen in central Puget Sound. During the 1980s, the first closures were seen in the eastern inlets of the South basin; shellfish closures occurred throughout much of south Puget Sound in the 1990s. An increase in the number of monitoring sites sampled over the decades is evident. Data from the 1970s indicated the high number of closures in 1978 in the Whidbey basin, however by the 1990s, few closures were observed here. The actual numbers of samples tested for toxins and closures in each basin as a percent of the total closures in all of Puget Sound are shown in Table 5. The greatest number of closures occurred in the Northwest basin in the 1950s (97% of all closures) and 1960s (99% of all closures), in the North basin in the 1970s (34% of all closures), in the Central basin in the 1980s (34% of all closures), and in the Central (36% of all
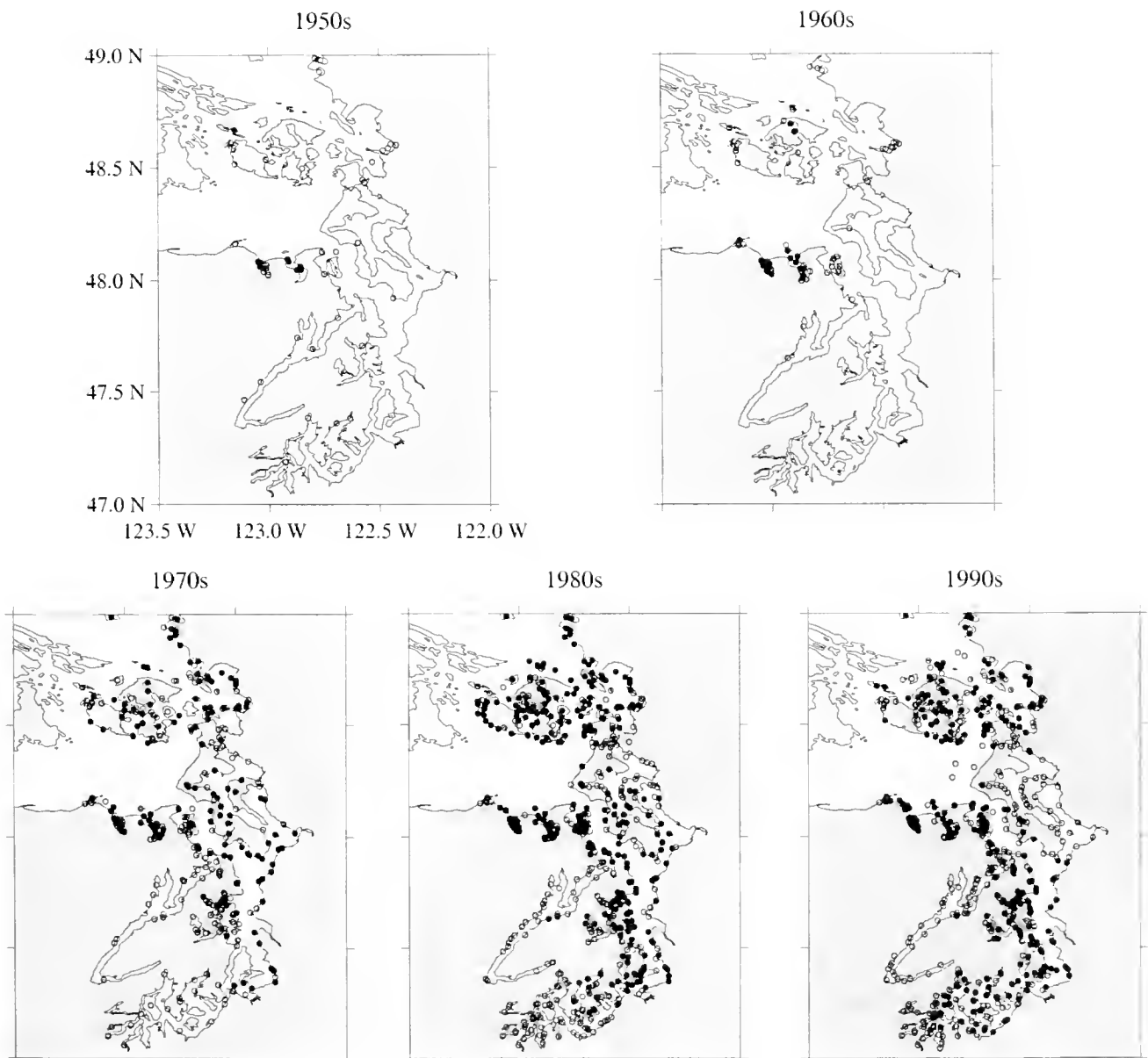
Figure 5. Closures because of PST in shellfish at all Puget Sound monitoring sites for each decade. Symbols represent maximum values for each decade shown as open circles (below 80 µg STXeq/100 g) or solid circles (greater than or equal to 80 µg STXeq/100 g). Data for the 1950s include only 1957–1959.

TABLE 5.

Number of samples analyzed for PSP toxins in each basin and closures in each basin as a percentage to total closures in Puget Sound.

Decade	Northwest Number	(%)	North Number	(%)	Widbey Number	(%)	Central Number	(%)	South Number	(%)	Total Measurements	Total Closures	Closures as a % of Total Measurements
1950s	130	97	53	3	1	0	13	0	4	0	201	33	16
1960s	433	99	202	1	1	0	17	0	ND[#]	ND	653	197	30
1970s	841	30	1302	34	423	22	607	14	24	0	3197	761	24
1980s	1793	23	3759	31	1704	4	3966	34	1080	9	12302	2706	22
1990s	2764	13	4859	16	1566	1	9070	36	4536	33	22795	2990	13
Highest level[*] (date)	3074 (9/17/90)		5968 (7/27/99)		30360 (9/28/78)		4822 (7/11/90)		10982 (10/22/98)				

[*] Highest level = μg STXeq 100g⁻¹, all in blue mussels

[#] ND = no data.

closures) and South basins (33% of all closures) in the 1990s. Whereas the highest percentage of total closures occurred in northern Puget Sound in the 1950s, the greatest percentage has more recently occurred in the central and south Puget Sound regions. Closures as a percent of total measurements made have decreased since the 1960s.

DISCUSSION

There is speculation that harmful algal bloom events are increasing in intensity, frequency, duration, and geographical location; however, the long-term monitoring data needed to support these ideas are often insufficient for trend analysis. Because of documented illnesses and deaths due to PSP beginning in the 1940s, Washington State has one of the longest monitoring histories for PSTs in the United States with the State of Maine having the next oldest monitoring program, established in 1958 (Shumway et al. 1988). Data collected in Washington State are compilations of PST measurements at shellfish harvest sites designated by the WDOH to have the greatest risk for human exposure to PSP. Although the location and frequency of monitoring at these sites have changed substantially over the years, we were able to use the data to establish trends for Puget Sound shellfish closures due to PSTs.

Spread of PSTs into Central and Southern Puget Sound

Until the last decade, the only Puget Sound basins with no measured PSTs were southern Hood Canal and the southernmost inlets of Puget Sound (Rensel 1993, Determan 2000). Since the 1980s, the frequency of PST detection has increased in southern basins of Puget Sound, an area that contains the region's most productive shellfish-growing beaches. Shallow sills that restrict the exchange of water between adjacent basins (Strickland 1983; see also Fig. 1) have likely hindered the movement of toxic dinoflagellates, especially because these cells generally do not prosper in mixing conditions that are characteristically found at sills. Alexandrium cells thrive in stratified environments, presumably due to the supply of nutrients, trace minerals, and natural humic substances that may serve as growth stimulants at the density interface (see, for example, Anderson 1997). Therefore, sills, which are found at several sites in Puget Sound (Fig. 1), have likely delayed the spread of Alexandrium cells to the South basin.

A geospatial map showing the first accounts of shellfish closures or PSP in each region of Puget Sound (Fig. 4) suggests that over time, toxigenic Alexandrium cells, cysts or both have made a slow progression from northern Puget Sound to the south. The numbers of cysts and cells likely have increased over the decades in the areas near sills, eventually reaching a critical mass that enabled their survival during transport over these natural barriers. Conditions for Alexandrium cell growth are ideal in south Puget Sound because of the many shallow, poorly flushed bays and inlets where thermally-caused stratification occurs during summer months, allowing ideal growth conditions for dinoflagellate cells to persist for weeks (Rensel 1993). However, the initial population of Alexandrium cells or cysts probably entered south Puget Sound only in recent years. The first detectable PSTs in south Puget Sound were noted in Carr Inlet (57 μg STXeq/100 g in blue mussels) in 1981. Some anecdotal evidence from the epidemiologic record also supports the gradual spread of toxigenic Alexandrium cells into south Puget Sound. Of the nine people who became ill after eating mussels from Carr Inlet in the summer 2000, one woman who was sick during that event previously had eaten shellfish from the same beach in Carr Inlet for more than 50 y with no PSP symptoms (Cox 2000).

A possible pathway of cells into Puget Sound was through the Strait of Juan de Fuca, into the Northwest basin and western San Juan Islands, then past the sill to the south of the San Juan Islands and Rosario Strait into Bellingham Bay. From the Northwest basin, cells may have been transported southward to the Whidbey basin, past the sill at Admiralty Inlet to central Puget Sound, and also past the sill at the entrance to Hood Canal to northern Hood Canal. Finally, from the Central basin, cells spread into south Puget Sound past the sills at Tacoma Narrows and the Nisqually (Fig. 1). The hydrographic separation of the eastern and western inlets of south Puget Sound (Ebbesmeyer et al. 1998) can explain the temporal lag in the first documented shellfish harvesting closures in Case Inlet in 1991 compared with Totten and Eld inlets in 1997 (Fig. 4).

The Initial Population of Alexandrium in Washington State

The first recorded PSP event in Washington State, at Sekiu in 1942 (Fig. 4), coincided with three deaths on the western coast of Vancouver Island, Canada. The next PSP episode in British Columbia was in the inland waters of the Strait of Georgia in 1961 when 61 people fell ill (Taylor & Horner 1994). It is possible that the source of the "seed" population of toxigenic A. catenella cells in Washington State originated from the inland or coastal waters of Canada. Indeed, the first documented PSP event in all of North America dates back to 1793, when four members of Captain

George Vancouver's crew became sick and one died of PSP during exploration of present day British Columbia (Quayle 1969). Unlike its neighbor to the north, Washington State had no recorded illnesses or deaths of humans with descriptions of PSP symptoms before 1942. *Alexandrium catenella* is the chief source of PSP off the west coast of British Columbia and eastern Vancouver Island (Taylor & Harrison, 2002) and evidence suggests that the earliest recorded PSP outbreaks were at least partially because of blooms of this dinoflagellate species (Quayle 1969). Because prevailing winds and currents are from the north during the summer months (Hickey 1989), when growth conditions for *Alexandrium* are optimal, and because the inlet to Puget Sound is at the north end of this fjord, a north to south transport would support the natural dispersal of algal cells from Canada. The routes of toxigenic cell dispersal in the Pacific Northwest could be defined in the future by a study of population genetics of *A. catenella* isolates from both British Columbia and Washington State.

Increased PST Levels

Because of increases in aquaculture activity as well as the measurement of PSTs in new areas of Puget Sound, the number of samples taken annually for PST testing has increased steadily from 1988 to the present time (Table 5). However, increased sampling frequency has not resulted in a higher percentage of closures during the latter decades (Table 3). The majority of closures during each decade was in July through November; a shift to more closures in earlier or later months has not been observed in recent years. In addition, no correlation between the highest toxin levels and total number of samples collected annually was observed (Table 5), suggesting that apparent increases in PST intensity are not due to increased sampling. Because mussels can accumulate higher levels of PSTs, the shift of reliance on oyster and clam samples in the monitoring program in the 1960s to mussel samples in the 1990s (Table 1) may account for some of the observed increase in toxin intensity. However, the normalized maximum values of PSTs in all shellfish have also increased over the past five decades (Fig. 2D), showing a statistically significant increase during the 1980s and 1990s compared with the three previous decades, supporting the fact that the increase in PST levels in Puget Sound shellfish was not due to the change in shellfish species monitored over the years.

PST Intensity Versus Human Population Growth

Over the last four decades, modern human development has extensively altered the shoreline habitats of Puget Sound (see the Department of Ecology, Water Quality Monitoring web page, http://www.ecy.wa.gov/programs/eap/mar_wat.html). A comparison of maximum PST averages per decade and population estimates (of all counties bordering Puget Sound) over the last 40 y shows a high level of correlation ($r^2 = 0.987$; Fig. 6). Although statistical correlation does not establish a causal link, it does suggest that some factor(s) associated with population growth may influence the magnitude of PSTs at any given site. Increased nutrients to our coastal environment may provide more favorable growth conditions for *Alexandrium* cells that populate a given basin. It has been speculated that the lack of nitrogen in surface and subsurface waters of Puget Sound has been a major factor limiting the further spread of PSTs into bays and inlets otherwise suitable for *A. catenella* (Rensel 1993). Land clearing, logging, aerial forest fertilizing by timber companies, direct sewage out-
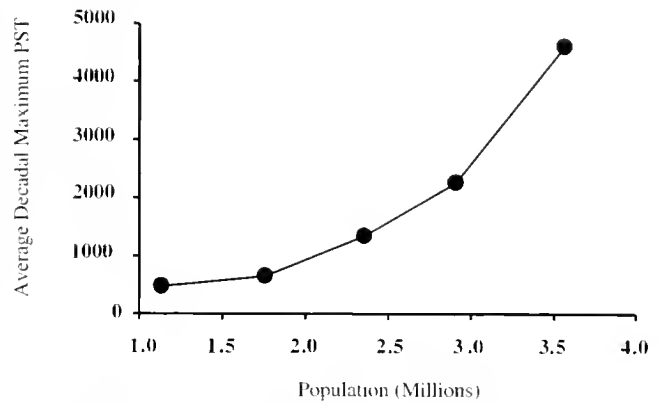
Figure 6. Maximum PST average per decade versus population estimates. Census data for counties bordering Puget Sound were obtained from the following site: http://www.census.gov/population/cencounts/wa190090.txt

falls, agricultural runoff, and even aquaculture operations have increased the amounts of nutrients, including nitrogen, that are supplied to the coastal ecosystems of Puget Sound (Howarth 2001). Inlets and fjords with low flushing rates that adjoin urbanized shorelines have the greatest sensitivity to nutrient addition (Mackay & Harrison 1997). The increased levels of PSTs in the semi-enclosed bays of south Puget Sound in recent years may, at least partially, be explained by increased eutrophication and generally poor circulation. Indeed, south Puget Sound is described by the Washington State Department of Ecology as one of the areas most susceptible to impacts of eutrophication (Cusimano 2002). Because the depth of south Puget Sound inlets is much shallower and flushing time is slower, nutrient inputs to surface waters provide ideal growth conditions for *A. catenella*.

Natural Events

Although the intensity of PSTs in shellfish has increased with time (Fig. 2), toxic events do not occur in each basin in every year. For example, shellfish closures have occurred in northern Hood Canal only in 1991, 1996, and 1997–1999. What sets those years apart from all other years? Environmental conditions such as water temperature, mixed layer depth, sunlight, and nutrients all work together to increase the chance of a toxic event in a particular basin and in any given year (Rensel 1993, Nishitani et al. 1988). In addition to microscale, basin-specific environmental factors that result in a periodicity of *Alexandrium* blooms, large-scale occurrences, such as the bloom that occurred in the Whidbey and Central basins in 1978, may have been motivated by global climatic events or shifts. In 1977, a large shift to a positive Pacific Decadal Oscillation occurred, with a resulting ecological response to the environmental changes. This period was marked by an enhancement of overall productivity that appeared to be closely related to changes in upper ocean mixed-layer depths and temperatures (Mantua et al. 1997). Indeed, an exceptionally deep surface layer of warm water was believed to have exacerbated the 1978 Whidbey basin bloom (Erickson & Nishitani 1985). Toxin levels of that magnitude have not been measured since that year in Whidbey basin, giving credence to the possibility that some unique, large-scale environmental factors influenced the occurrence of this event. The linkage of harmful algal bloom magnitude and frequency to climatic regime shifts has been suggested in recent

studies (Epstein et al. 1998, Hayes et al. 2001). The specific co-variance of levels of PSP toxins in shellfish with strong El Niño/Southern Oscillation events (Erickson & Nishitani 1985) and other environmental parameters such as the condition of oysters in Willapa Bay (Ebbesmeyer et al. 1995) has been suggested.

Effective Monitoring

Although greater numbers of closures have been observed over time in many of the basins of Puget Sound, the percentage of closures relative to the total sites monitored in a given basin has decreased in all but south Puget Sound (Table 3). Although PSP toxins pose a serious threat to commercial and recreational shell-fishing operations, the large number of sites monitored by WDOH allows the agency to pinpoint areas within a basin that are safe for harvest. This rigorous monitoring has resulted in a greater proportion of open than closed sites for shellfishing in the Puget Sound region where the risk for PSP is extreme. Increased HAB events and interest in commercial shellfish operations in all regions of Puget Sound and wide-scale, year-round recreational harvest op-portunities will likely result in a mandate for the WDOH to sustain its rigorous sampling efforts. In the future, improved monitoring methods (e.g., molecular probes for cells and rapid analytical as-says for toxins) will be essential for cost-effective and timely management of the fishery in Puget Sound.

CONCLUSIONS

The following conclusions can be obtained from our study. 1) There has been a significant increase in the magnitude of PSTs in Puget Sound shellfish with time. 2) The geographical scope of shellfish closures caused by high levels of PSTs in Puget Sound has increased over the past four decades. The first recorded shell-fish closures in the Northwest basin in the 1950s, the Central basin in the 1970s, and the South basin in the 1980s are likely due to the spread of *A. catenella* cysts and/or cells from north to south. 3) Shellfish closures in south Puget Sound may have been delayed until recent years by the physical blockage of cell movement by sills to the north. Hydrographic blockage may also explain the delayed appearance of PSTs in the southwestern finger inlets of south Puget Sound. 4) Increased shellfish closures caused by PSTs over the past few decades are not just the result of greater numbers of samples collected over time. 5) Global climate changes, such as the Pacific Decadal Oscillation and increased eutrophication in nearshore areas, are possible explanations for the increased mag-nitude of PSTs in shellfish today.

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FEEDING SOUTHERN ROCK LOBSTER, *JASUS EDWARDSII* HUTTON, 1875, PHYLLOSOMATA IN CULTURE: RECENT PROGRESS WITH LIPID-ENRICHED *ARTEMIA*

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ABSTRACT *Jasus edwardsii* phyllosoma larvae were successfully grown in static culture with antibiotics from newly hatched to stage V with high survival. Feeding phyllosomata on *Artemia salina* Linnaeus, 1758, enriched with (1) a triacylglycerol (TAG)-rich A1 DHA Selco–*Chaetoceros muelleri* Lemmermann, 1898, nutrient source or (2) a formulated ethyl ester (EE)-rich nutrient source was compared with the more novel approach of using a formulated mussel powder–polar lipid diet attached to mesh. Individuals showed an increase to stage V in dry mass (0.1–1.5 mg) and total length (2.1–6.1 mm). Survival of *Artemia*-fed phyllosomata was high (92–98% from stages II–III; 49% mean total survival). Animals fed the mussel powder–polar lipid diet had low molt success, although the presence of faecal trails confirmed they were consuming the diet. Total lipid remained generally constant in *Artemia*-fed phyllosomata from newly hatched to stage V (155 mg g⁻¹ dry mass); this was notably higher than observed for previous feeding trials. The major lipid class in all phyllosomata samples was polar lipid, followed by sterol, with TAG as a minor component only, and EE not detected. The main fatty acids were 18:1(n-9)c, 18:2(n-6), 16:0, eicosapentaenoic acid [20:5(n-3)], 18:0, 18:1(n-7)c, arachidonic acid [20:4(n-6)], and docosahexaenoic acid [DHA; 22:6(n-3)]. Levels of the essential polyunsaturated fatty acids (PUFA), namely, arachidonic acid, eicosapentaenoic acid and, in particular, DHA, decreased, on both a relative and absolute basis, from newly hatched to stage V, although phyllosomata fed the EE-rich *Artemia* diet showed higher essential PUFA content with oil content. This experiment further validates that lipids and fatty acids are important nutritional component in rock lobster larvae and that feeding phyllosomata with lipid-enriched *Artemia* maintains excellent growth and survival in early stages. Strategies will be needed, however, to either overcome the issue of low DHA, in particular, delivered by *Artemia* (because of retroconversion), or to supply DHA by alternate means at later stages.

KEY WORDS: *Artemia,* enrichment, fatty acids, *Jasus edwardsii,* lipids, lobster, phyllosoma

INTRODUCTION

Rock lobster in Australasia has recently attracted the interest of a number of research institutions for its potential as a valuable aquaculture species. The fishery for southern rock lobster, *Jasus edwardsii* Hutton, 1875, boasts a value of over A$200 million in Australia (Punt & Kennedy 1997) and NZ$100 million in New Zealand (Breen & Kendrick 1997). As wild fishing pressure escalates (Booth & Phillips 1994), future exploitation of the rock lobster marketplace will logically be realized through aquaculture (Phleger et al. 2001).

As an aquaculture species, rock lobster possesses the allure of potentially high financial reward. Equally great is the challenge for research scientists because the larval phase, including metamorphosis from phyllosoma to puerulus, is extensive (Phillips & Sastry 1980, McWilliam & Phillips 1997), currently requiring close to a year in culture (Tong et al. 2000). To conquer this challenge, several vital aspects of culture of rock lobster phyllosomata can be identified as follows: (1) exploration of feeding capabilities of phyllosomata (Johnston & Ritar 2001, Nelson et al. 2002a) to determine appropriate format of feed presentation; (2) determination of nutritional requirements to focus further the feed format; (3) a suitable aquarium design (Kittaka & Booth 2000, Ritar 2001) to optimize exposure of animals to the food source while minimizing microbial loading (Igarashi et al. 1990, Diggles et al. 2000).

This study examines the second aspect (noted above), nutrition, and in particular the requirements for lipids. To focus this aspect,

features of lipid nutrition under examination include: (1) total lipid content, the mg g⁻¹ of the lipid provided in the diet and that incorporated into larvae; (2) the lipid classes, examination of the delivery, and incorporation of types of lipids, such as triacylglycerol (TAG), polar lipid (PL) and ethyl ester (EE); and (3) the profile of fatty acids (FA), which are components of lipid classes.

Building on the studies of lipids and FA in wild phyllosomata (Phleger et al. 2001) and potential prey items (Nichols et al. 2001), we have examined enrichment of *Artemia* with essential polyunsaturated fatty acids (PUFA) (Phleger et al. 2001, Nelson et al. 2002b, Smith et al. 2002) and feeding of these TAG-enriched *Artemia* to phyllosomata (Nelson et al. 2003). The evidence amassed to date from these studies indicates that wild phyllosomata largely obtain, and therefore may require, lipid in a PL form rather than in a TAG form. However, *Artemia* store their lipid enrichment as TAG (McEvoy et al. 1996, Sorgeloos et al. 1998, Harel et al. 1999). With this in mind and because phyllosomata do consume static food items (e.g., mussel pieces) (Kittaka 1997b, Matsuda & Yamakawa 2000, Nelson et al. 2002a), the present study was performed to provide phyllosomata a diet presented at a feed station (i.e., formulated diet attached to aquaria), a format currently receiving attention (Cox & Johnston 2003). A comparison was made for feed-station fed larvae to animals fed *Artemia*, enriched with either a TAG-rich product or with a novel docosahexaenoic acid (DHA)-rich EE product, by examining the effects on *J. edwardsii* phyllosomata survival, growth and lipid composition.

METHODS

Artemia *Enrichment*

Decapsulated *Artemia* cysts (INVE, Great Salt Lake Prime Gold) were hatched at 28 ± 1°C in 50-L white fiberglass cones in

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0.2-μm filtered brackish water (27 ± 1 g kg^{-1}), with vigorous aeration and a 150 W light suspended 0.5 m above the water. After 24 h, *Artemia* nauplii were removed from the hatching cones, rinsed in freshwater for 2 min and transferred into 1000-L tanks of filtered seawater (0.2 μm, 34 ± 1 g kg^{-1}, 27 ± 1°C). *Artemia* were fed twice daily with a rice pollard–soy flour–wheat flour brine shrimp diet (Eyre Peninsula Aquafeeds, South Australia) at a rate to maintain a Secchi depth of 25–30 cm. The environmental parameters remained stable for the duration of the on-growing period; salinity (35.7 ± 0.2 g kg^{-1}), pH (8.3 ± 0.0), dissolved oxygen (7–7.2 mg L^{-1}), and temperature (26.9 ± 0.1°C). After 5 days, 80,000 *Artemia* with a total length of 1.5 ± 0.2 mm were removed from the on-growing container, rinsed in freshwater for 2 min and transferred to the 50-L white fiberglass cones containing 10 L of filtered seawater to achieve a density of 4 mL^{-1}.

Artemia were enriched for 24 h with 0.6 g L^{-1} of three nutrient sources (i.e., *Artemia* enrichment diets):

(1) A1 DHA Selco (INVE Group, Belgium).
(2) The microalga *Chaetoceros muelleri* Lemmermann 1898.
(3) Ethyl ester–mussel: a mixture of New Zealand Greenshell mussel (*Perna canaliculus* Gmelin, 1791) powder (NIWA Research, Auckland, New Zealand)–DHA (66%) EE oil (CSIRO Marine Research, Hobart, Australia)–AA (39%) TAG marine oil (Sun-TGA40S, Suntory Limited, Osaka, Japan)–Greenshell mussel polar lipid (NIWA Research, Auckland, New Zealand) (56:30:10:4 by mass).

C. muelleri were cultured and the cell density was measured daily as described in Wilkinson (2000). The nonalgal enrichment diets were prepared daily by homogenizing ingredients suspended in seawater.

Experimental Aquaria

Three-hundred phyllosomata aquarium^{-1} were grown from newly-hatched to stage V in 3-L plastic static aquaria on three diet treatments; each treatment was conducted in triplicate. The diet treatments consisted of:

(1) *Artemia* enriched with A1 DHA Selco and *Artemia* enriched with *C. muelleri* (1:2 v/v).
(2) *Artemia* enriched with the Ethyl ester–mussel nutrient source (as described above).
(3) Mussel powder–polar lipid feed station diet [Greenshell mussel powder–Greenshell mussel polar lipid–lyprinol (from Greenshell mussel) (NIWA Research, Auckland, New Zealand)–sodium alginate (81:10:5:4 by mass)] affixed to 8 × 17 cm meshes (bird netting) with 10% CaCl$_2$ solution.

The water in aquaria was changed daily. After recording any molts/mortalities, the contents of each aquarium were poured through a 1,000-μm screen, retaining the phyllosomata while the uneaten feed and debris went to waste. The aquaria were cleaned, refilled with seawater, and larvae were washed back in. Phyllosomata were provided with new diets once daily in the afternoon. *Artemia* were fed to phyllosomata at a rate of 3 *Artemia* mL^{-1}. Oxytetracycline was added to the water at 20 mg L^{-1} daily. After each molt, all animals were counted and 10 phyllosomata aquarium^{-1} were measured for total length, carapace length and carapace width utilizing a dissecting microscope, digital camera and Scion Image Beta 4.0.2 software (Scion Corporation, Frederick, MD).

Lipid Extraction

Artemia and phyllosomata samples were filtered through 4.7-cm Whatman glass filters (GF/F) and rinsed with 0.5 M ammonium formate. Sample numbers of phyllosoma taken for lipid analyses were as follows: 400 newly hatched (sampled at start before distribution of larvae to aquaria); from each aquarium 50 stage II, 35 stage III, 25 stage IV, and 15 stage V; all midstage. Samples were lyophilized to determine dry mass and quantitatively extracted overnight using a modified Bligh and Dyer (1959) one-phase methanol:chloroform:water extraction (2:1:0.8 v/v/v). The phases were separated by the addition of chloroform:water (final solvent ratio, 1:1:0.9 v/v/v methanol:chloroform:water). The total solvent extract was concentrated using rotary evaporation at 40°C.

Lipid Classes

An aliquot of the total solvent extract was analyzed using an Iatroscan MK V TH10 thin-layer chromatography–flame-ionization detector (TLC-FID) analyzer (Tokyo, Japan) to quantify individual lipid classes (Volkman & Nichols 1991). Samples were applied in duplicate to silica gel SIII chromarods (5-μm particle size) using 1-μL micropipettes. Chromarods were developed in a glass tank lined with pre-extracted filter paper. The primary solvent system used for the lipid separation was hexane:diethyl ether:acetic acid (60:17:0.1), a mobile phase resolving nonpolar compounds such as wax ester (WE), TAG, free fatty acids (FFA) and sterols (ST). A second nonpolar solvent system of hexane:diethyl ether (96:4) was also used to resolve hydrocarbons, WE, TAG, and diacylglyceryl ether (DAGE). After development, the chromarods were oven dried and analyzed immediately to minimize absorption of atmospheric contaminants. The FID was calibrated for each compound class (phosphatidylcholine, cholesterol, cholesteryl oleate, oleic acid, squalene, TAG [derived from fish oil], WE [derived from orange roughy oil], and DAGE [derived from shark liver oil]; 0.1–10 μg range). Peaks were quantified on an IBM-compatible computer using DAPA Scientific software (Kalamunda, Western Australia). TLC-FID results are generally reproducible to ±5–10% of individual class abundances (Volkman & Nichols 1991).

Fatty Acids

An aliquot of the total lipid was *trans*-methylated to produce fatty acid methyl esters (FAME) using methanol:chloroform:conc. hydrochloric acid (10:1:1, 80°C, 2 h). FAME were extracted into hexane:chloroform (4:1, 3 × 1.5 ml) and treated with *N,O*-bis-(trimethylsilyl)-trifluoroacetamide (BSTFA 50 μL, 70°C, overnight) to convert ST and alcohols to their corresponding TMSi ethers.

Gas chromatographic (GC) analyses were performed with a Hewlett Packard 5890A GC (Avondale, PA) equipped with an HP-5 cross-linked methyl silicone fused silica capillary column (50 m × 0.32 mm i.d.), an FID, a split/splitless injector, and an HP 7673A auto sampler. Helium was the carrier gas. After addition of methyl nonadecanoate and methyl tricosanoate internal injection standards, samples were injected in splitless mode at an oven temperature of 50°C. After 1 min, the oven temperature was raised to 150°C at 30°C min^{-1}, then to 250°C at 2°C min^{-1}, and finally to 300°C at 5°C min^{-1}. Peaks were quantified with Waters Millennium software (Milford, MA). Individual components were identified using mass spectral data and by comparing retention time data with those obtained for authentic and laboratory standards. GC results are subject to an error of ±5% of individual

component area. GC-mass spectrometric (GC-MS) analyses were performed on a Finnigan Thermoquest GCQ GC-mass spectrometer (Austin, TX) fitted with an on-column injector. The GC was fitted with a capillary column similar to that described above.

RESULTS

Morphometrics

The increase in total length and mass in phyllosomata was similar between the two *Artemia* diet treatments, with a good fit of exponential trend lines observed ($R^2 \geq 0.999$; Fig. 1). Phyllosomata fed the A1 DHA Selco–*C. muelleri–Artemia* diet treatment showed a greater increase in total length (2.1 to 6.1 mm) and mass per individual (0.1 to 1.5 mg dry mass) from stages I to V than did larvae fed ethyl ester–mussel-enriched *Artemia* (total length: 5.9 mm; mass per individual: 1.2 mg dry mass; Fig. 1). Percentage survival was >68% between each stage and was highest from stages II–III (92–98%; Table 1). Total survival to stage V was high for animals fed *Artemia* enriched with either the A1 DHA Selco–*C. muelleri* (57%) or ethyl ester–mussel (42%) nutrient sources. There were no differences in intermolt period for *Artemia*-fed phyllosomata among treatments. Intermolt periods were 9, 11, 12,

TABLE 1.

Intermolt period (days) and percentage survival of phyllosomata fed different diets.

	Diet		
	A1 Selco–*C. muelleri*[b]	Ethyl Ester–Mussel[b]	Mussel Powder–Polar Lipid[c]
Survival[a]			
I–II	81.8 ± 4.8	76.2 ± 15.9	–
II–III	97.5 ± 2.2	92.3 ± 3.5	93.5 ± 7.2
III–IV	87.3 ± 6.0	86.0 ± 6.7	51.3 ± 3.6
IV–V	81.1 ± 14.5	68.3 ± 6.8	–
Total	56.5 ± 12.6	41.5 ± 11.2	–
Intermolt period			
I–II	9	9	–
II–III	11	11	10
III–IV	12	12	–
IV–V	13	15	–

[a] Presented as mean ± SD; $n = 3$
[b] Enriched *Artemia*.
[c] Feed station.
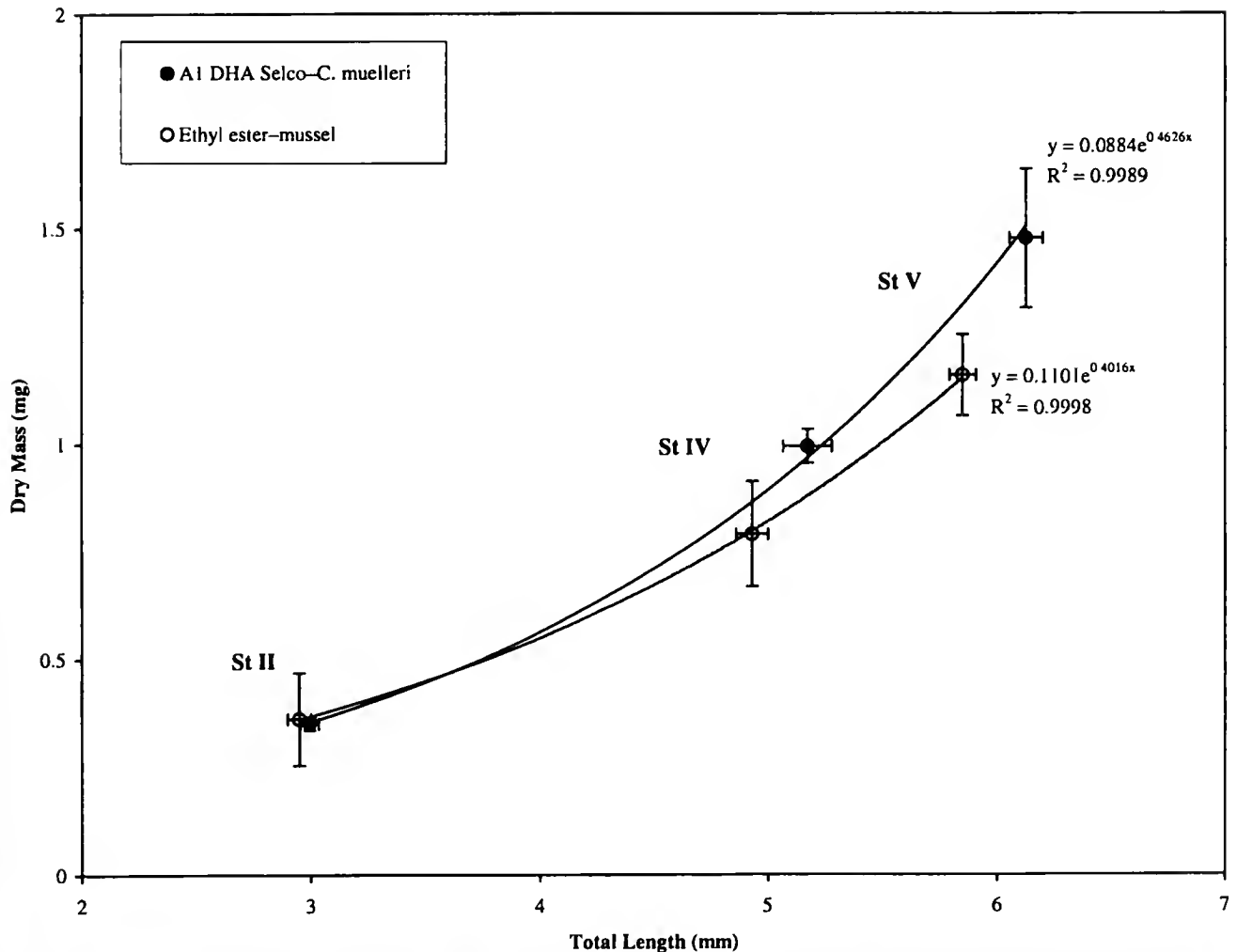
Figure 1. Dry mass as a function of total length of *J. edwardsii* phyllosomata from stages I to V on two diet treatments of *Artemia* enriched with either A1 DHA Selco–*C. muelleri* or ethyl ester–mussel nutrient sources. Presented as mean ± SD; fitted with exponential trend lines.

and 13–15 days to commencement of molt for stages I–II, II–III, III–IV, and IV–V, respectively.

Phyllosomata fed the Mussel powder–polar lipid diet failed to molt to stage II on the feed station diet alone. Two animals remained alive at stage I for 30 days, at which time they were put on the Ethyl ester–mussel-enriched *Artemia* diet. At day 41 they successfully molted to stage II, and were sampled at day 56. After sampling stage II animals at day 15, phyllosomata fed A1 DHA Selco–*C. muelleri*-enriched *Artemia* were divided and half were put on the Mussel powder–polar lipid diet. After 10 days, these animals molted to stage III, were sampled at day 30, but failed to molt to stage IV. After sampling stage IV animals at day 37, phyllosomata fed ethyl ester–mussel-enriched *Artemia* were divided and half were put on the Mussel powder–polar lipid diet. They did not molt to stage V, but were sampled concurrently with *Artemia*-fed phyllosomata at day 56.

Lipid Content and Classes

The two nutrient sources were lipid-rich with A1 DHA Selco higher than EE–mussel (960 and 410 mg g^{-1} dry mass, respectively; Table 2). A1 DHA Selco was dominated by TAG (88%) and ethyl ester–mussel by EE (55%), with TAG the second most abundant lipid class (28%). Lipid content of *Artemia* enriched with A1 DHA Selco–*C. muelleri* and ethyl ester–mussel was identical

(250 mg g^{-1} dry mass). TAG was the major lipid class (46–51% of total lipid), followed by PL (37–40%). ST (5–6%), FFA (4–10%), diacylglycerol (DG; 0.7–1.9%), and WE (0.1–0.3%) were minor components.

In *Artemia*-fed phyllosomata, although lipid per individual generally increased from newly hatched to stage V (8 to 180 µg), the absolute lipid content remained generally constant in *Artemia*-fed phyllosomata from newly hatched to stage V (Table 2). Total lipid was 155 mg g^{-1} dry mass in newly hatched phyllosoma and increased slightly from stage I to stage II (207 and 173 mg g^{-1} for A1 DHA Selco–*C. muelleri* and ethyl ester–mussel *Artemia*-fed phyllosomata, respectively) and to stage IV (157 and 176 mg g^{-1}). By stage V, total lipid decreased to the starting (newly hatched) value in ethyl ester–mussel *Artemia*-fed phyllosomata (156 mg g^{-1}) and was slightly lower in animals fed A1 DHA Selco–*C. muelleri*-enriched *Artemia* (128 mg g^{-1}). PL comprised the major lipid class in all phyllosoma samples (73–87% of total lipid), followed by ST (4–8%; mainly cholesterol), FFA (2–8%), DG (2–4%), and WE (0–0.5%). Minor TAG was detected (0–0.4%).

Stage IV phyllosomata fed the Mussel powder–polar lipid diet were similar to those fed either *Artemia* diet, with 156 mg g^{-1} dry mass of lipid and PL the dominant lipid class (83%; Table 2). ST were comparatively higher (10%). Compared with other phyllosoma, PL (73%) and DG (1%) were lower in stage III Mussel powder–polar lipid feed station-fed animals, with a proportionate

TABLE 2.

Percentage lipid class composition of nutrient sources, enriched *Artemia*, feed station, and phyllosomata.

	Wax Ester	Ethyl Ester	Triacylglycerol	Free Fatty Acid	Diacylglycerol	Sterol	Polar Lipid	Lipid as mg g^{-1} Dry Mass	Lipid Mass Indiv^{-1} Dry Mass (µg)
Nutrient souces									
A1 DHA Selco	0.0 ± 0.0	–	88.2 ± 0.8	1.6 ± 0.1	–	0.8 ± 0.0	9.5 ± 0.9	958.5 ± 18.7	–
Ethyl ester–mussel	0.7 ± 0.0	55.1 ± 5.8	27.9 ± 0.7	5.4 ± 1.6	0.1 ± 0.0	1.4 ± 0.4	9.3 ± 3.1	411.9 ± 44.2	–
Artemia									
A1 DHA Selco–*C. muelleri*	0.1 ± 0.0	–	45.9 ± 0.4	10.0 ± 0.2	1.9 ± 0.3	5.6 ± 0.0	36.5 ± 1.0	259.7 ± 12.8	–
Ethyl ester–mussel	0.3 ± 0.2	–	50.5 ± 0.2	3.5 ± 0.5	0.7 ± 0.1	5.2 ± 0.0	39.7 ± 0.9	254.2 ± 0.3	–
Feed station									
Mussel powder–polar lipid	2.8 ± 0.1	–	12.5 ± 0.3	26.2 ± 0.1	0.4 ± 0.1	3.5 ± 0.3	54.6 ± 0.9	191.0 ± 11.4	–
Phyllosomata									
Newly hatched	1.7 ± 0.1	–	0.2 ± 0.1	9.9 ± 0.1	–	9.9 ± 0.1	78.3 ± 0.1	154.6 ± 9.4	8.0 ± 1.1
A1 DHA Selco–*C. muelleri*[a]									
II	–	–	–	5.5 ± 5.0	1.5 ± 0.4	4.3 ± 1.7	88.7 ± 6.8	207.5 ± 19.6	72.7 ± 6.1
IV	0.3 ± 0.0	–	0.2 ± 0.1	4.7 ± 0.6	3.9 ± 0.7	6.9 ± 0.2	84.1 ± 1.4	157.2 ± 13.9	156.0 ± 8.0
V	0.3 ± 0.1	–	0.3 ± 0.1	2.1 ± 0.2	3.0 ± 0.3	7.1 ± 0.6	87.2 ± 0.9	127.8 ± 9.5	188.9 ± 25.2
Ethyl ester–mussel[a]									
II	–	–	–	8.1 ± 3.4	1.5 ± 0.0	5.5 ± 0.4	84.8 ± 3.7	173.0 ± 17.5	61.3 ± 13.3
IV	0.5 ± 0.3	–	0.2 ± 0.1	4.9 ± 0.8	3.0 ± 0.4	6.3 ± 0.4	85.1 ± 1.1	175.6 ± 18.4	137.3 ± 8.3
V	0.5 ± 0.1	–	0.4 ± 0.1	2.3 ± 0.5	3.2 ± 0.2	7.5 ± 0.4	86.1 ± 1.3	156.0 ± 19.3	180.0 ± 13.3
Mussel powder–polar lipid[b]									
III	2.7 ± 0.2	–	1.1 ± 0.1	9.2 ± 1.2	0.8 ± 0.3	12.8 ± 0.8	73.4 ± 0.9	54.2 ± 7.5	46.7 ± 10.8
IV	1.0 ± 0.3	–	0.5 ± 0.3	2.7 ± 0.3	2.2 ± 0.9	10.1 ± 1.9	83.4 ± 2.5	155.5 ± 86.4	133.3 ± 57.0

Presented as mean ± SD; $n = 3$; (–), below detection.

[a] Enriched *Artemia*.

[b] Feed station (molted from stage II to III only).

increase in ST (13%) and WE (3%). Lipid content was a third that of other *Artemia*-fed samples (54 mg g⁻¹ dry mass).

Fatty Acids

The FA in the two nutrient sources differed markedly (Table 3). In A1 DHA Selco, dominant FA in decreasing order of proportional abundance of total FA were: palmitic acid (16:0; 17%), EPA (15%), oleic acid [18:1(n-9)c; 14%], palmitoleic [16:1(n-7)c, 9%], DHA (8%), myristic acid (14:0; 7%) and linoleic acid [18:2(n-6); 5%]. The ethyl ester–mussel nutrient source was dominated by PUFA (75%), with major FA as DHA (37%), AA (13%), EPA (12%) and 16:0 (7%).

The major FAs in enriched *Artemia* were as follows: 18:1(n-9)c (32–36%), 18:2(n-6) (23–27%), 16:0 (9–11%), *cis*-vaccenic acid [18:1(n-7)c; 4%], stearic acid (18:0; 4%), and 16:1(n-7)c (2–4%; Table 3). *Artemia* enriched with ethyl ester–mussel had higher essential PUFA (3% AA, 6% EPA, 7% DHA) than those enriched with A1 DHA Selco–*C. muelleri* (1% AA, 2% EPA, 1% DHA). The Mussel powder–polar lipid diet was dominated by 16:0 (19%), EPA (14%), and DHA (14%), with AA at 3% of total FA. Compared with *Artemia*, levels in the Mussel powder–polar lipid diet of 18:0 fatty aldehyde (6%), 20:1(n-9)c (4%) and minor C₂₂ PUFA (3%) were elevated, and levels of 18:1(n-9)c (3%) and 18:2(n-6) (2%) were lower.

In *Artemia*-fed phyllosomata, the major FA were similar to those found in the enriched *Artemia* and in decreasing order of abundance were: 18:1(n-9)c (23–27% of total FA), 18:2(n-6) (17–

22%), 16:0 (9–11%), 18:0 (7–9%), EPA (7–11%), 18:1(n-7)c (4–6%), DHA (4–6%), and AA (2–5%; Table 4). These phyllosomata experienced a decrease in essential PUFA, on both a relative (Table 4) and absolute basis (Fig. 2), from newly hatched to stage V. In phyllosomata fed ethyl ester–mussel-enriched *Artemia*, there was a concurrent drop in levels of AA (5–4%, 4 to 3 mg g⁻¹ dry mass), EPA (21–9%, 14 to 6 mg g⁻¹), and DHA (14–5%, 9 to 3 mg g⁻¹), with a similar, although more pronounced, decrease in animals fed A1 DHA Selco–*C. muelleri*-enriched *Artemia* (AA: 3%, 2 mg g⁻¹ dry mass; EPA: 8%, 5 mg g⁻¹; DHA: 4%, 2 mg g⁻¹). Conversely, levels increased in 18:1(n-9)c (8 to 23–24%) and 18:2(n-6) (1–18%). The FA profile of animals fed the Mussel powder–polar lipid diet closely reflected the diet, being dominated by 16:0 (12–15%), EPA (8–14%), DHA (6–9%), and AA (5–6%). Compared with *Artemia*-fed phyllosomata, levels in the Mussel powder–polar lipid-fed animals of 18:1(n-9)c (9–18%) and 18:2(n-6) (5–13%) were lower, while in stages III and IV Mussel powder–polar lipid-fed animals, levels of 20:2(n-6) (2–3%) and 22:1(n-9) (3–4%) were higher.

DISCUSSION

A major feature of previous Australian feeding trials with southern rock lobster phyllosoma has been comparatively poor survival. This trial, however, represents a turning point in Australian rock lobster phyllosomata nutritional research, with greater than 80% survival of *Artemia*-fed phyllosomata through each stage from newly hatched to stage V. We believe that a primary differ-

TABLE 3.

Percentage fatty acid composition of nutrient sources, enriched *Artemia*, and feed station.

| | Nutrient Sources | | Artemia | | Feed Station |
	A1 DHA Selco	Ethyl Ester–Mussel	A1 DHA Selco–*C. muelleri*	Ethyl Ester–Mussel	Mussel Powder–Polar Lipid
14:0	6.9 ± 0.2	0.7 ± 0.1	1.3 ± 0.1	0.5 ± 0.0	3.7 ± 0.3
16:1(n-7)c	8.8 ± 1.0	0.8 ± 0.1	3.9 ± 0.1	2.0 ± 0.0	5.3 ± 0.4
16:0	16.6 ± 0.0	7.2 ± 0.8	11.0 ± 0.2	9.1 ± 0.1	18.5 ± 1.2
18:4(n-3)	2.7 ± 0.0	1.5 ± 0.0	0.2 ± 0.0	0.3 ± 0.0	2.1 ± 0.1
18:2(n-6)	5.1 ± 0.2	3.6 ± 0.3	27.2 ± 0.3	22.9 ± 0.3	1.9 ± 0.1
18:1(n-9)c/18:3(n-3)	14.1 ± 0.8	3.5 ± 0.7	35.8 ± 0.2	31.8 ± 0.6	2.6 ± 0.1
18:1(n-7)c	3.0 ± 0.1	0.5 ± 0.0	4.1 ± 0.0	3.7 ± 0.1	2.6 ± 0.1
18:0	3.5 ± 0.2	3.4 ± 0.3	4.2 ± 0.0	4.4 ± 0.1	5.1 ± 0.0
18:1 Falde	0.1 ± 0.0	–	–	0.7 ± 0.8	6.4 ± 0.1
20:4(n-6)	0.9 ± 0.0	13.2 ± 0.6	0.5 ± 0.0	3.4 ± 0.0	2.7 ± 0.1
20:5(n-3)	14.9 ± 0.6	12.3 ± 0.3	2.4 ± 0.3	6.3 ± 0.0	13.9 ± 0.4
20:4(n-3)	0.1 ± 0.0	0.1 ± 0.0	0.9 ± 0.0	1.4 ± 0.1	2.1 ± 0.1
20:1(n-9)c	0.4 ± 0.4	0.4 ± 0.5	0.0 ± 0.0	0.8 ± 0.1	3.6 ± 0.2
22:6(n-3)	7.9 ± 0.3	37.1 ± 0.7	0.8 ± 0.1	7.0 ± 0.6	13.5 ± 0.9
C₂₂ PUFA	–	0.7 ± 0.0	–	–	2.9 ± 0.3
Other	14.8	15.0	7.6	5.7	13.0
Sum SFA	31.8 ± 0.2	15.9 ± 0.4	18.9 ± 0.3	16.5 ± 0.1	31.6 ± 1.6
Sum MUFA	30.7 ± 1.5	8.5 ± 1.2	47.0 ± 0.6	40.8 ± 0.1	18.1 ± 0.3
Sum PUFA	37.4 ± 1.3	74.7 ± 0.2	33.7 ± 0.6	42.4 ± 0.5	43.1 ± 1.8
Sum (n-3)	28.3 ± 1.0	52.9 ± 0.9	3.7 ± 0.3	14.0 ± 0.7	32.8 ± 1.5
Sum (n-6)	7.0 ± 0.1	20.6 ± 1.0	28.2 ± 0.5	27.5 ± 0.5	6.5 ± 0.1
Ratio (n-3)/(n-6)	4.1	2.6	0.1	0.5	5.0
Ratio EPA/AA	15.8	0.9	5.2	1.9	5.1
Ratio DHA/EPA	0.5	3.0	0.3	1.1	1.0

Presented as mean ± SD; *n* = 3; (–), below detection, AA, arachidonic acid; EPA, eicosapentaenoic acid; DHA, docosahexaenoic acid; SFA, saturated fatty acids; MUFA, monounsaturated fatty acids; PUFA, polyunsaturated fatty acids; Other includes components present at <2%: i15:0, a15:0, 15:0, i16:0, C₁₆ PUFA, 16:1(n-9)c, 16:1(n-7)t/16:2, 16:1(n-5)c, 16:0 Falde (fatty aldehyde), 16:1 Falde, i17:0, a17:0, 17:1, 17:0, 18:3(n-6), i18:0, 18:1(n-7)t, 18:1(n-5)c, 18:0 Falde, i19:0, 19:1, 20:3(n-6), 20:2(n-6), 20:1(n-11)c, 20:1(n-9)c, 20:1(n-7)c, 20:0, C₂₁ PUFA, 21:0, 22:5(n-6), 22:4(n-6), 22:5(n-3), 22:1(n-11), 22:1(n-7), 22:0, 24:1, 24:0.

TABLE 4.

Percentage fatty acid composition of phyllosomata from feeding trial.

	Newly Hatched	A1 DHA Selco–C. muelleri[a]			Ethyl Ester–Mussel[a]			Mussel Power–Polar Lipid[b]		
		II	IV	V	II	IV	V	II	III	IV
16:1(n-7)c	4.1 ± 0.2	2.7 ± 0.1	1.8 ± 0.0	3.1 ± 0.2	1.6 ± 0.0	0.9 ± 0.0	1.6 ± 0.2	2.1	2.1 ± 0.4	0.9 ± 0.0
16:0	12.2 ± 0.4	10.7 ± 0.5	10.0 ± 0.0	11.3 ± 0.1	10.4 ± 0.0	9.0 ± 0.2	10.1 ± 0.3	15.4	15.1 ± 2.2	12.2 ± 1.0
17:0	1.6 ± 0.0	0.8 ± 0.0	0.8 ± 0.0	0.9 ± 0.0	0.8 ± 0.0	0.6 ± 0.0	0.7 ± 0.1	4.6	1.3 ± 0.2	0.9 ± 0.1
18:2(n-6)	0.7 ± 0.0	17.4 ± 0.4	21.7 ± 0.2	18.3 ± 0.3	17.1 ± 0.1	20.1 ± 0.0	18.3 ± 0.3	9.7	5.4 ± 0.8	13.0 ± 2.4
18:1(n-9)c/18:3(n-3)	8.1 ± 0.2	26.5 ± 0.4	27.4 ± 0.2	23.5 ± 0.3	24.8 ± 0.3	24.9 ± 0.1	22.7 ± 0.5	14.5	9.4 ± 1.8	17.6 ± 3.4
18:1(n-7)c	4.9 ± 0.1	5.9 ± 0.1	4.7 ± 0.0	5.8 ± 0.1	5.6 ± 0.1	4.0 ± 0.0	4.5 ± 0.1	3.9	4.1 ± 1.0	3.9 ± 0.5
18:0	8.3 ± 0.0	7.4 ± 0.1	7.2 ± 0.1	8.5 ± 0.2	8.3 ± 0.1	7.5 ± 0.1	8.7 ± 0.1	13.3	10.9 ± 1.2	11.6 ± 1.0
20:4(n-6)	5.1 ± 0.1	2.4 ± 0.1	2.0 ± 0.0	2.6 ± 0.1	4.7 ± 0.2	4.3 ± 0.1	4.3 ± 0.1	6.1	5.2 ± 0.8	5.4 ± 0.3
20:5(n-3)	21.0 ± 0.2	10.1 ± 0.4	6.9 ± 0.1	8.2 ± 0.2	10.8 ± 0.1	8.8 ± 0.3	8.6 ± 0.2	10.2	14.1 ± 2.4	8.7 ± 0.5
20:2(n-6)	1.7 ± 0.0	0.0 ± 0.0	–	1.5 ± 1.3	0.0 ± 0.0	0.8 ± 1.5	1.8 ± 1.6	–	2.6 ± 0.2	2.1 ± 1.8
20:1(n-11)c	0.3 ± 0.0	1.7 ± 0.1	2.3 ± 0.0	0.8 ± 1.3	1.8 ± 0.0	1.7 ± 1.5	0.8 ± 1.4	–	–	1.1 ± 1.9
22:6(n-3)	13.5 ± 0.3	5.3 ± 0.3	4.0 ± 0.1	3.5 ± 0.1	5.4 ± 0.4	6.3 ± 0.2	4.9 ± 0.2	5.6	8.6 ± 1.5	5.9 ± 0.2
22:1(n-9)	0.8 ± 0.1	0.1 ± 0.0	1.6 ± 0.2	1.8 ± 0.3	0.1 ± 0.0	1.7 ± 0.2	1.9 ± 0.1	0.5	3.9 ± 2.6	3.2 ± 1.8
Other	17.5	9.0	9.7	10.3	8.6	9.2	10.8	14.0	17.1	13.4
Sum SFA	27.6 ± 0.3	22.2 ± 0.3	21.1 ± 0.2	24.6 ± 0.4	22.8 ± 0.1	20.1 ± 0.3	23.7 ± 0.9	35.6	34.8 ± 5.0	29.9 ± 3.4
Sum MUFA	23.8 ± 0.3	40.2 ± 0.4	40.8 ± 0.1	38.0 ± 1.0	37.0 ± 0.4	35.8 ± 1.4	34.4 ± 2.0	24.8	23.1 ± 0.7	29.8 ± 2.0
Sum PUFA	44.4 ± 0.7	37.3 ± 0.7	37.9 ± 0.2	35.7 ± 0.3	39.8 ± 0.6	43.0 ± 0.4	39.8 ± 0.8	39.6	37.6 ± 4.8	37.5 ± 1.6
Sum (n-3)	36.6 ± 0.5	16.5 ± 0.8	11.8 ± 0.2	12.4 ± 0.1	16.9 ± 0.5	15.9 ± 0.5	14.3 ± 0.3	18.3	24.0 ± 4.0	15.6 ± 0.3
Sum (n-6)	8.5 ± 0.1	20.4 ± 0.3	25.4 ± 0.3	24.1 ± 1.4	22.5 ± 0.1	27.2 ± 1.6	26.8 ± 1.9	21.3	15.2 ± 1.3	23.2 ± 2.6
Ratio (n-3)/(n-6)	4.3	0.8	0.5	0.5	0.8	0.6	0.5	0.9	1.6	0.7
Ratio EPA/AA	4.1	4.3	3.5	3.1	2.3	2.0	2.0	1.7	2.7	1.6
Ratio DHA/EPA	0.6	0.5	0.6	0.4	0.5	0.7	0.6	0.6	0.6	0.7

Presented as mean ± SD, $n = 3$.

[a] Enriched *Artemia*.

[b] Feed station (molted from stage II to III only).

[c] $n = 1$.

(–), below detection.

AA, arachidonic acid; EPA, eicosapentaenoic acid; DHA, docosahexaenoic acid; SFA, saturated fatty acids; MUFA, monounsaturated fatty acids; PUFA, polyunsaturated fatty acids; (–), below detection; Other includes components present at <2%: 14:0, i15:0, a15:0, 15:0, i16:0, C_{16} PUFA, 16:1(n-9)c, 16:1(n-7)t/16:2, 16:1(n-5)c, 16:0 Falde (fatty aldehyde) i17:0, a17:0, 17:1, 17:0, 18:3(n-6), 18:4(n-3), i18:0, 18:1(n-7)t, 18:1(n-5)c, 18:0 Falde, i19:0, 19:1, 20:3(n-6), 20:4(n-3), 20:1(n-11)c, 20:1(n-7)c, C_{21} PUFA, 2 22:4(n-6), 22:5(n-3), 22:5(n-6), 22:1(n-11), 22:1(n-7), 24:1, 24:0.

ence between this and the majority of previous trials has been the daily use of antibiotics in static culture. Although static culture and antibiotics are less appropriate for medium to large-scale culture of phyllosomata (Ritar 2001), they have been used in raising phyllosomata to pueruli (Matsuda & Yamakawa 2000). Additionally, the growth results from this trial, although similar to a previous trial, had much tighter standard deviations (Nelson et al. 2003). This suggests that larvae from this trial had more similar environmental parameters resulting from both aquarium design and use of antibiotics. The successful use of antibiotics in static culture in this experiment highlights the fact that because the vital aspects of phyllosomata culture (i.e., feeding capabilities, nutritional requirements, aquarium design and microbial loading) are intrinsically linked, advances cannot be readily made sequentially, and should ideally be performed concurrently. In previous trials, it has been difficult to test the effectiveness of feeding phyllosomata on *Artemia*, including using different enrichments, when experiments may be confounded by the adverse effects of microbial loading and aquarium design. The higher survival and good growth in this trial suggest that enriched *Artemia* may be adequate for early stage phyllosomata.

The dominance of TAG in enriched *Artemia* illustrates the propensity for readily incorporating TAG from nutrient sources, as well as metabolizing EE to TAG for assimilation into their tissues. Similar results were observed when providing *Artemia* with a high-PL diet (Nelson, unpublished). These lipid class results are comparable to previous trials using 5-day old *Artemia* (Nelson et al. 2002b, Smith et al. 2002, Nelson et al. 2003).

A distinction between this trial and an earlier trial (Nelson et al. 2003) is the detection of TAG in phyllosomata, albeit at low amounts and the higher relative proportion of DG. Although the difference is small, the presence of these short-term energy storage molecules is consistent with improved larval health. In a prior feeding trial, total lipid content dropped markedly in phyllosoma to below 100 mg g^{-1} by stage IV, and was also accompanied by poorer survival (Nelson et al. 2003). This result contributed to the hypothesis that phyllosoma, like puerulus (Jeffs et al. 2001), may be better served by use of PL, rather than TAG (Nichols et al. 2001, Nelson et al. 2003). Animals in the present trial did not experience the same marked decrease in lipid content. This finding may be the result of a number of reasons. First, if lipid is critical to survival, a drop in total lipid is associated with the poorer survival in previous trials. Maintenance of lipid at above 100 mg g^{-1} in the present trial may therefore be linked with good survival. Second, because animals had high survival, but still did not have total lipid equal to wild phyllosomata (250 mg g^{-1} lipid dry mass
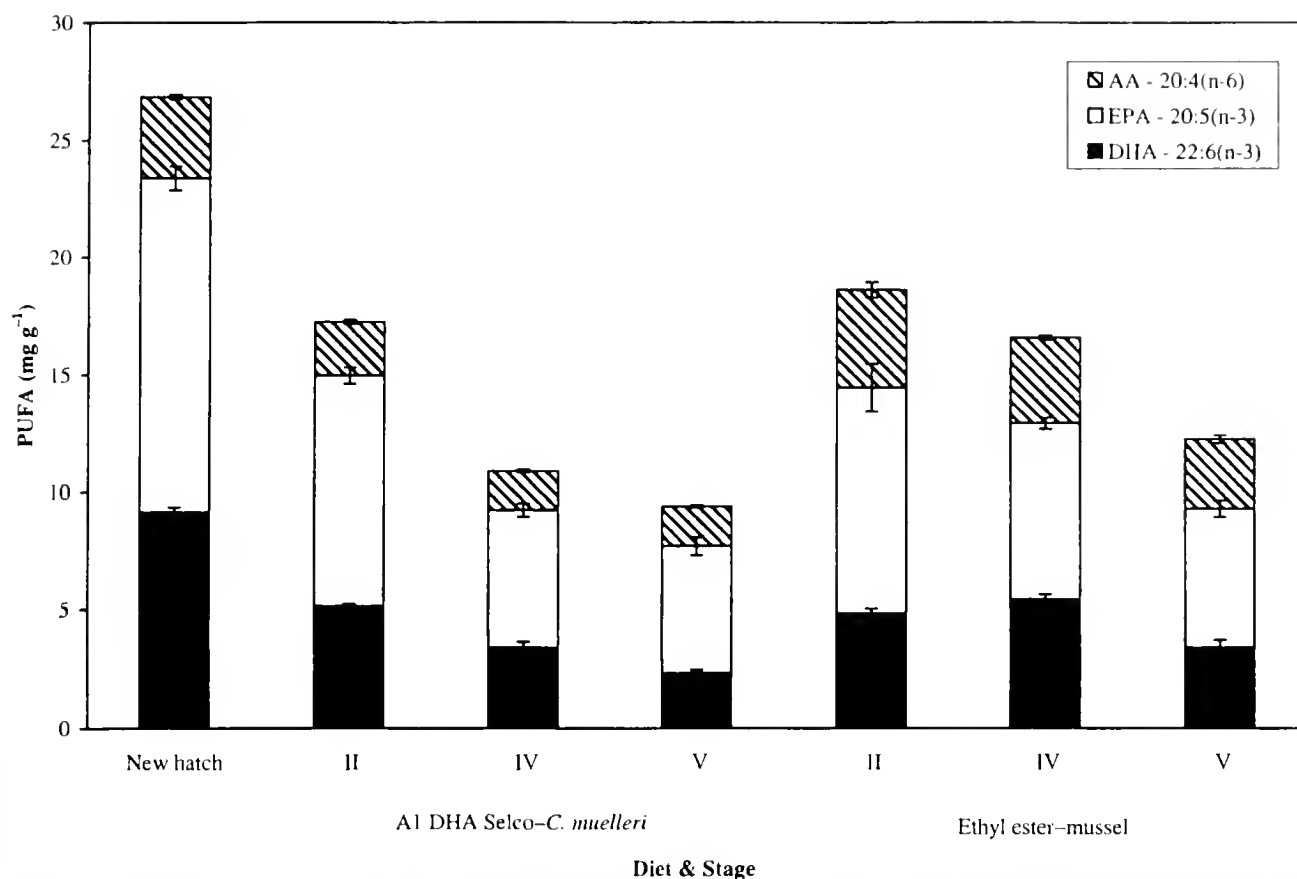
Figure 2. Content (mg g^{-1}) of the essential long chain-polyunsaturated FA arachidonic acid (AA), eicosapentaenoic acid (EPA), and docosahexaenoic acid (DHA) in *J. edwardsii* phyllosomata from stages I to V on two diet treatments of *Artemia* enriched with either A1 DHA Selco–*C. muelleri* or ethyl ester–mussel nutrient sources. Presented as mean ± SD.

at stage V) (Phleger et al. 2001), the class of lipid provided (i.e., TAG in feeding trial versus largely PL in wild) was less effective. Thirdly, aquarium design and microbial loading can affect metabolism of lipids in larvae. For example, in previous trials, conducted in flow-through aquaria without antibiotics, and the present trial, conducted in static aquaria with antibiotics, *Artemia* that were similarly enriched with DHA Selco–*C. muelleri* were fed to phyllosomata. Animals from the present trial had 189 mg g^{-1} lipid dry mass at stage V, while animals in the previous trial had 50 mg g^{-1} lipid dry mass at stage V (Nelson et al. 2003). The animals in the previous trial either did not store lipid, or used more lipid as energy, while under the strain of microbes and/or swimming. Although *Artemia* supported excellent survival for larval stages I–V in the present trial, the *Artemia* diet may still not sufficiently condition phyllosomata for later stages; *Artemia* may not be providing adequate total lipid for growth and high survival, especially if the aspects of aquarium design and microbial loading are not addressed.

The current emphasis in phyllosoma culture in Australia is the use of *Artemia* for feeding stages I–V. This concept stems from developments with other aquaculture species, such as marine finfish, where it has been impossible to grow them during the early part of their life cycle without using live, motile feed (Olsen 1997, Castell et al. 1998). With rock lobster, complete rearing of phyllosoma to puerulus was achieved by feeding on *Artemia*, fish larvae and/or mussel tissue (Kittaka 1997b, a, Kittaka & Abrun-

hosa 1997, Matsuda & Yamakawa 2000). Mussel gonad has been identified as the key to this success (Kittaka 1997b), used exclusively after the third instar (Kittaka 1997a). In culture, phyllosomata have been observed ingesting inanimate food particles, such as lobster, prawn and mussel pieces at late stages (Thomas, unpublished). Early stage animals have likewise been observed consuming pieces of mussel, jellyfish and other inanimate foods (Mitchell 1971, Nelson et al. 2002a, Cox & Johnston 2003). Phyllosomata in the present study were no exception. The larvae were observed consuming the Mussel powder–polar lipid feed station diet, a diet with which we attempted to build on the success of using mussel gonad. Additional evidence of feeding was the presence of faecal trails, and molting, considering that phyllosomata do not molt when not feeding (Abrunhosa & Kittaka 1997). Nevertheless, since phyllosomata fed the Mussel powder–polar lipid diet failed to molt properly beyond more than one stage, there is perhaps a necessary component either not present in sufficient amounts, or lost by leaching, in the feed station diet that contributes to molting. Therefore, the use of *Artemia* up to the third instar (Kittaka 1997a) remains valuable for phyllosomata. However, to improve conditioning of larvae, the potential use of co-feeding of *Artemia* (Dhert et al. 1999), along with a PL source, should be examined, particularly for later stage animals.

Of note is the decrease in essential PUFA from newly hatched to stage V phyllosomata. On a relative basis, *Artemia*-fed phyllosomata and wild-caught animals at stage V had similar levels of

AA (trial, 3–4%; wild, 2–3%) and EPA (trial, 8–9%; wild, 7–9%), with markedly lower DHA in cultured animals (trial, 4–5%; wild, 16–17%) (Phleger et al. 2001). Results from a previous feeding trial are similar for relative levels of these FA (3–6% AA; 8–9% EPA; 2–4% DHA) (Nelson et al. 2003). However, because the larval lipid remained above 189 mg g⁻¹ lipid dry mass at stage V, on an absolute basis this trial represents a marked improvement for incorporation of essential PUFA. The fact that the amount of total lipid remained the same to stage V, but there was a drop in the level of essential PUFA, in particular DHA, highlights the importance of these FA. Higher absolute concentrations of these FA may be associated with enhanced survival and growth in this feeding trial compared with previous trials (Nelson et al. 2003, Hart et al., unpublished). Total lipid and levels of essential PUFA in phyllosomata fed ethyl ester–mussel-enriched *Artemia* were higher than in larvae fed A1 DHA Selco–*C. muelleri*-enriched *Artemia*. Because there was no direct association of enhanced FA profiles with survival and growth for phyllosomata from the two *Artemia* diet treatments, and the majority of lipid provided to phyllosomata through enriched *Artemia* was TAG, we propose that the improved survival and growth in this trial may result from the presence of lipid in the diet (as described above) in combination with better health. Furthermore, we suggest it is likely that the PUFA profiles will have a more significant effect if provided in a PL form. These results also support the suggestion that co-feeding of *Artemia* and a PL source should be trialed to improve larval condition.

In conclusion, the use of antibiotics and static culture has enabled a clearer picture of the effects of nutrition on larval health. Our experiment demonstrated that lipid-enriched *Artemia* support excellent growth and survival in early stages of phyllosomata, and we are now better placed to take nutrition of phyllosoma forward. The results suggest that the class of lipid provided via *Artemia* may not adequately condition larvae, nor supply sufficient quantities of the essential PUFA, in particular DHA, for later stages. Thus, for successful culture of phyllosomata, the development of a formulated diet, which can provide the nutritional requirements in the right form to enhance long term conditioning of the larvae, is likely to be vital, particularly for later stage larvae.

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THE RELATIONSHIP BETWEEN HEMOLYMPH CHEMISTRY AND MOULT INCREMENT FOR THE SOUTHERN ROCK LOBSTER, *JASUS EDWARDSII* HUTTON

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ABSTRACT   Growth data are essential to rock lobster fisheries stock assessment. At present, predictions of growth for a given year are based on data from previous years with the accuracy of the estimates being unknown until measures of growth are obtained in the year in question. This article tests the hypothesis that premoult hemolymph lipid concentration is a predictor of moult increment for the southern rock lobster, *Jasus edwardsii*, in the laboratory. The study was undertaken to develop a nonlethal means of moult increment prediction, which could then be used in the field. Premoult carapace length had no effect on percent moult increment ($P >$ 0.05) in the laboratory. Both phospholipid and triglyceride were significantly correlated with percent moult increment. Phospholipid showed the highest coefficient at $r^2 = 0.66$. Our data suggest that hemolymph phospholipid level has the potential to predict moult increment. However, the hemolymph lipid/moult increment data were gathered over a short time period and within a relatively controlled environment. Further field studies are essential to better understand the relationship between hemolymph lipid level and moult increment in wild populations of this species.

KEY WORDS: *Jasus edwardsii*, lipid, moult increment, moulting, growth

INTRODUCTION

Growth data are essential to rock lobster fisheries stock assessment. At present, predictions of growth for a given year are based on data from previous years with the accuracy of estimates being unknown until measures of actual growth are obtained for the year in question.

The shedding of all hard parts at ecdysis complicates measurements of growth in rock lobsters and other crustaceans. No structures are retained (*sensu* fish otoliths) from which age at size, and therefore growth information, may be gathered. During recent work in South Africa, Cockcroft (1997) suggested that growth may be estimated from hepatopancreatic lipid level having found a significant relationship between moult increment and percent hepatopancreatic lipid during premoult in *Jasus lalandii*. The finding of this relationship was a significant advance, although it was still necessary to kill the animal to gather the data, a step that might be avoided by isolation of an equally useful hemolymph component. During a recent study, Musgrove (2001) found that hemolymph protein, in combination with hemolymph pigment level and moult stage, was useful in distinguishing between lobsters at high and low growth sites within the South Australian fishery. He was able to show that grouping serum protein data by pigment stage with reference to the major pigment, astaxanthin, allowed the differentiation of lobsters at the beginning and those at the end of intermoult. Given the correlation between serum protein and % dry weight, differences in lobster condition between high and low growth sites could be examined more thoroughly using this method. Hemolymph protein has been used successfully in other studies as a measure of condition (Leavitt & Bayer 1977, Musgrove 2001) but has not been shown to be useful in predicting moult increment. Given Cockcroft's work, premoult hemolymph lipid appeared to be the most likely to show a predictive relationship with moult increment. If hemolymph lipid could be used in place of total hepatopancreas lipid to predict moult increment, the necessity to kill the lobster would be avoided and multiple samples may be taken over time from the same individual.

Phospholipids are the major circulating lipid and triglycerides the major storage lipid in crustaceans. Both are found in the hemolymph and hepatopancreas (Chang & O'Connor 1983). The hepatopancreatic lipid component of *Jasus lalandii* is largely triglycerides (neutral lipids) with phospholipids (polar) of less importance (<14%) Cockcroft (1997). In the hepatopancreas, ingested neutral lipids are cleaved to mono or diglycerides, which are then converted to phospholipids. These are expelled into the hemolymph and transported to various tissues, either for use as membrane components or conversion to triglycerides and storage (Chang & O'Connor 1983).

The hepatopancreas of rock lobsters increases in size and lipid content through the moult cycle, reaching a maximum just before ecdysis (Musgrove 2000b, Cockcroft, 1997) it may also be expected that other chemical compounds would show similar patterns. Thus, as the hepatopancreas reached maximum storage during late premoult (Musgrove 2001, Mercaldo-Allen 1991), so hemolymph lipid would reach maximum concentration. Furthermore, as Cockcroft (1997) found that hepatopancreas lipid was an indicator of moult increment in the field, so may hemolymph lipid be, as phospholipid would be used for both the cell membranes of the expanding hepatopancreas and, after conversion to triglyceride, as the main lipid store.

This article tests the hypothesis that premoult hemolymph lipid concentration is a predictor of moult increment for the southern rock lobster, *Jasus edwardsii*, in the laboratory and examines the relationship between hemolymph and hepatopancreatic lipid content and tissue weight. The study was undertaken to develop a non-lethal means of moult increment prediction, which could then be used in the field.

MATERIALS AND METHODS

Laboratory Experiment 1: Relationship Between Moult Increment and Premoult Hemolymph Level

Forty lobsters (mean CL: 89.88 ± 0.60 mm, mean weight 364.6 ± 6.54g) were individually housed in 30-L plastic tanks in a flow through system (0.4 L/h/tank) for 185 days. Each tank was independently supplied with air and water of a constant temperature

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(18°C, which was similar to the average summer temperature in the area of capture). Day length was set at 12 h and the lights covered with red cellophane to minimize disturbance. Lobsters were fed *ad libitum* daily on a mixed diet of artificial pellets (four pellets/feed, Geddes et al. 2000) and cockles (four cockles/feed, *Donax deltoides*) in a rotation. Daily consumption was assessed by eye from day 52 and categorized as 0, <25%, 25–50%, and >50%. Excess food was removed and tanks cleaned each morning, taking care to minimize disturbance to the lobsters.

Hemolymph samples (0.5 mL) were taken fortnightly from each lobster by pericardial puncture for analysis of hemolymph serum. Once the pigment stage of each hemolymph sample had been noted (Musgrove 2001) it was snap-frozen (−196°C) for later analysis. Pigment stage refers to the color of the hemolymph, which changes from light blue through beige to deep orange during the moult cycle, the beige becoming visible during intermoult (Musgrove 2001). If the lobster was immediately premoult, samples were taken before and after ecdysis. Pleopod samples were also taken periodically to track moult stage by examination of setal development (Musgrove 2000).

Laboratory Experiment 2: Relationship Between Moult Increment and Premoult Hemolymph Level in a Less-Controlled Environment

Seven premoult lobsters were selected from animals that had been kept in an outside tank for several months with other species (echinoderms, other decapods) and fed two to three times a week on blue mussels (*Mytilus* sp.). The tanks were at ambient temperature (about 16°C) and contained abundant limestone rocks, *Macrocystis* sp., *Ulva* sp. and other aquatic macrophytes. The selected lobsters were measured (range, 65.3 to 103.8 mm CL), moult staged (after Musgrove 2000) and placed in plastic cages within the aquaria. They were fed mussels *ad libitum* 3 to 4 times a week. Pleopods were taken regularly to keep track of the moult stage and, during late premoult (Stage D₃) a 0.2-mL hemolymph sample was taken, pigment staged, then snap frozen. Once each lobster had hardened (i.e., at intermoult) it was re-measured. The data were then compared with those collected from laboratory experiment 1.

For experiments 1 and 2, blood was taken during the afternoon to standardize postprandial effects on hemolymph lipid (*sensu* Dall 1981). Lobsters were fed after extraction was completed. In both cases, lobsters were not observed to feed during daylight.

Field Study: Relationship Between Tissue Lipid and Hemalymph Lipid Level

One hundred and thirty nine rock lobsters were collected from the wild fishery as described by Musgrove (2001) and hemolymph samples taken as described above within 3 h of capture, the pigment stage noted and the sample snap-frozen (−196°C) for later serum lipid analysis. A pleopod was also taken for moult stage determination by examination of setal development (Musgrove 2000). The lobsters were then frozen (−30°C) and retained for dissection and tissue analysis.

Within two weeks of collection, lobsters were rapidly thawed and the abdominal tissue and hepatopancreas removed, weighed then dried to constant weight (60°C, 72 h). The tissue was then allowed to cool to room temperature in a desiccator over silica gel, reweighed (to nearest 0.1 mg) and dry weight and percent dry weight calculated.

Hemolymph Serum Analysis

All whole hemolymph samples from the laboratory study and a random selection of samples from the field collection ($n = 139$) were analyzed for triglyceride and phospholipid. The clotted hemolymph was thawed then broken up gently with a glass stirring rod and the sample centrifuged (Hettich EBA12 centrifuge, 15 min, 17,280g) to extract the serum. Serum aliquots were analyzed on a Cobas Mira Autoanalyser for triglyceride and phospholipid using commercially produced test kits (Roche). To test for phospholipid the triglyceride kit (Roche, No. 07 3679 1) was modified as follows. 250 units phospholipase C (Sigma No. P4014) were added to a 30-mL bottle of triglyceride reagent. The modified reagent was then incubated with the serum sample for 15 min at 37 C (cf 6 min for triglyceride) to convert the serum phospholipids to diglycerides, which were then converted to glycerol by the lipase in the kit. The incubation time was chosen by incubating a lecithin solution (2 mM) to give a result equivalent to 2 mM triglyceride. Accuracy was maintained for all tests using commercially available quality controls (Nycomed Farmer).

Data Analysis

If data were normally distributed or could be normalized analyses were performed using analysis of covariance (ANCOVA) or analysis of variance (ANOVA) with the GLM module (General Linear Models) on SPSS. If data could not be normalized, the Kruskel Wallis nonparametric ANOVA or the Wilcoxon Rank Sign were used. In all cases significance was accepted at $P = 0.05$.

RESULTS

Laboratory Experiments 1 and 2

Percent Moult Increment, Tank Placement, and Feeding Regimen

Premoult CL had no effect on % moult increment ($P > 0.05$, ANCOVA), and there was considerable overlap between the ranges of % moult increment recorded in the outside tanks (2.4 to 8.0% of premoult CL, $n = 7$) and those inside (0.8 to 5.2%, $n = 9$). The slopes of the percent moult increment: lipid regressions were the same for inside and outside tanks ($P > 0.05$, ANCOVA). For this reason, data from inside and outside tanks were pooled for further analyses.

Hemolymph Serum Lipid and Moult Increment

Both lipid fractions were significantly correlated with percent moult increment (Table 1). Phospholipid showed the highest coefficient at $r^2 = 0.66$. Both phospholipid and triglyceride showed a progressive increase with pigment stage (Fig. 1) until PS3.0 to 4.0 then declined to PS4.5.

TABLE 1.

Relationship between percent moult increment and haemolymph lipid (μmol^{-1}) for phospholipid, triglyceride, and TP (triglyceride + phospholipid).

Parameter	α	β	r^2	F	P
Phospholipid	0.0715	2.072	0.66	30.062	<0.001
Triglyceride	0.1140	2.248	0.403	11.123	0.005
TP	−0.0786	1.765	0.641	27.781	<0.001

The regression model is Log % moult increment = $\alpha[\log (\text{lipid})]^\beta$, $n = 16$.
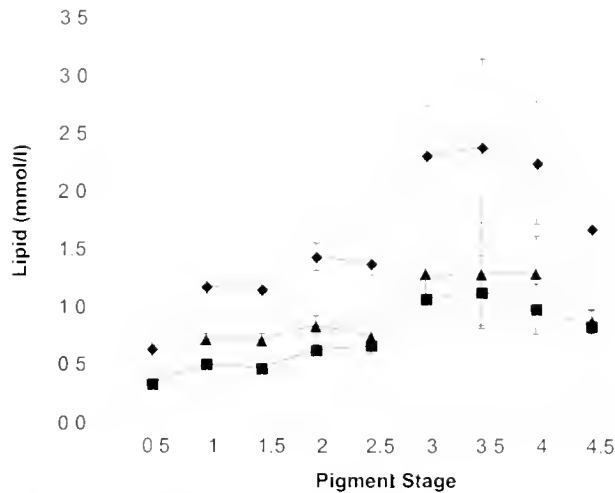
Figure 1. Laboratory: mean hemolymph serum triglyceride (mmol/L) ±SE, phospholipid (mmol/L) ±SE, and triglyceride plus phospholipid (TP) (mmol/L) ±SE vs pigment stage. Closed diamond, TP; closed square, triglyceride; closed triangle, phospholipid.

Figure 3. Field: mean hemolymph serum triglyceride (mmol/L) ±SE, phospholipid (mmol/L) ±SE, and triglyceride plus phospholipid (TP) (mmol/L) ±SE vs pigment stage. Closed diamond, TP; closed square, triglyceride; closed triangle, phospholipid.

Feeding Rates

Feeding rate increased 4-fold after the moult in those lobsters for which there was data (Fig. 2). There were no sexual differences in feeding rate ($P > 0.05$).

Field Study

Hemolymph Serum Lipid

The field serum lipid data showed a progressive increase in lipid content with pigment stage (Fig. 3) in a similar fashion to that found in the laboratory, although in this case the peak occurred at PS4.

Hemolymph Lipid and Hepatopancreas Weight

Hemolymph lipid increased with hepatopancreas dry weight on both a total weight and a percentage basis (Fig. 4a and b) up to PS4. However, although hemolymph lipid was significantly correlated with tissue weight during intermoult (Table 2), the relationship declined after PS2-2.5.

Laboratory Experiments and Field Study Comparisons

Moult Stage and Pigment Stage

The relationship between moult stage and pigment stage was similar in the laboratory and the field (Fig. 5). In the following

Figure 2. Mean feeding rates (±SE) for 10 weeks before and after ecdysis. Four daily consumption categories (0, <25, 25–50, >50%) were used and assigned numbers from 1 to 4 ($n = 18$).

analysis, comparisons are made between pigment stage-specific laboratory and field hemolymph lipids. Before this was done, analysis was undertaken to check that the same pigment stages had similar distributions of moult stages in the laboratory and the field. To facilitate the analysis each moult stage was assigned a number (1–11).

The laboratory distribution of moult stages within each pigment stage was similar to that in the field (Mann-Whitney U, Zar 1984). The only significant difference was in PS 4 (U = 37.5, $P = 0.014$, mean moult stage$_{laboratory}$ = 9.46 ± 0.39, mean moult stage$_{field}$ = 8.15 ± 0.249), otherwise $P \geq 0.212$.

Hemolymph Serum Lipid

Pigment stage-specific total lipid of laboratory animals was greater than that in the field until PS3.5 (Mann-Whitney U, $P \leq 0.05$; Fig. 6). The patterns in the relative importance of the two lipid fractions were also different. In the field, the proportion of phospholipid increased until PS 2.5 (Fig. 7) then fell until PS 4.5, in contrast to the laboratory where the peak was reached during PS1. Both laboratory and field showed the same trends after PS2.5.

DISCUSSION

The key result to come out of this study is the potential use of hemolymph lipid in the prediction of percent moult increment. Although further field studies are needed to be sure of the result, this outcome is potentially very useful because hemolymph lipid measurement does not require killing the lobster. Questions remain as to whether higher growth sites, showing higher serum protein content would also have higher moult increments. In this regard, significant differences were reported in mean serum protein level between sites by Musgrove (2001). The differences occurred mainly during intermoult, which is the period when hemolymph lipid is significantly correlated with both serum protein and hepatopancreas percentage dry weight. This may suggest a relatively higher degree of lipid accumulation at those sites, pointing to a higher moult increment. Dall (1981) suggested that the principal function of digestive gland lipid in *Nephrops norvegicus* was
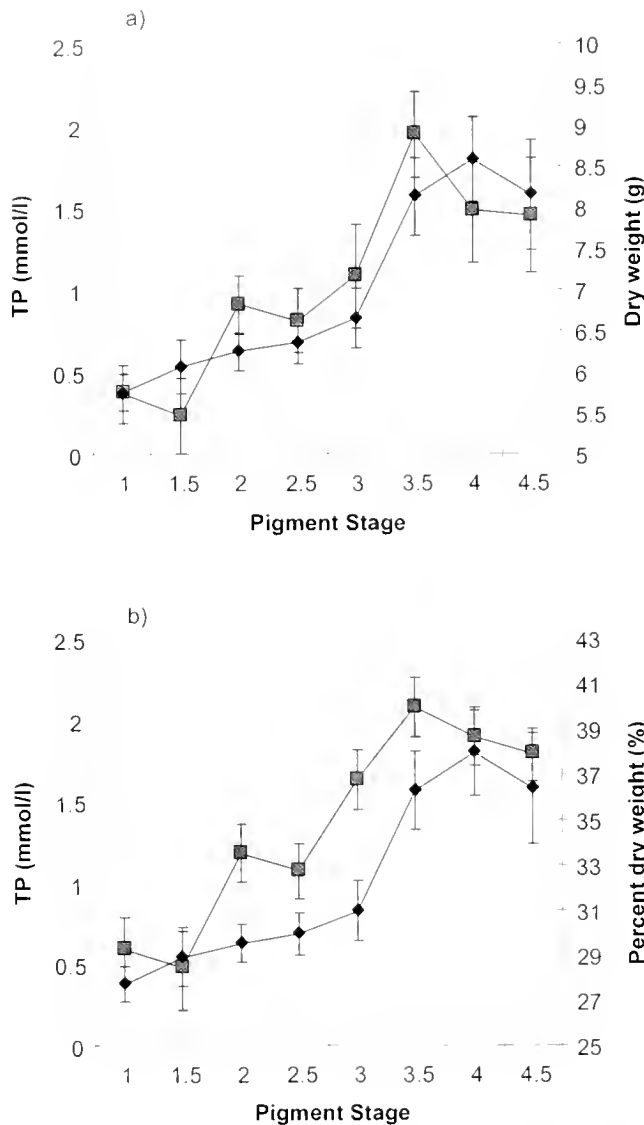
Figure 4. Comparison of (a) hepatopancreatic mean dry weight (g, ±SE), (b) mean percent dry weight (±SE), and triglyceride plus phospholipid (TP) vs pigment stage. Mean dry weight (g) standardized for carapace length using GLM analysis of SPSS. Data are displayed for a 97.9-mm CL lobster. Closed diamond, TP; closed square, weight (g).

in the moulting process so one might expect that lipid accumulation in *Jasus edwardsii* would be similarly focused.

Cockcroft (1997) also found a significant relationship between moult increment and hepatopancreas lipid level, for *Jasus lalandii*. He reported that moult increment was positively related to peak %lipid values occurring during late premoult in the hepatopancreas, similar to the present study, where the significant relationship was between hemolymph lipid (μmol/L) and % moult increment. Furthermore, he suggested a "window" period of reserve accumulation, essential for growth. This occurs from intermoult to early premoult, especially the former, as suggested for *J. edwardsii* by the relative increase in feeding rate after ecdysis. The period of reserve accumulation (PRA) would probably lead up to a "reserve saturation point" as suggested by Anger (1987) for crustacean larvae. Cockcroft found that lobsters starved during PRA, then feed during premoult, showed severely reduced growth rates and

TABLE 2.

Field data regression statistics for pigment stage-specific percent dry weight and total dry weight versus total lipid (T + P).

Percent or Total Weight	PS	r^2	F	P	n
Percent	1	0.722	17.33	<0.001	25
	1.5	0.887	118.29	<0.001	17
	2	0.599	29.93	<0.001	24
	2.5	0.685	41.39	<0.001	21
	3	0.448	8.10	.017	12
	>3	0.067	1.93	0.176	29
Total Weight	1	0.532	24.96	<0.001	25
	1.5	0.767	49.50	<0.001	17
	2	0.389	13.37	0.001	24
	2.5	0.282	7.47	0.013	21
	3	0.000	2.9^{-04}	0.987	12
	>3	0.078	2.29	0.142	29

Regression model is Lipid $= \alpha$ Weight$^\beta$ except for percent dry weight at pigment stage 1, where the best fit was given by the cubic model (lipid $= \alpha + \beta_1$ weight $+ \beta_2$ weight$^2 + \beta_3$ weight^3)

even shrinkage. Those starved prior to moulting but fed during PRA moulted with similar growth increments to those of control lobsters, which were fed throughout. Therefore, it is this PRA that is critical to future growth, influencing both moult increment and intermoult period (Cockcroft, 1997).

The question is, why should the percent moult increment be correlated with the lipid level in the hemolymph at PS4.5, when it was not related to the hepatopancreas percent dry tissue at that stage? At PS4.5 about 95% of lobsters were beyond D_1'''. The rigorous investigation of this question is outside the framework of this study but it may be that the apparent decoupling of the relationship between hepatopancreas weight and hemolymph lipid at the later pigment stages is due to a mobilization of lipid reserves from the hepatopancreas to the hemolymph in preparation for the energetic demands of ecdysis. The correlation may arise because the higher the level of stored lipid in the hepatopancreas, the greater the reserve that may be mobilized in readiness for ecdysis.
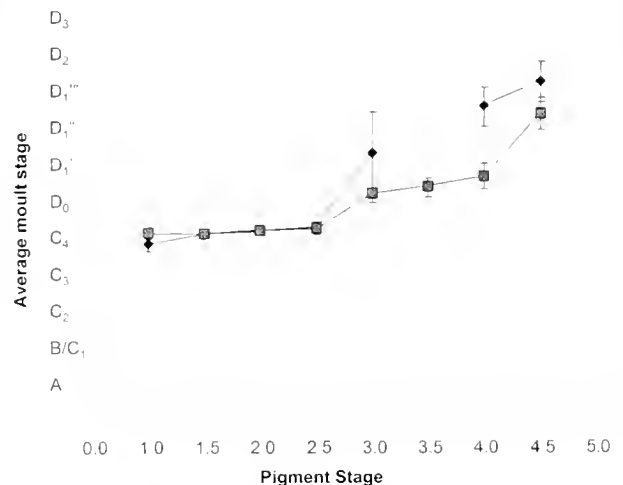
Figure 5. Mean moult stage (±SE) within each pigment stage for laboratory experiments (pooled, n = 40) and field study (n = 135). There were no lobsters at PS3.5 in the laboratory sample. Closed diamond, laboratory; closed square, field.
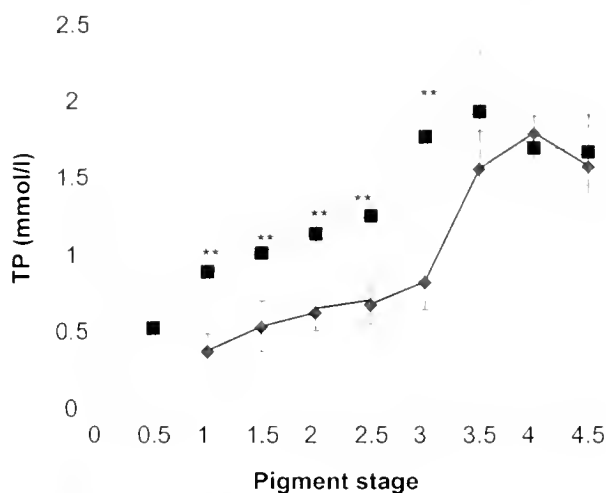
Figure 6. Mean triglyceride and phospholipid (TP; mmol/L) ±SE: field vs laboratory data by pigment stage. Significant differences (Mann–Whitney U test) between laboratory and field data are indicated by asterisks (**$P \leq 0.001$). Closed square, laboratory TP; closed diamond, field TP.

The importance of phospholipid in the relationship fits in with Chang and O'Connor's (1983) contention that phospholipid is the main circulating lipid in crustaceans. Bligh and Scott (1966) reported that 65% of the total lipid in the hemolymph of the lobster, *Homarus americanus*, was phospholipid, with the remainder almost equally divided between triglycerides and sterols. The latter has a primarily structural role (Fraser, 1989). Free fatty acids comprised only about 2.4% of the total lipid. O'Connor and Gilbert (1969) reported similar results for the land crabs, *Gecarcinus lateralis* and *Cardiosoma guanhumi*.

Finally, the relative levels of hemolymph protein and lipid in the laboratory and the field suggest that the rate of accumulation differs, particularly in the early stages of the moult cycle. One would assume that these differences occur because captive lobsters did not have to hunt for food, more nutrients being directed to muscle accumulation and lipid storage earlier in the moult cycle.

Our data suggest that hemolymph phospholipid level has the
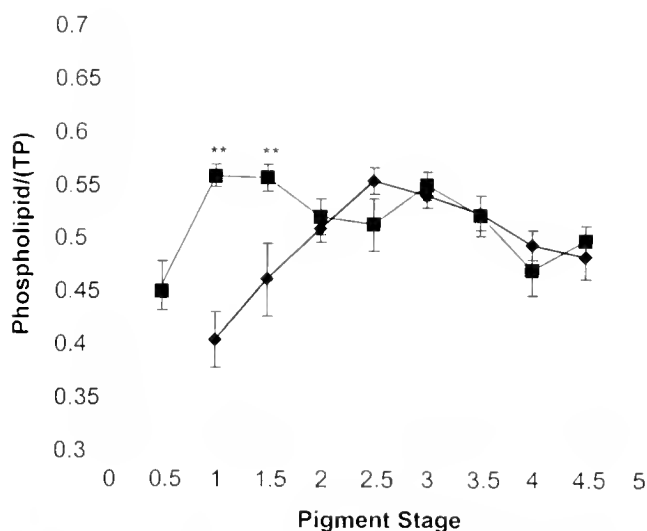
Figure 7. Hemolymph serum phospholipid/(triglyceride plus phospholipid, mmol/L) ±SE: field vs laboratory data by pigment stage. Significant differences (Mann–Whitney U test) between laboratory and field data are indicated by asterisks (**$P \leq 0.001$). Closed square, laboratory TP; closed diamond, field TP.

potential to predict moult increment. However, the hemolymph lipid/moult increment data were gathered over a short time period and within a relatively controlled environment. Further field studies are essential to better understand the relationship between hemolymph lipid level and moult increment in wild populations of this species.

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BLUE CRAB MORTALITY IN THE NORTH CAROLINA SOFT-SHELL INDUSTRY: BIOLOGICAL AND OPERATIONAL EFFECTS[1]

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ABSTRACT The rapid growth of the soft-shell blue crab (*Callinectes sapidus*) industry in North Carolina and elsewhere has outpaced the generation of certain information to address key management and operational issues concerning this fishery. The specific objectives of this study were to quantify: 1) mortality rates of white-line versus red-line peeler crabs; 2) size-specific mortality rates of crabs in shedding systems; 3) mortality rates of peeler crabs as a function of crab source (purchased or self-caught), system type (closed versus open), and gear (hard crab pot vs. peeler pot); 4) any relationship between peeler crab mortality and water quality parameters, such as dissolved oxygen, temperature, salinity, and nutrients; 5) the effects of crab sex on peeler mortality rates; 6) the effects of female crab presence or absence and molt stage on time-to-molt and survival of male peelers; and 7) the effects of crab density on time-to-molt and survival of male peelers. We addressed these objectives during May–October 2001 through collaboration with 11 different commercial crab shedders located throughout coastal North Carolina. Study locations represented a broad spectrum of water quality while simultaneously providing replicated closed and open systems and replicated use of purchased versus self-caught crabs. Both large-scale seafood producers and small backyard operations were represented in this study. The key findings were 1) significantly higher mortality of white-line than red-line peelers; 2) relatively high mortality rates of peelers in shedding systems ranging from an average of 10–30% per tank per day, but no effect of crab size on mortality rates; 3) no relationship between mortality of peelers and water quality parameters, such as dissolved oxygen (DO), temperature, salinity, and nitrates; 4) significantly higher mortality of peelers purchased by crab shedders than peelers caught by shedders; 5) no significant difference in peeler mortality between closed and open systems or between those crabs captured by hard crab pots or peeler pots; 6) decreasing peeler mortality with increasing density of peelers in holding tanks; 7) significantly higher mortality rates for male than female peelers and significantly lower time-to-molt for males than females; 8) no significant increase in male peeler mortality or time-to-molt in the presence of red-line females; and 9) a significant decrease in a male red-line peeler's time-to-molt in the presence of a red-line female and intermolt male. Implementing best management practices in the soft crab industry could encourage crabbers to take better care of peeler crabs by always placing them in a cooler on ice immediately after capture or underneath wet burlap sacks. The benefits of best management practices will likely include a reduction in the mortality rate of peeler crabs in shedding systems, increased financial profits for crabbers who sell peelers that are now more likely to survive in shedding systems, and improved profits of shedding system operators who purchase peeler crabs. It is important to reduce mortality in North Carolina's soft-shell blue crab industry because 1) soft-crab landings are increasing rapidly and becoming a larger component of overall landings, (2) approximately 23% crabs placed in shedding systems die, and 3) there is an urgent need to conserve the blue crab spawning stock given the recent 80% decline and a highly significant stock-recruitment relationship for the blue crab in North Carolina. The information from this study should lead to improvements in shedding technology, better fishery management, and improved profits.

KEY WORDS: blue crab, *Callinectes sapidus*, crab mortality, density-dependence, management issues, peeler crab, soft-shell industry

INTRODUCTION

Soft-shell blue crabs (*Callinectes sapidus*, Rathbun 1884) are produced by aquaculture operations ("shedding operations") that hold premolt ("peeler") crabs until they molt (Ary and Poirrier 1989). Commercial fishermen sell soft-shell ("soft crabs") crabs for nearly seven times more per pound than intermolt ("hard") crabs (Oesterling 1995). In North Carolina, the soft-shell crab industry has become an increasingly important component of the blue crab fishery, the state's most valuable fishery in terms of total landings, value, processing, participation, employment, and amount of gear used (Henry and Mckenna 1998). Recently, a significant decline in the state's blue crab population and commercial landings (Eggleston et al. 2002) has increased financial pressure on commercial fishermen, who look to production of soft crabs as a means of economic survival. Although most shedding operations are profitable, large financial losses are also common

because of the high mortality of peelers (Dell Newman, crab shedder and commercial fisherman, Swan Quarter, NC, personal communication; Connie Ingraham, crab shedder, Wilmington, NC, personal communication). Thus, the value of the soft crab industry is directly dependent on mortality rate of peeler crabs. Several field and laboratory studies have documented the effects of water quality on crab survival and molting success (Ary and Poirrier 1989, Das and Stickle 1993, Lakshmi et al. 1984, Weis et al. 1992); however, few, if any, published studies have quantified mortality of peeler crabs in the soft crab industry or identified sources of mortality. This study quantified how blue crab mortality in soft-shell shedding operations varied with biologic and operational factors.

Blue Crab Molting

The time period between molting in blue crabs varies from days to months depending upon crab size. For example, the smallest juvenile stages of crabs (6–10 mm carapace width, CW) molt on the order of days, whereas sub-adult crabs with a carapace width of 80–100 mm CW molt on the order of weeks, and crabs > 100 mm CW molt on the order of months (Milliken and Williams 1984). Shedding of the exoskeleton (i.e., ecdysis) occurs when

crabs secrete a new exoskeleton within the old one. The old exoskeleton then cracks along suture-lines and the crab exits the old shell with a soft-shell that is larger than the old one. Four or five hours after molting, the soft shell gradually hardens. Crabs in the soft-shell industry are collected shortly after molting and before the shell hardens. When crabs begin to secrete their new shell, a white-line becomes visible inside the cuticle of the crab's last appendage or swimmeret. This white line indicates that the crab will molt within two weeks. As molting time nears, the indicator line gradually changes color: a pink line peeler will molt within 1 wk, and a red-line peeler will molt within 3 days (Oesterling 1984).

North Carolina's Soft-Shell Blue Crab Industry

Soft crab landings in North Carolina have made up 1.6% of the total blue crab landings for the past 8 y (~6,166,160 lbs), but the value of this fishery has averaged 6.6% of the total during that same time and increased to nearly 10% during 2001 (~$3,336,990; North Carolina Division of Marine Fisheries 2002). The increase in value of the soft crab fishery in North Carolina may be attributable to drastic declines in the blue crab population and in hard crab catch (Eggleston et al. 2002), and increasing local, regional, and worldwide demand (Oesterling 1995).

In North Carolina, peeler crabs are trapped as by-catch in the hard crab fishery using hard crab pots or targeted directly using peeler pots. Hard crab pots are constructed of 3.8-cm wire mesh, fitted with at least two escape rings of 5.9-cm inside diameter (North Carolina Division of Marine Fisheries 2002), and are baited with dead fish. Peeler pots are constructed of 2.54-cm mesh and are not fitted with escape rings because there is no size limit on peeler crabs (North Carolina Division of Marine Fisheries 2002). Peeler pots are either unbaited or are baited with a mature male crab whose urine may attract prepubertal female peeler crabs (Ryan 1966). Prepubertal female peeler crabs are attracted to male blue crab urine because they are only able to copulate during a brief period of 2–3 h after ecdysis.

Two types of shedding systems, open and closed re-circulating, are used primarily in North Carolina. In open systems, water is pumped into shedding tanks from a nearby source such as a creek or bay, and drains back into the same water source. In closed systems, tanks are either filled with well water and aquarium salt added, or water is trucked in from the nearest suitable source. Water drains into a biologic filter tank and is continuously pumped back into shedding tanks. Nitrogen fixing bacteria in filter tanks reduce the toxicity of ammonia in the water by reducing it to nitrite and then to nitrate (Wheaton 1977).

Management and Operational Issues

The rapid growth of the soft-shell blue crab industry in North Carolina and elsewhere has outpaced the generation of certain information to address key management and operational issues concerning this fishery. Input on key management and operational issues concerning the soft-shell crab industry in North Carolina were provided through direct communication with the North Carolina Division of Marine Fisheries (NC DMF) and through a series of public workshops that sought input from commercial crabbers as a part of the North Carolina Fisheries Resource Grant Program, administered through North Carolina Sea Grant. Specific management questions are described below.

1. Do white-line peelers in the soft-shell blue crab industry

suffer relatively high mortality rates caused by long holding periods (e.g., held for weeks) compared with red-line peeler stages (e.g., held for days)? (Henry and McKenna 1998).
2. Are overall mortality rates of crabs in shedding operations relatively high, and does crab mortality vary with crab size? (S. McKenna, NC DMF, personal communication). Currently, there is no size limit on peeler crabs in North Carolina.

Specific questions raised by soft-shell crab shedders in NC during public workshops are described below.

1. Do peelers purchased by shedders suffer higher mortality than those they caught?
2. Is peeler crab mortality caused by low dissolved oxygen and high temperatures?
3. Is peeler crab mortality higher in closed than open systems?
4. Is peeler crab mortality elevated for crabs captured in hard crab pots as opposed to peeler pots?
5. Does peeler mortality increase with the crab density in holding tanks?
6. Is peeler crab mortality and time-to-molt higher in males than females? Is male peeler crab mortality disproportionately high in the presence of female peelers?

Such untested questions are the basis of at least one current regulation in North Carolina. For example, the NC DMF prohibits harvest of white-line peelers after June 1 each fishing season because of assumed high mortality during summer months. Moreover, much of the hypothesis testing in the present study was driven by the collective observations of commercial crab shedders.

The overall objectives of this study were to address the management and operational questions raised above by quantifying: 1) mortality rates of white-line versus red-line peelers; 2) size-specific mortality rates of crabs in shedding systems; 3) mortality rates of peelers as a function of crab source (purchased or self-caught), system type (closed versus open), and gear (hard crab pot vs. peeler pot); 4) the relationship, if any, between peeler mortality and water quality parameters such as dissolved oxygen, temperature, salinity, and nutrients; 5) the effects of crab sex on peeler mortality rates; 6) the effects of female crab presence or absence and molt stage on time-to-molt and survival of male crabs; and 7) the effects of crab density on time-to-molt and survival of male crabs. This information should lead to improvements in shedding technology, better fishery management, and improved profits.

METHODS AND MATERIALS

Study Locations

Data were collected in collaboration with 11 different commercial crab shedders throughout coastal North Carolina (Fig. 1) from May until October 2001. Study locations were selected to represent a broad spectrum of water quality while simultaneously providing replicated closed and open systems and replicated use of purchased versus self-caught crabs (Chaves 2002). Both large-scale seafood producers and small backyard operations were represented in this study. Seven locations used closed systems, three used open systems, and one location used both an open and a closed system (Chaves 2002).

Crab Collection

Crabs were captured by commercial fishermen using hard crab and peeler crab pots. After capture, peeler crabs were either stored
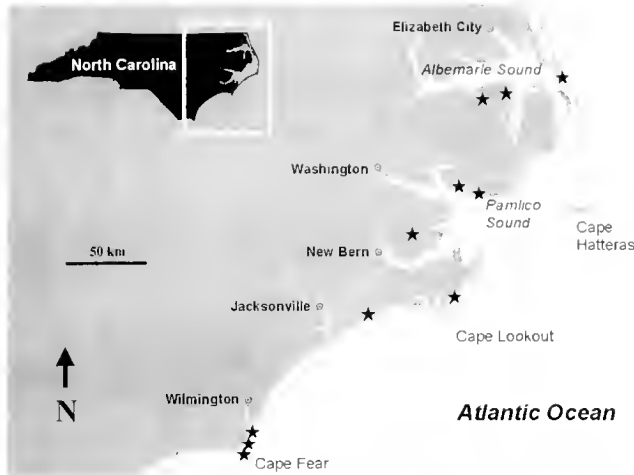
Figure 1. Map of North Carolina showing study locations (stars).

in wooden baskets on the deck of a boat or were placed in coolers on ice. Once landed at the dock, crabs were placed in nearby shedding tanks or trucked to shedding operations up to 200 km away. At each of the 11 crab shedding study locations, premolt crabs were sexed and their carapace width measured (mm CW). Crabs were separated according to peeler stage (red-line vs. white-line) and then equally distributed among four experimental tanks measuring 1.2 m wide × 2.4 m long and 20 cm deep. The crab sizes used ranged from 5–17.2 cm CW. A total of 49 experiments were conducted. A single experiment could last for ~6 days or ~21 days for red-line or white-line peelers, respectively, to allow crabs enough time to molt. Some shedders conducted experiments in either closed or open systems, using only purchased or self-caught crabs. Other shedders would conduct simultaneous experiments with red-line and white-line peelers that were self-caught. Others might switch systems and crab source from one month to the next. For example, a shedder might conduct an experiment with only purchased crabs in a closed system in one month, followed by an experiment using only self-caught crabs in an open system the next month. Each experiment at a specific location was treated as a single independent replicate, since a new grouping of crabs from varying sources was placed in the shedding tanks at the initiation of each experiment, and the experimental methods were standardized across locations. In the following Methods and Results sections, the objectives of the study are described within the topics of operational (system, crab source, gear, water quality) and biologic (crab molt stage, sex, density, size) considerations.

Operational Considerations

Effects of Water Quality on Crab Mortality

To quantify the effects of water quality on crab mortality, the following water quality parameters were measured daily at ~0800 h: dissolved oxygen (DO) (mg/L), temperature (°C), salinity (parts per thousand; ppt), pH, and concentrations of nitrite (mg/L), nitrate (mg/L), and ammonia (mg/L). A weighted mean calculated from the number of crabs that died in each experimental tank per day divided by the number of crabs in the tank on that day was used. Thus, the response variable in all cases dealing with crab mortality

was a weighted percent mortality/day. Tanks with red-line crabs were monitored for 6 days and tanks with white-line crabs were monitored for up to 21 days. If all crabs in a tank shed or died before the 6- or 21-day period, the experiment was terminated. The experimental unit was each tank, and four replicate tanks were used at each of the 11 sites.

Statistical analyses used a multiple regression model with crab source (purchased vs. self-caught) as the independent variable and water quality parameters as independent continuous variables. In this case, crab source was highly significant (see below), which confounded mortality associated with the source of crabs and water quality parameters. Thus, in subsequent statistical analyses, the data were first divided into separate categories of self-caught versus purchased crabs. In assessing the effects of water quality on crab mortality, we then used a backward, stepwise multiple regression model. Alpha to enter and remove factors from the model was 0.10. A Levene's Median test assessed constant variance among the responses and a Kolmogorov–Smirnov test tested for normality. In cases where the data failed to meet the assumptions, the data were transformed using ArcSine or log10 transformations, which were successful in all cases.

Effects of Shedding System, Crab Source, Gear Type, and Crab Density on Crab Mortality

The mean daily crab percent mortality in closed versus open systems, between self-caught versus purchased crabs, and between crabs caught in hard crab pots versus peeler pots was compared with three separate one sample t tests. The relationship between mean daily crab density and mean daily crab proportional mortality pooled across 23 experiments using self-caught crabs and 26 experiments using purchased crabs was examined with a linear least squares regression model.

Biologic Considerations

Effects of Crab Sex, Size, and Peeler Stage on Crab Mortality.

To quantify the effects of crab sex and peeler stage on crab percent daily mortality, we compared the mortality of male versus female crabs, and white-line peelers versus red-line peelers using an analysis of covariance (ANCOVA) model with crab sex and peeler stage as factors and crab size as a covariate. The data were normally distributed and variances were homogeneous.

Effects of Female Crab Presence on Mortality and Time-to-Molt in Male Crabs

The effects of female crab presence and their molt stage on the time-to-molt and mortality of male crabs were examined in a closed shedding system in Swan Quarter, NC. Twenty tanks, measuring 1.2 m wide × 2.4 m long and 20 cm high were filled with estuarine water from Albemarle Sound to a height of 15 cm. Once the tanks were filled, the pump was turned off and water was not allowed to circulate between tanks, thereby preventing any potential pheromone contamination across tanks. Tanks were aerated by aquarium air pumps. Crabs were purchased from several fishermen and randomly assigned to one of the following three treatments: 1) one red-line male per tank [control]; 2) one red-line male and one intermolt female per tank; and 3) one red-line male and one red-line female per tank. All crabs were visually examined hourly to record the time that they molted or died. When a red-line male crab

molted or died, the trial was stopped. Each male red-line peeler was an experimental unit and each treatment was replicated seven to nine times. We tested whether there was a treatment effect on a male crab's time-to-molt and percent daily mortality with a one-way analysis of variance (ANOVA). The data were log-transformed to meet assumptions of normality and homogeneity of variance.

Effects of Increasing Male Crab Density in the Presence of a Red-Line Female on Mortality and Time-to-Molt of Male Crabs

The effect of increasing male crab density on percent daily mortality and time-to-molt in male crabs was also tested at Swan Quarter, North Carolina. Four treatments were randomly interspersed among tanks: 1) one red-line male per tank [control]; 2) one red-line male and one red-line female per tank; 3) one red-line male, one red-line female, and one intermolt male per tank; and 4) one red-line male, one red-line female, and three intermolt males per tank. Each male red-line peeler was an experimental unit and the response variables were time-to-molt and percent daily mortality. Each treatment was replicated five to seven times.

Effects of Crab Sex on Time-to-Molt

Time-to-molt of male versus female crabs in the absence of other crabs was quantified in separate experiments at Swan Quarter, North Carolina. This experiment was conducted to determine if males simply took longer to shed than females regardless of any other factors such as presence of females or increasing crab density. Each tank contained a single male or female red-line crab. The response variable was time-to-molt in hours. Each crab was an experimental unit and each treatment was replicated 10 times.

The LIFETEST procedure in SAS was used to compare the distribution of male's time-to-molt in the presence and absence of red-line females and other male crabs. The data was right censored (experiments ended before a response could be observed) due to the early termination of several trials when male crabs died before molting. The censored data points can not be left out of the analysis because crabs that take longer to molt are also more likely to die. The LIFETEST uses both censored and uncensored times to molt when comparing distributions of times to molt for various treatments. An uncensored data point is an actual observation of the time-to-molt, but the time-to-molt for censored data points is a calculation based on the distribution of times to molt among non-censored data points. Chi-Square tests were used to detect differences in mortality between treatments, and ANOVA was used to detect differences in the time-to-molt between male and female crabs.

RESULTS

Operational Considerations

Effects of Water Quality on Crab Mortality

Water quality was somewhat poorer in closed than open recirculating systems (Table 1). For example, DO was lowest (2.9 mg/L) and nitrates highest (77.4 mg/L) in closed systems. Nevertheless, most of the water quality values were well within tolerance limits of blue crabs (Manthe et al. 1983). The percent daily mortality of self-caught and purchased peeler crabs did not vary significantly with any of the water quality parameters recorded (multiple regression; self-caught: all $P > 0.08$, purchased: all $P > 0.16$).

Effects of Crab Source, Shedding System, Gear Type, and Crab Density on Crab Mortality

Mortality of peeler crabs was significantly higher for purchased than self-caught crabs (t test; $t = -2.22$, df = 1,50, $P = 0.03$; Fig. 2). Shedding system type (i.e., open vs. closed) did not significantly affect the mortality of self-caught (t test; $t = 1.23$, df = 1,48, $P = 0.22$) or purchased crabs (t test, $t = 0.32$, df = 1,44, $P = 0.75$). For self-caught crabs, there was no difference in crab mortality between crabs caught by peeler pots or those caught by hard crab pots (peeler pots: mean daily percent mortality = 2%, SE = 0.007, $n = 16$; hard crab pots: mean daily percent mortality = 3%, SE = 0.006, $n = 8$; $t = 0.54$, df = 1,22, $P = 0.60$). We were unable to test the effects of gear type on mortality of purchased crabs because all purchased crabs came from hard crab pots.

Surprisingly, the percent daily mortality of red-line male peelers decreased with increasing density of peelers held in shedding tanks for both self-caught and purchased crabs (Fig. 3). The declining trend in percent daily peeler mortality with density was significant for self-caught crabs (linear least-squares regression: $F = 14.27$, df = 1,17, $P < 0.01$; Fig. 3A), and marginally significant for purchased crabs ($F = 4.05$, df = 1,19, $P = 0.06$; Fig. 3B).

Biological Considerations

Effects of Crab Sex, Size, and Peeler Stage on Crab Mortality

Mortality rates of self-caught crabs were unaffected by crab sex and the covariate of crab size (two-way ANCOVA; sex: $F = 3.06$,

TABLE 1.

Means and ranges of water quality parameters measured.

	DO (mg/L)	Temp (°C)	Sal (ppt)	Nitrite	Nitrate	Ammonia	pH
Closed systems							
Mean	5.9	25.4	20.1	0.2	21.7	0.5	7.4
Low value	3.0	17.4	3.7	0	0	0.1	7.1
High value	8.2	30.8	39.8	0.5	77.4	3.2	8.0
Open systems							
Mean	6.7	26.4	17.3	0.1	3.6	0.3	7.4
Low value	3.6	19.5	5.0	0.0	0.0	0.2	6.9
High value	9.7	31.8	35.9	0.2	8.3	0.5	7.8

Nitrate, nitrite and ammonia are presented as mg/L.
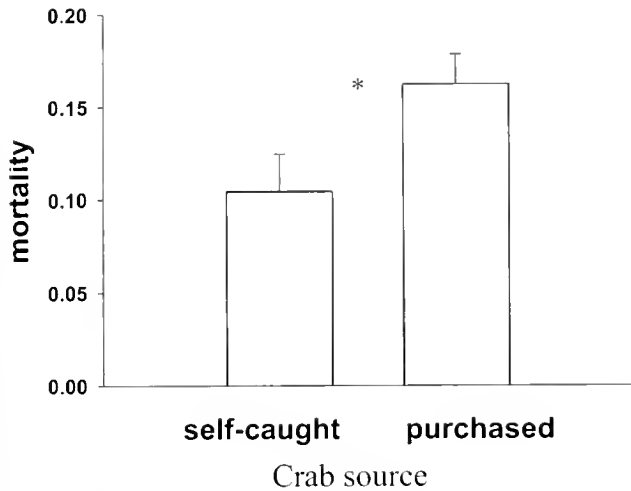
Figure 2. Mean daily proportional mortality (+ SE) of self-caught ($N = 24$) and purchased ($N = 27$) peeler crabs. Asterisk denotes significant difference. See text for details of statistical test.

df = 1.20, P = 0.10; size: F = 3.03, df = 1.20, P = 0.10); however, self-caught white-line peelers experienced significantly higher mortality rates than self-caught red-line peelers (molt-stage: F = 5.4, df = 1.20, P = 0.03; Fig. 4A). Mortality rates of purchased crabs were not affected by crab size (one-way ANCOVA; F = 0.02, df = 1.44, P = 0.88); however, purchased male peelers experienced significantly higher mortality rates than purchased female peelers (F = 10.04, df = 1.44, P < 0.01; Fig. 4B). It was not possible to determine the effect of crab stage on purchased crabs because no white-line peelers were purchased.

Effects of Female Crab Presence on Mortality and Time-to-Molt in Male Crabs

There was no significant difference in time-to-molt between male red-line peelers held alone, held with intermolt females, or held with red-line females (ANOVA; F = 0.13; df = 3,23; P = 0.718; Fig. 5A). There was also no significant difference in mortality between males that were held alone, held with intermolt females, or held with red-line females (Chi-square test; χ^2 = 4.14, df = 1,44, P = 0.13).

Effects of Increasing Male Crab Density in the Presence of a Red-Line Female on Mortality and Time-to-Molt of Male Crabs

Time-to-molt in male peelers varied significantly according to whether a red-line female and intermolt male were also present (ANOVA; F = 13.06; df = 2,10; P < 0.01). In this case, time-to-molt was significantly shorter among males held with one red-line female and one green male compared with the control group of a single red-line male held alone (Ryan's Q-multiple comparison test; Fig. 5B). There was no significant difference in daily percent mortality of male peelers in the presence or absence of red-line female and intermolt male crabs (Chi-square test; χ^2 = 3.06, df = 3,50, P = 0.38).

Effects of Crab Sex on Time-to-Molt

The average time-to-molt for male crabs was significantly shorter than for female crabs (ANOVA; F = 14.21, df = 1,19, P < 0.01, Fig. 5C).
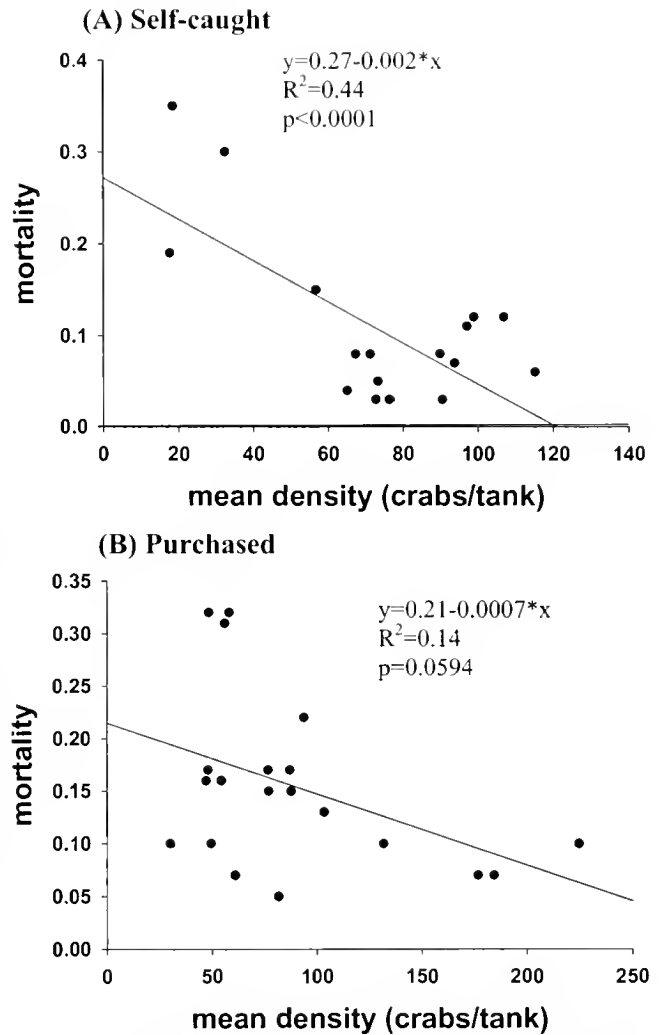
Figure 3. Relationship between mean daily proportional mortality of (A) self-caught ($N = 18$) versus (B) purchased ($N = 20$) peeler crabs and mean density per tank.

DISCUSSION

The soft-shell blue crab industry is one of the fastest growing fisheries in North Carolina. In this study, we collaborated with a team of commercial crab shedders across 11 different locations spanning the entire North Carolina coast to address key management and operational questions intended to better manage the blue crab resource, improve shedding technology, and increase profits. The key findings were as follows: 1) significantly higher mortality of white-line than red-line peelers; 2) daily mortality rates of 10–30% per tank (primarily poorly handled red-line peelers) in shedding systems, but no effect of crab size on mortality rates; 3) no relationship between mortality of peelers and water quality parameters, such as DO, temperature, salinity, and nitrates; 4) significantly higher mortality of peelers purchased by crab shedders than peelers caught by the shedders; 5) no significant difference in peeler mortality between closed and open systems, or between those crabs captured by hard crab pots or peeler pots; 6) decreasing peeler mortality with increasing density of peelers in holding tanks; 7) significantly higher mortality rates for male than female

(A) Self-caught
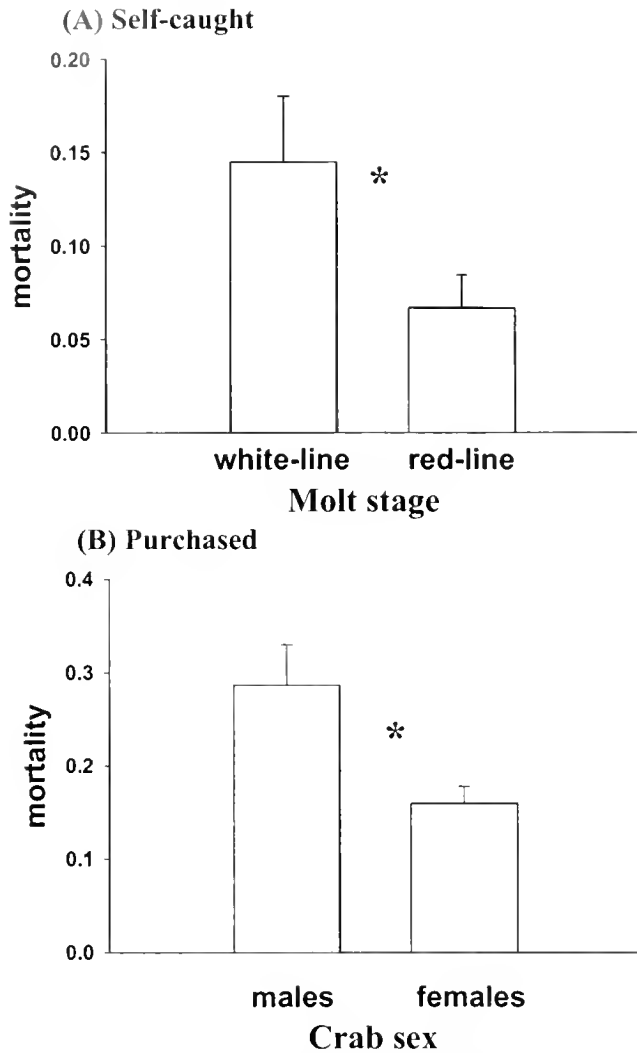
(B) Purchased

Figure 4. Mean daily proportional mortality (+ SE) of (A) self-caught white-line (n = 15) versus (B) red-line peelers (N = 37), and (B) purchased males (N = 21) versus females (n = 25). Asterisk denotes significant difference. See text for results of statistical tests.

peelers, and significantly lower time-to-molt for males than females; 8) no significant increase in male peeler mortality or time-to-molt in the presence of red-line females; and 9) a significant decrease in a male red-line peeler's time-to-molt in the presence of a red-line female and intermolt male.

Blue Crab Management Issues

White-Line Peelers

Mortality of self-caught white-line peelers was significantly higher than self-caught red-line peelers. We were unable to assess the effect of molt stage on purchased peelers because commercial shedders do not shed white-line peelers for fear of high mortality rates. White-line peelers in this study probably experienced higher mortality rates than red-line peelers because of the relatively long periods of time required for them to molt, in which they are more likely to suffer from accumulated stress, as compared with red-line peelers. Most crabbers will feed white-line peelers if they will eat,

(A)

(B)

(C)

Crab sex and molt stage
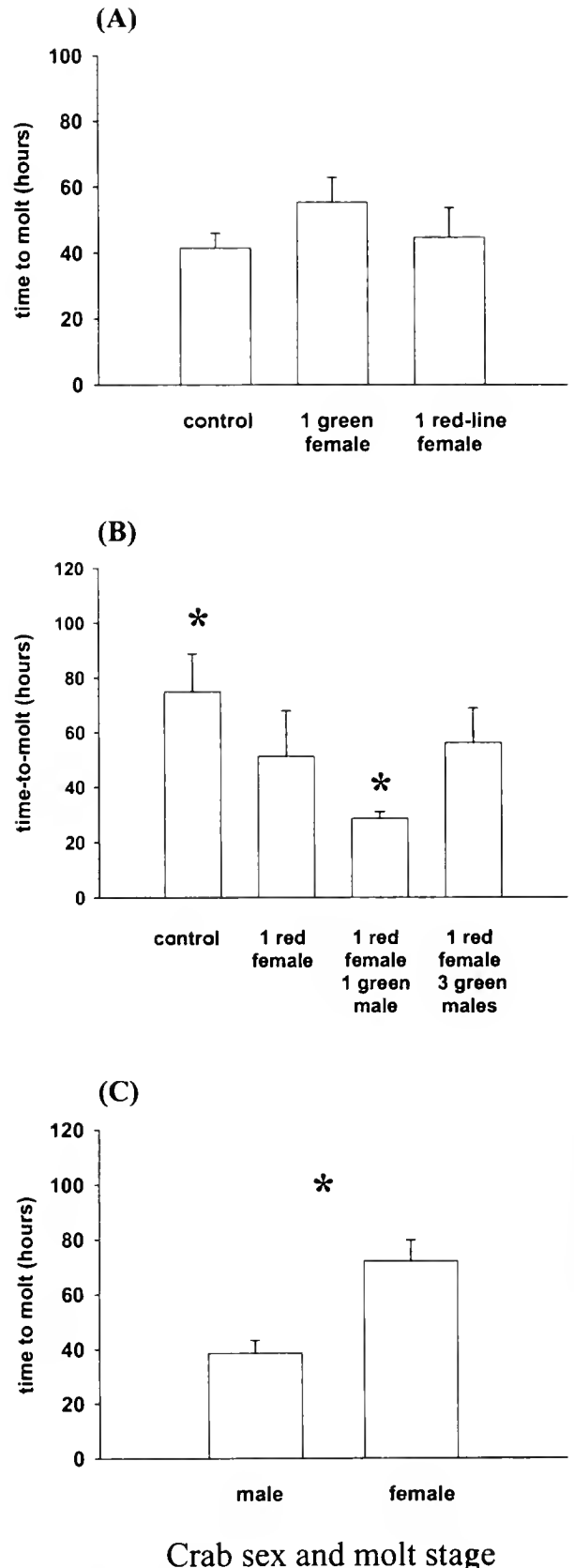
Figure 5. Mean (+ SE) time-to-molt for male red-line peelers as a function of crab sex and molt stage. Green = intermolt crabs. N = 7–9. Asterisk denotes significant differences between treatments. See text for results of statistical tests.

Depending upon temperature and where a crab is in the molt cycle, white-line peelers cease to feed somewhere between 10–21 days before molting (pers. obs.). The peeler crab fishery in NC is regulated on the assumption that male white-line peeler mortality is very high during summer months, and that to keep them would be a wasteful practice. Our results support this management practice; however, it is important to note that the mortality of self-caught white-line peelers was similar to that of purchased red-line peelers (compare Fig. 2 and 4A), highlighting the importance of crab source as a key determinant of peeler mortality. Oesterling (1984) suggested that it is not economical to keep white-line peelers for more than 10 days, and various coastal Sea Grant extension agents have urged crabbers that harvest peelers to take better care of them after capture.

Overall and Size-Specific Crab Mortality Rates.

Mortality rates of red-line peelers placed in shedding tanks in this study averaged 15% per day. This daily mortality rate is extremely high when compounded over the typical 5-day duration of shedding, and is dramatically higher than natural mortality rates. For example, the annual mortality rate for sub-adult and adult blue crabs is 50% (Eggleston 1998). For a hypothetical, yet realistic example of how mortality in shedding systems is compounded over time, assume that 100 crabs are placed in a shedding tank on Day 1. On Day 2, ~15% of the crabs would have died and 50% would have molted successfully and been sold. This would leave 35 crabs on Day 2. Assuming these same daily percentages of death and successful molting, we would have five dead crabs and 17 crabs molting successfully on Day 3, a total of two dead crabs and seven molting successfully on Day 4, and one dead crab and two molting successfully on Day 5, after which there would be one crab left. Thus, out of a starting population of 100 peelers in the shedding table, an average of 23 (23%) would die over 5 days. The cumulative mortality for white-line peelers would likely be even higher since they are generally held two to four times longer than red-line crabs before they molt. Depending upon method of harvest and crab source, a high mortality of peelers could be expected immediately after crabs are stocked, with a large decrease in mortality as stronger crabs shed.

We did not detect any effect of crab size on crab mortality rates, contrary to popular belief that male peeler crab mortality increases with crab size, especially when males become very large. There may not have been enough contrast in our data to reveal a positive relationship between crab size and mortality since male peelers >16 cm CW were rarely observed. The belief among crabbers that male peelers experience high mortality is so prevalent that many have given large male soft-shell crabs the nickname "miracle crabs."

Operational Considerations

Source of Peelers

Crab source was always the single greatest source of variation in crab mortality rates, with highest mortality in purchased peelers. Variability in mortality because of crab source is likely caused by different handling methods used by crabbers who shed their own peelers versus crabbers who sell peelers. Crabbers who shed their own peelers put forth great effort to ensure the survival of their peeler crabs, such as carrying a cooler with ice and wet burlap bags in their boats, which keep crabs cool and moist. Crabbers who sell peelers usually place peeler crabs in a wooden basket on the deck of their boat, unprotected from bright sun, wind, and extreme heat. Purchased peeler crabs may also experience significantly higher mortality rates than self-caught peelers because they are more likely to travel greater distances from the point of capture to shedding systems. Long travel times may cause crabs to dehydrate, but also increase the likelihood that crabs will experience large changes in salinity from the point of capture to shedding tanks. Although blue crabs are euryhaline organisms that can survive in a broad range of salinities from 0 ppt to over 40 ppt, sudden large changes in salinity (i.e., > 10 ppt in less than 24 h) may exceed a crab's ability to osmoregulate the tissues in its body, causing mortality (Engel and Thayer 1998). Although we were unable to record changes in salinity from the point of capture to a given shedding system, these salinity changes may be another source of stress, in addition to poor handling, leading to significantly higher mortality rates of purchased than self-caught peelers and is worthy of further research. If large changes in salinity prove to be an important source of stress for peelers, then shedders capable of regulating salinity in their systems could alter the salinity to accommodate the peelers they buy. A crabber who sells poorly treated peelers is unaffected by high mortality rates that may occur in shedding systems because market demand for peeler crabs ensures that they will receive top dollar for these peelers, despite their relatively poor treatment.

One of the most obvious ways to reduce crab mortality in North Carolina's soft-shell crab industry is for shedders to capture their own crabs rather than rely on purchasing peelers. The majority of all peeler crabs in North Carolina shedding systems, however, are purchased crabs (Tony Roughton, Seafood dealer and commercial fisherman, Columbia, NC, personal communication), and the data in this study indicate that purchased peeler crab mortality is 11% greater than self-caught crabs. The 11% difference in mortality rates of self-caught and purchased peelers could easily equate to a financial loss of over $776,044 per year for crab shedders alone (see Appendix for calculations). The relatively high mortality of purchased crabs could probably be reduced greatly by taking better care of peelers on the boat, such as using coolers or wet burlap sacks to keep crabs cool, moist, and out of the sun and wind, and reducing the travel times between initial harvest, and placement in shedding systems. If the mortality rate of purchased crabs were reduced to that of self-caught crabs, the soft crab industry would increase in value by 11% ($776,044) without any increases in harvest size.

Effects of Crab Density on Crab Mortality

Mortality of both self-caught and purchased crabs decreased as crab density increased, in contrast to expectations. It is possible that crabs abandon aggressive behavior that causes mortality once density is increased to a certain level, as evidenced by some species of fish that abandon territorial behavior if density surpasses a certain threshold (Dr. Jon Shenker, Fl. Inst. Tech., personal communication). Many shedders feel that peelers can be stocked at extremely high densities (>250 crabs/tank) without any harmful effects as long as good aeration is maintained in the tanks and all crabs placed in the tank are red-line peelers. Failure to carefully examine the molt stage of each crab placed in tanks allows the

accidental entry of both intermolt and white-line peelers, which are known to cannibalize red-line peelers and soft crabs.

Isolating crabs from each other may be a highly effective method of reducing crab mortality. One of the shedders in this study reduced mortality in his system by placing 100 plastic mesh cylinders in each tank to isolate crabs from each other. The cylinders were originally designed to eliminate cannibalism, but the shedder felt that the reduction in mortality was much greater than what would have been caused by cannibalism alone. Crab mortality at the study location where the cylinders were used was consistently the lowest in the entire state during our study. Eliminating physical interaction between crabs may greatly reduce crab stress, and therefore reduce mortality. Although the use of mesh cylinders in shedding tanks also reduced the density of crabs that could be held in a shedding table, the lack of a decline in mortality with decreasing crab density in this study suggests that creating physical barriers between crabs by using mesh cylinders explains the reduction in mortality of peelers. Most shedders that we spoke with were reluctant to try isolating crabs in tanks because they felt that cylinders would greatly reduce the capacity of shedding tanks during large runs of peeler crabs when tanks are stocked at densities of 200–300 crabs. These peeler runs only occur two or three times each year, however, and it is not likely that peeler supply would exceed the capacity of 100 crabs per tank during the rest of the shedding season.

Biologic Considerations

Effects of Crab Sex

Crab sex had a significant effect on the mortality of purchased crabs but not on the mortality of self-caught crabs. Commercial crab shedders that purchased crabs report that they always observed higher male mortality than female mortality, whereas shedders that caught their own peelers reported that they never observed higher male mortality than female mortality. Several crabbers have suggested that high male mortality associated with purchased crabs is the result of aggressive encounters that males experience in hard crab pots. We could not statistically test for a significant interaction between crab sex, crab source, and gear type on crab mortality because nearly all purchased crabs were caught in hard crab pots, and nearly all self-caught crabs were caught in peeler pots. Nevertheless, relatively high male crab mortality did coincide with the use of hard crab pots and not with peeler pots. Female peeler crab mortality may not be affected by the use of hard crab pots because female peeler crabs that enter hard crab pots are usually cradled with a pre-copulatory embrace by a male crab immediately after entry into a crab pot (personal observation). The male crab protects the female from other crabs and attempts to mate with her (Dell Newman, commercial fisherman, Swan Quarter, NC, personal communication). Alternatively, when a male peeler crab enters a hard crab pot, he is not protected from aggressive encounters with intermolt crabs, and may experience injuries or sub-lethal stress that will not become manifest until he is placed in a shedding system and dies. In a peeler crab pot, intermolt crabs are rarely present, so males and females do not encounter aggressive intermolt crabs. Whether peeler crabs face sub-lethal aggressive encounters with aggressive intermolt crabs in hard crab pots is unknown, but further research in this area may explain the higher mortality of purchased male than female peelers.

Effect of Female Crab Presence and Increasing Male Density on Mortality and Time-to-Molt in Male Crabs

Male red-line peelers held with a relatively low density of intermolt male crabs experienced significantly shorter times to molt than control crabs, but time-to-molt did not differ significantly between male red-line peelers held with a relatively high density of intermolt males and the control treatment of no conspecifics. This result was contrary to the expectation that increasing male density would lead to longer times-to-molt. The biologic explanation for the decreasing time-to-molt of male peelers with a low density of intermolt male crabs is unclear and warrants further experimentation.

Male red-line peelers experienced significantly shorter times to molt than female red-line peelers, contrary to our expectation that time-to-molt would be equal among males and females or that males might experience longer times to molt than females. The findings in this study differ from the opinions of many crab shedders that report male red-line peeler crabs in shedding tanks take longer on average to molt than female red-line peeler crabs. Further investigation might reveal the extent to which intermolt periods differ between male and female blue crabs. In this study, male peeler crabs showed no ability to regulate their time-to-molt in response to different situations (i.e., the presence of red-line female, different levels of conspecific density).

CONCLUSIONS

Our study revealed sources of mortality in the North Carolina soft-shell blue crab industry that fisherman are capable of eliminating. The survival of self-caught peeler crabs is significantly higher than for purchased crabs. Implementing best management practices in the soft crab industry could encourage crabbers to take better care of peeler crabs by always placing them in a cooler on ice immediately after capture or underneath wet burlap sacks. The benefits of best management practices will likely include a reduction in the mortality rate of peeler crabs in shedding systems, increased financial profits for crabbers who sell peelers that are now more likely to survive in shedding systems, and improved profits of shedding system operators who purchase peeler. It is important to reduce mortality in North Carolina's soft-shell blue crab industry because 1) soft-crab landings are increasing rapidly and becoming a larger component of overall landings; 2) approximately 23% crabs placed in shedding systems die; and 3) there is an urgent need to conserve the blue crab spawning stock given the recent 80% decline and a highly significant stock-recruitment relationship for the blue crab in NC (Eggleston et al. 2002).

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APPENDIX

Estimation of Annual Financial Loss for North Carolina's Soft-Shell Blue Crab Industry Using Purchased Peeler Crabs

Assume 2,565,434 purchased peelers are placed in shedders each year in North Carolina. Assume each dead crab represents a loss of $2.75 (purchase price = $0.75; lost revenue = $2.00). If one assumes a mortality rate of 16% (this study), then 410,469 dead crabs die at a cost of ~$1,128,790 per year.

SEX-SPECIFIC RESPONSE TO DISTURBANCE IN A FIDDLER CRAB

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ABSTRACT Fiddler crabs are organism with an extreme sexual dimorphism. Male crabs have an enlarged claw used for sexual display and combat but not for feeding, which place them in foraging disadvantage when are compared with females. Given that avoiding disturbance (e.g., predators or human activity), courting, and feeding are incompatible behaviors, males should have different time budget to balance all the activities. In this study we experimentally evaluated the hypothesis that males of the Southwestern Atlantic fiddler crab *Uca uruguayensis* have a sex-specific response to disturbance. We performed an experiment where we applied an artificial disturbance (created by addition of flags). During a tidal cycle we found that males were more affected by disturbance than females. During the ebb tide, more males than females remained into their burrows because of the artificial disturbance. After disturbance (i.e., when flags were removed) the male-to-female sex ratio on the surface increased in disturbed plots. However, once disturbance was interrupted the male-to-female sex ratio on previously disturbed plots differed from the observed in control plots, being smaller during the ebb tide and larger during the flood tide. The latter might indicate that male crabs increase their foraging effort to compensate the time they loss for feeding as consequence of disturbance. Disturbance also affected the proportion of courting males, but when disturbance was removed courtship returned to initial values of activity, which indicates that the cost of stop courting may be higher than cost of stop feeding. However, after 27 days of experimental disturbance comparison of body condition (dry weight in relation to their carapace width) showed no effect of disturbance, suggesting that males were able to compensate the decrease in feeding time.

KEY WORDS: *Uca uruguayensis*, fiddler crabs, disturbance, sex-specific response

INTRODUCTION

Fiddler crabs are interesting animals for studying the effect of sexual dimorphism on their behavior. Male fiddler crabs show an enlarged claw used for courtship displays (Crane 1975, Christy & Salmon 1984); its size gives them an advantage in combats, burrow acquisitions (see Hyatt & Salmon 1979), and probably in mate acquisition. However, given that the enlarged claw is not used for feeding, male feeding efficiency is lower, which leads to different foraging strategies in males and females (Valiela et al. 1974, Weissburg 1993). Furthermore, the color of the claw makes males more conspicuous and visually detectable than females (Crane 1975), and its size hinders escape from predators (Iribarne and Martínez 1999). Thus, if the visual detectability by predators is related to predation rate (Utne-Palm 2000) male fiddler crabs may have higher predation risk than females because of their conspicuousness and their longer time they spend on the surface (although some field studies show the opposite situation; e.g., Bildstein et al. 1989, P. Ribeiro, unpublished data).

Fiddler crabs are intertidal organisms with surface activity (feeding or courtship) only during low tide remaining inside their burrows during high tide (Crane 1975, Wolfrath 1993). During the courtship season both sexes feed mostly during the first hours after the tide ebbs and then, males court (by waving their large chelae) until the tide start to flood when they go back to feed (Crane 1975, Wolfrath 1993). However, males usually keep feeding longer than females before sheltering into their burrows while tide is flooding (Wolfrath 1993). This difference may be the product of lower feeding efficiency and/or higher energetic investment by males during waving.

When a disturbance occurs (i.e., predators, such as shorebirds, human activity) most fiddler crabs shelter into their burrows as a generalized antipredatory response (Frix et al. 1991, Iribarne & Martinez 1999). However, the trade off between feeding, mating,

and survival may not be the same for males and females. Therefore, we expect the response to disturbance to be sex specific. For example, given the visual conspicuity of the enlarged claw, males should show an earlier response than females to disturbances or potential predators. However, given that males need to feed longer (Valiela et al. 1974), receding early inside burrows may involve a higher cost in terms of food acquisition and loss of mating opportunities.

In this work, we experimentally evaluate the hypothesis that fiddler crabs show a sex-specific response to disturbance by studying the SW Atlantic fiddler crab *Uca uruguayensis* (Nobili 1901). We predict the following responses to disturbance: (1) more males than females will shelter into their burrows during ebb tide, given that the time available for feeding is still long and they may be, for their conspicuousness, at a higher risk of predation than females; (2) more females than males will shelter into their burrows during flood tide because time available for feeding is now short; (3) there will be an overall reduction in the time allocated to courtship during the whole tidal cycle; and (4) if males do not reduce the time allocated to courtship, their body condition will be affected.

MATERIALS AND METHODS

The study was conducted at the Mar Chiquita coastal lagoon (Argentina, 37°32′ to 37°45′S and 57°19′ to 57°26′W) from February 4 to March 3, 2000 (Austral summer). *Uca uruguayensis* occurs in the upper levels of the tidal flats, adjacent to the border of extensive marshes dominated by the cordgrass *Spartina densiflora* (Spivak et al. 1991). We marked 16 plots (each 2 m long and 6 m width) parallel to the shoreline with four 50-cm height (30-mm diameter) iron stakes. Plots were arranged at the same tidal level and were separated from each other by 2 × 2 m areas. Eight plots were subjected to disturbance (disturbed) and the remaining eight plots were kept as controls (control). Treatments were systematically assigned. Disturbance was applied by means of thirty flags consisting in iron stakes (30 cm high, homogeneously dis-

persed) with black and red nylon stripes (30 cm long, 2 cm width) added on their tips. Nylon stripes were easily waved by the wind and when approached to a crab induced it to shelter into its burrow. Control plots were without these nylon stripes but we walked on them to keep the same effect of setting up the nylon stripes as in the treatment plots. In all cases observation of crab behavior (focal census) were conducted using a 10×50 binoculars, 8 m from the plots and 5 min after the setting or extraction of the nylon stripes, to allow crabs reinitiate their activities after disturbance caused by the experimental setup.

To assure that crabs were entering inside their burrows in response to disturbance, we quantified in 12 plots the density of crabs on the surface. In each plot we sampled one transect of 2-m long by 0.2-m wide counting the number of crabs. Then, in six of these plots we placed the nylon flags and quantified the number of crabs again following the same procedure. Finally, the nylon stripes were extracted and crabs were quantified again. Data was square root transformed to comply with the assumptions and two-factor repeated measures ANOVA (Neter et al. 1991) was used to evaluate the density of crabs on the surface in relation to treatment (disturbed-control) and the disturbance state (before-during-after; as the repeated measures factor).

To evaluate the effect of disturbance on the sex ratio (males to females) and on the proportion of courting males we carried out an experiment encompassing a complete diurnal tidal cycle. The experiment began 4 h before low tide and finished 4 h after low tide. In disturbed plots, we applied two intervals of disturbance (from 4 to 2 h before low tide and from 0 to 2 h after low tide; thereafter during disturbance) and two intervals where disturbance was removed (from 2 to 0 h before low tide and from 2 to 4 h after low tide; thereafter after disturbance). To measure the sex ratio and the proportion of courting males we performed focal censuses. For this we started by randomly taking a male crab and then we successively located the most near crab, which was sexed (a simple task because of the sexual dimorphism). This procedure was systematically performed to reach a minimum quantification of 20 male crabs. For males we noted if they were feeding or courting (denoted by the waving movement of the enlarged claw). These observations were performed for both periods of disturbance and for both periods where the disturbance was removed. Given that 4 hours since low tide represent the moment where crabs unplug (when ebbing) or plug their burrows (when flooding), the 4 h of disturbance affected the 50% of the available surface time. To fit parametric assumptions (Neter et al. 1991) the sex ratio was transformed to the square root of data and the proportion of courting males was transformed with the arc-sin of the square root of data. A three-factor repeated measures ANOVA (Neter et al. 1991) was used to evaluate the effect of treatment (disturbed-control), tidal state (ebb-flood; repeated measures factor) and disturbance state (during-after; repeated measures factor) on 1) sex ratio on the surface and 2) the proportion of courting males.

We conducted a 27-day experiment to evaluate the effect of disturbance on the body condition of crabs. For this, during the diurnal tidal cycle of all days of this period we applied two intervals of disturbance and two intervals where the disturbance was removed (similarly as was explained before). After 27 days, 10 adult males and 10 adult females (carapace width larger than 9 mm) were sampled from each plots (a total of 80 crabs of each sex), measured (maximum carapace width, precision 0.02 mm), and then dried at 70°C for 48 h and weighed (precision 0.001 g). Carapace width was log transformed to fit linearity of model

(Neter et al. 1991). Differences in dry weight between treatments (disturbed–control) in relation to carapace width were evaluated with ANCOVA (Neter et al. 1991). Given the allometric growth of the enlarged claw of males, regression equations of dry weight in relation to carapace width of males and females are not parallel $(F_{1,314} = 59.03, MS_{effect} = 0.1534, P < 0.01)$, thus we made the analysis for each sex separately.

RESULTS

There was an interactive effect of treatment and disturbance state on the density of crabs $(F_{2,20} = 10.13, MS_{effect} = 12.14, P < 0.001)$. The density of crabs on the surface before disturbance was applied did not differed between the plots to be disturbed and the plots to be maintained as controls (disturbed plots: $x = 20.83$, SE = 3.71; control plots: $x = 19.58$, SE = 5.35). However, the density of crabs on the surface in disturbed plots was lower than in control plots during the disturbance (disturbed plots: $x = 1.25$, SE = 0.51; control plots: $x = 18.33$, SE = 2.26). Once disturbance was interrupted, crab density on the surface returned to initial values (disturbed plots: $x = 16.67$, SE = 2.55; control plots: $x = 23.75$, SE = 1.93).

An interactive effect between Treatment, Disturbance State and Tidal State affected the male to female ratio on the surface $(F_{1,14} = 9.32, MS_{effect} = 0.2535, P < 0.01$; Fig. 1A). During disturbance the male to female sex ratio was higher in control plots than in disturbed ones. Nevertheless, the male to female sex ratio increased in disturbed plots after disturbance. During the ebb tide,
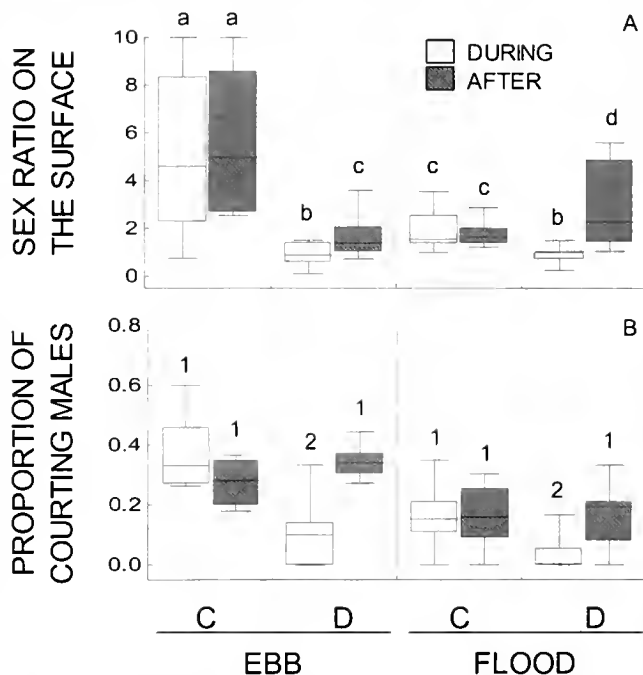
Figure 1. Effects of disturbance on the behavior of the fiddler crab *Uca uruguayensis*. (A) Male-to-female sex ratio on the surface and (B) proportion of courting males. Limits of boxes represent the 0.75 and 0.25 percentiles, lines represent the 0.01 and 0.99 percentiles, and the line inside boxes is the median. Different lowercase letters indicate differences from multiple comparisons for three factors interaction. Different numbers indicate differences from multiple comparisons for the Treatment X Disturbance State interaction. Significance is at $P < 0.05$. C = control plots, D = disturbed plots, EBB = ebb tide, FLOOD = flood tide, DURING = during disturbance, and AFTER = after disturbance.

the increase in the male to female ratio after disturbance was not large enough to surpass the values observed at control plots. During the flooding tide, however, the male to female ratio observed after disturbance exceeded the value observed at control plots.

The proportion of courting males was higher during the ebb tide than during the flood tide ($F_{1,14} = 25.19$, $MS_{effect} = 0.7094$, $P < 0.001$: Fig. 1B). However, it was lower during the disturbance at disturbed plots than either after the disturbance or at control plots (interaction effect between Treatment and Disturbance state $F_{1,14} = 22.18$, $MS_{effect} = 0.5884$, $P < 0.001$: Fig. 1B). There were not significant interactions between Treatment and Tidal State ($F_{1,14} = 0.06$, $MS_{effect} = 0.0016$, $P > 0.8$) nor between Tidal State and Disturbance state ($F_{1,14} = 0.02$, $MS_{effect} = 0.0002$, $P > 0.9$) nor between Treatment, Tidal State and Disturbance state ($F_{1,14} = 2.36$, $MS_{effect} = 0.0370$, $P > 0.1$) on the proportion of courting males.

There were no effect of disturbance on the dry weight of both males ($F_{1,157} = 0.43$, $MS_{effect} = 0.0019$, $P > 0.5$; Disturbed plots, Slope = 2.20, SE = 0.04, Elevation = 2.03, SE = 0.04; Control plots, Slope = 2.48, SE = 0.03, Elevation = 2.34, SE = 0.04) and females ($F_{1,157} = 2.60$, $MS_{effect} = 0.0020$, $P > 0.1$; Disturbed plots, Slope = 0.91, SE = 0.01, Elevation = 0.80, SE = 0.01; Control plots, Slope = 0.99, SE = 0.01, Elevation = 0.88, SE = 0.01).

DISCUSSION

Artificial disturbances are useful for the study of behavioral responses of organisms by simulating natural environmental conditions (e.g., Bell 2001, Sloman et al. 2001). Responses to disturbances are helpful for understanding how organisms face critical trade offs under changes in their environment. Our disturbance experiments show that the fiddler crab *Uca uruguayensis* has a sex-specific response. Disturbance decreased the male to female sex ratio on the surface, indicating that more males shelter into their burrows in response to disturbance. However, during the ebb tide and after the disturbance the male-to-female sex ratio in disturbed plots was lower than in control plots. This pattern was the opposite to the observed during the flood tide, which suggest that the proclivity of crabs to shelter and stay inside their burrows may depend on the time available for feeding before the tide flood their habitat. Given that males need to feed for longer periods as a consequence of the sexual dimorphism (Valiela et al. 1974, Weissburg 1992), the cost of stop to feed may be higher for them when the remaining feeding time is short.

Our study encompassed the effect of disturbance at a temporal scale of days in relation to the body weight and at a temporal scale of hours in relation to the behavioral avoiding response. Other works analyzed the effect at a lower, practically immediate, temporal scale where they look at the direct effect of the disturbance in the avoidance response of crabs. Frix et al. (1991), found that both male and female fiddler crabs *Uca pugilator* and *U. pugnax* shelter into their burrows at similar rates when simulated predators approach them indicating, in fact, that both sexes may perceive a similar risk of predation. However, females descend further into their burrows than males. This pattern could be expected if the

female is the most preyed sex, as is recognized to happen in the Frix et al. (1991) study case (see also Bildstein et al. 1989). For the case of *Uca uruguayensis*, we did not investigated if females and males shelter at similar rates, but instead, we know that during the disturbance males spend less time on the surface. The disproportionate effect on males that we have observed may be expected from a high predation rate on male crabs. However, there are not evidences of high shorebird predation in our study site (Bogazzi et al. 2001); but in nearby population of *U. uruguayensis* (Samborombón Bay; 36°22′S, 56°45′W) predation by migratory shorebirds is intense (Iribarne and Martínez 1999). Nevertheless, the occurrence and nature of sex-specific predation pressure is likely to be dependent on the predatory species present at the locale and their abundance because some predators prefer females whereas other prefers males (Iribarne & Martínez 1999). In any case, it was observed that the overall effect of predation is not male-biased (Ribeiro et al. unpublished data). Thus, the male-biased response to disturbance in this species is not related with the extant sex-specific predation pressure. This response, therefore, might have evolved under other selective forces than extant predation pressure. This scenario can occur with a higher relative abundance of shorebirds that specialize on males, such as the Ruddy Turnstone *Arenaria interpres* (Iribarne & Martinez 1999).

Disturbance also decreased the proportion of courting males, which after disturbance returned to values similar to those observed in controls. This is contrary to the expectation that males may increase their foraging effort if they loss the opportunities to do it by evading the disturbance (or potential predators). This response suggests that courtship is risky when disturbance is in action, given that the male waving display may enhance their vulnerability to predator (P. Ribeiro, unpublished data)

However, despite males lose a larger proportion of time available for feeding than females, their body condition was not affected as consequence of disturbance. This might be because disturbance was not so severe or the experimental period was not long enough. Alternatively this result may indicate that crabs were able to successfully compensate in some way for the time they loss as consequence of disturbance, which is potentially available for feeding. Given that males are less efficient foragers than females (Valiela et al. 1974, Weissburg 1992) the mechanism solving this trade off should incorporate changes in their foraging effort and changes in the mechanisms of food delivery and extraction (Weissburg 1993). The fact that the proportion of males increased after disturbance and that it was higher in disturbed plots than in control ones during the flooding tide strongly suggest that males are increasing their foraging effort after disturbance.

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GEOGRAPHICAL EXPANSION OF A NONINDIGENOUS CRAB, *CARCINUS MAENAS* (L.), ALONG THE NOVA SCOTIAN SHORE INTO THE SOUTHEASTERN GULF OF ST. LAWRENCE, CANADA

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ABSTRACT   The European green crab, *Carcinus maenas*, was first observed in the western Atlantic in the 19th century (from New Jersey to Massachusetts, USA). A northward expansion along the coast of New England has been observed in the first half of the second century. The green crab was observed in Canadian waters in Passamoquoddy Bay in 1951. The species has gradually invaded the Bay of Fundy in the 1950s, and the Atlantic coast of Nova Scotia from the 1960s to the mid 1990s, and reached the southern Gulf of St. Lawrence in the mid 1990s. Further westward expansion in the southern Gulf of St. Lawrence has been confirmed along the eastern coast of Prince Edward Island in 1997 and more recently in the Northumberland Strait at the border between Nova Scotia and New Brunswick.

KEY WORDS: *Carcinus maenas*, green crab, geographical expansion, nonindigenous crab, northwestern Atlantic, southern Gulf of St. Lawrence

INTRODUCTION

Accidental and voluntary introduction of species has occurred as a result of expanded human settlement and international trade. Over the past 200 years, the invasions were mainly due to shipping activities. Various species of invertebrates with free-swimming larvae were accidentally introduced into many coastal areas when ships using ballast water appeared around 1880 (Carlton 1985). Ruiz et al. (2000) suggested that about 298 species of invertebrates and algae have been introduced in marine and estuarine regions in North America. Crustaceans and mollusks constitute *ca* 50% of the intruders. The green crab, *Carcinus maenas* (Linnaeus, 1758), is a good example of a species that is now well established in estuarine habitats around the world.

Carcinus maenas was originally distributed along the eastern Atlantic coast, from Norway to Mauritania including southern Iceland (Broekhuysen 1936, Crothers 1968, Grosholz & Ruiz 1996). This species was recorded on the northeastern American coast in 1817 (Say 1817). Sporadic introductions in Brazil, Hawaii and Panama Bay were also recorded in the second half of the 19th century (Smith 1880). Australian occurrences were first documented about a hundred years ago in Port Phillip Bay, Victoria (Thresher 1997). The crab has since expanded its distribution from South Australia to New South Wales in the late 1970s (Zeidler 1978) and on the east coast of Tasmania in 1993 (Gardner et al. 1994). The green crab was first recorded in South Africa near Cape Town in 1983 and is now well established (LeRoux et al. 1990). The species also colonized the San Francisco Bay area (California, USA) in 1989 to 1990 (Cohen et al. 1995). The present green crab distribution on the eastern Pacific coast lies between Morro Bay (South California, USA) (Grosholz et al. 2000) and Esperanza Inlet on the west coast of Vancouver Island (British Columbia, Canada) (Glen Jamieson, pers. comm.). This rapid and irregular expansion, which occurred from 1997 to 1999, could be related to

an El Niño event during the same period (Behrens Yamada & Hunt 2000). According to these investigators, the green crab's range expansion is limited off the northwestern American coasts since then because of a declining recruitment.

On the northeast American coast, the green crab was first documented in New York and New Jersey in 1817 and slowly migrated northward towards New England where it was reported in Casco Bay (Maine, USA) in the early 1900s (Rathburn 1905). Through the following 50 years, the species has colonized various estuarine habitats along the coast of Maine up to the Bay of Fundy in Canada (Scattergood 1952, Glude 1955, MacPhail et al. 1955).

The green crab is a voracious predator of a wide range of invertebrates (Elner 1981) with preferences for bivalve species (Ropes 1968) (e.g., American oysters [*Crassostrea virginica*], soft-shell clams [*Mya arenaria*], blue mussels [*Mytilus edulis*] and northern quahogs [*Mercenaria mercenaria*]). Aquaculture stakeholders in the southern Gulf of St. Lawrence (SGSL) expressed serious concerns about a potential threat to cultured and wild shellfish populations in the Canadian maritime provinces.

The purpose of this paper is to document the northeast expansion of the green crab in eastern Canadian waters, from the Passamaquoddy Bay area in New Brunswick (NB) along the shores of Nova Scotia (NS) to the SGSL. The possible effects on the shellfish aquaculture industry are also discussed.

MATERIALS AND METHODS

Museum Archives and Interviews

Unpublished museum records were examined from the Nova Scotia Museum of Natural History (NSMNH) (Halifax, NS), the Atlantic Reference Centre (ARC) (St. Andrews, NB) and the Canadian Museum of Nature (CMN) (Ottawa, Ontario) to complete the history of occurrence of *C. maenas* along NS and NB coasts.

Interviews were carried out among twelve eel fishermen and four fishery officers in the fall of 1998. Eel fishermen were chosen because they were fishing in potential green crab habitats, and fishery officers for their frequent contacts with various fishermen.

Interviews were held in northern NS and western Cape Breton Island (CBI) to obtain information on the year and location of the first green crab occurrence in commercial catches.

Survey

Annual observations on the presence and absence of green crabs were made during the summer period (June to September) from 1997 to 2001. Forty-six stations (estuary and river systems) were chosen at an interval of 30–50 km along (1) the coast between the southwestern region of Bras d'Or Lakes and the tip of CBI; (2) between the western coast of CBI and Shippagan along the NB coast; and (3) around Prince Edward Island (PEI), (Table 1 and see Fig. 2). A frozen mackerel was placed in a modified pearl lantern net (30 × 30 cm with two openings) and immersed to the bottom at each observation site for a duration of 15–30 min to determine the green crab presence.

RESULTS AND DISCUSSION

Northward Expansion of C. maenas Along the New England Coast, USA

Rafinesque (1817), as stated in Fowler (1912), reported the presence of the green crab off the coasts of Long Island, New York and New Jersey in 1817, and Say (1817) confirmed the presence of the green crab in estuarine habitats off the Atlantic coast of the United States in 1817. Smith (1880) stated that the range of *C. maenas* was limited in northwestern Atlantic waters in 1871 and 1872. At the time, the crab seemed to be found in great numbers and well established in Great Egg harbor (New Jersey), on the southern coast of Long Island (New York), in Long Island Sound (Connecticut), and in Vineyard Sound, Buzzards Bay and Provincetown (Massachusetts) (Fig. 1). Rathburn (1905) reported that the

crab reached Maine's Casco Bay area (Eagle Harbor, Harpswell and New Meadows River) in 1905. Green crab observations, however, in Maine at that time were scarce and the species was not considered a regular member of the community before 1935 (Scattergood 1952). According to Scattergood (1952), the northern limit of its distribution was near Winter Harbor (Maine) from 1939 to 1942 (Fig. 1). Dow and Wallace (1952) reported that the presence of green crabs at Lakeman's Harbor on Spruce Island near Jonesport was observed in 1919 by a lobster fisher. There were no further reports until 1948 and by 1951, green crabs were abundant in Jonesport and also found in Lubec in Passamaquoddy Bay (Fig. 1).

Expansion of C. maenas in the Bay of Fundy

A specimen of *C. maenas* was discovered in 1951 in the estuary of the Digdeguash River (Fig. 1) in Passamaquoddy Bay near Oven Head (NB) (Scattergood 1952, MacPhail 1953). Five green crabs were also collected the same year at the mouth of Magaguadavic River, near St. George (ARC unpublished records). A small number of crabs were found a year later in the western Bay of Fundy. Crabs were observed, however, in great numbers in the entire Passamaquoddy Bay in Pocologan Harbor and in the Lepreau Basin (NB) by spring of 1953 (MacPhail et al. 1955). They then spread eastward in the Bay of Fundy (Welch 1968) where it was reported in Sandy Cove on the northern shore of St. Marys Bay (NS) and at the mouth of the Pereau River in the Minas Basin (NS), both in 1953 (MacPhail et al. 1955) (Fig. 1). By 1958, green crab populations were established in Minas Basin (Bousfield & Leim 1960, NSMNH unpublished records). The eastward range expansion in NS was confirmed with the presence of one crab in Wedgeport in 1954 (MacPhail et al. 1955). A survey made from Cape Fourchu to Three Fathom Harbor (between Lawrencetown

TABLE 1.

Confirmed sighting of green crab (*Carcinus maenas*) in the southern Gulf of St. Lawrence and adjacent area during the summer period (June to September) between 1997 and 2001. The station number corresponds to the numbers in Figure 2.

St.	Site	97	98	99	00	01	St.	Site	97	98	99	00	01
1	NB Shippagan	−	−	−	−	−	24	Wagmatcook	+	+	+	+	+
2	Tracadie	−	−	−	−	−	25	Dundee	+	+	+	+	+
3	Escuminac	−	−	−	−	−	26	PEI Cap Traverse	−	−	−	−	−
4	Richibuctou	−	−	−	−	−	27	Clyde River	−	−	−	−	−
5	Pointe-du-Chêne	−	−	−	−	−	28	Vernon Bridge	−	−	+	+	−
6	Murray Corner	−	−	−	−	−	29	Belle River	−	−	−	−	−
7	Baie Verte	−	−	−	−	−	30	Murray Harbor	−	+	+	+	+
8	NS Pugwash	−	−	−	−	−	31	St. Mary's Bay	−	+	+	+	+
9	Sand Point	−	−	−	−	−	32	Cardigan River	+	−	+	+	+
10	Caribou River	−	−	+	+	+	33	Bay Fortune	−	+	+	+	+
11	Merigomish	−	+	+	+	+	34	East Lake	−	−	+	+	+
12	Malignant Cove	+	−	+	+	+	35	Naufrage	−	−	+	+	+
13	Bayfield	−	+	+	+	+	36	St. Peters	−	−	−	−	−
14	Strait of Canso	+	+	+	+	+	37	Winter Bay	−	−	−	−	−
15	Mabou	+	+	+	+	+	38	North Rustico	−	−	−	−	−
16	Inverness	−	+	+	+	+	39	French River	−	−	−	−	−
17	Margaree Harbor	+	+	+	+	+	40	Indian River	−	−	−	−	−
18	Petit Etang	+	−	+	+	+	41	Bideford River	−	−	−	−	−
19	Pleasant Bay	+	+	+	+	+	42	Cascumpec Bay	−	−	−	−	−
20	South Harbor	−	−	−	−	−	43	Anglo Tignish	−	−	−	−	−
21	Ingonish	+	+	+	+	+	44	Miminegash	−	−	−	−	−
22	Indian Brook	+	+	+	+	+	45	Baie Egmont	−	−	−	−	−
23	Baddeck	+	+	+	+	+	46	Summerside	−	−	−	−	−
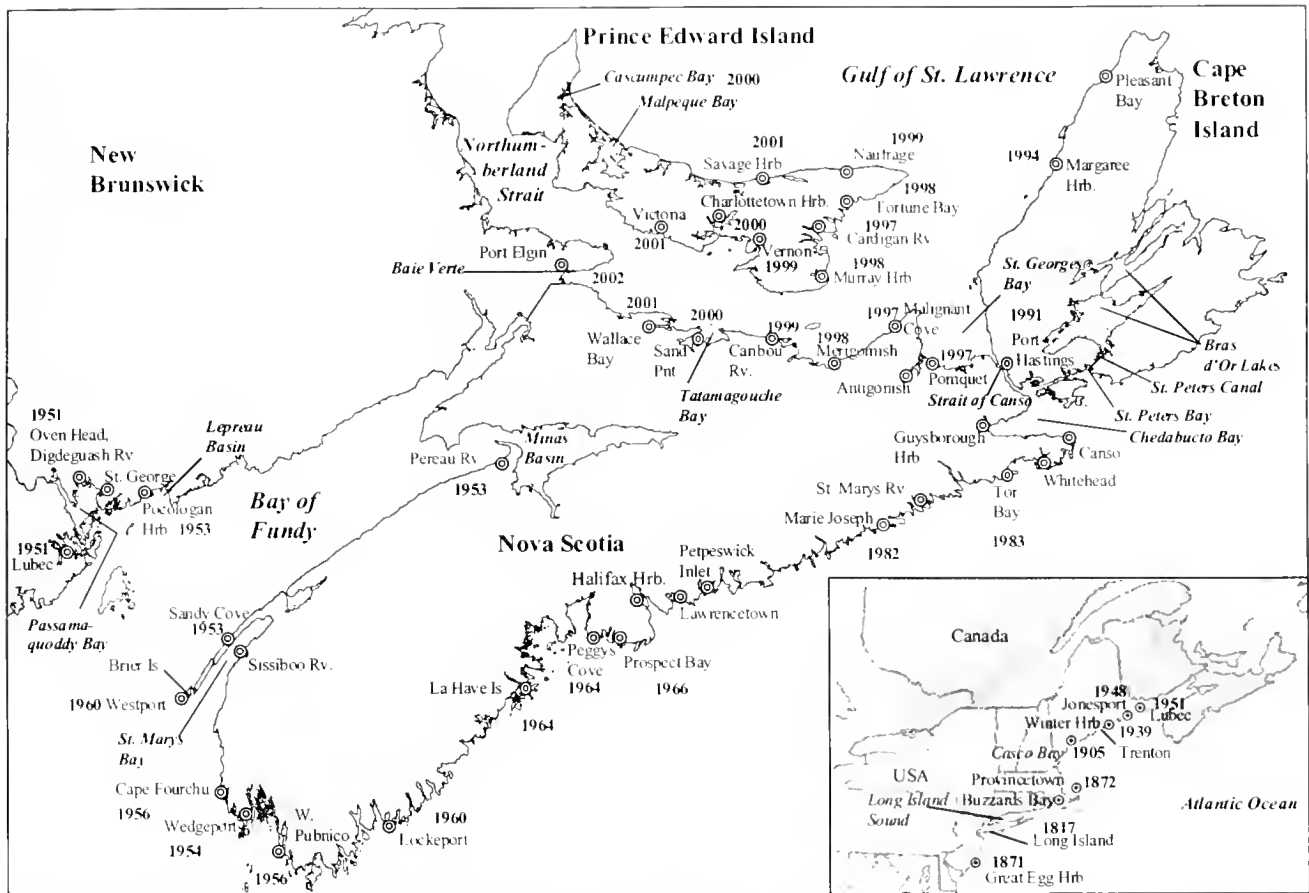
Figure 1. Historical records of sighting of *Carcinus maenas* from the eastern USA to the eastern Canadian coasts. Date indicates the earliest confirmed sighting.

and Petpeswick Inlet) by Bousfield (1958) in 1956, revealed that *C. maenas* was present only in the Cape Fourchu and West Pubnico areas in few numbers (CMN unpublished records). According to the NSMNH records, the presence of the green crab was confirmed in Westport on Brier Island in 1960. MacPhail et al. (1955) reported a low catch rate of green crabs (i.e., an average of two crabs a day) at the mouth of the Sissiboo River in St. Marys Bay in the mid 1950s. From the first green crab sighting in the Bay of Fundy, the species expanded its range more than 400 km in 2 years. The crab density increased significantly from 1952 to 1953 in Passamaquoddy Bay (MacPhail et al. 1955).

Further Northward Expansion Along the Eastern Coast of Nova Scotia

The spread of the green crab around the southwestern end of NS began at the latest in 1954 to 1956. The presence of the crab was reported in Lockeport on the southeastern coast of NS in 1960 (Anonymous 1961). Green crabs were considered by fishermen to be abundant starting from 1964 in the La Have Islands area only 4 years after their arrival on the southeastern coast of NS (ARC unpublished records). They were collected from Peggys Cove to Prospect Bay from 1964 to 1966, respectively (NSMNH unpublished records). After reaching Peggys Cove in the mid 1960s, the abundance of green crabs decreased considerably and the rate of expansion further north seemed to have diminished possibly due to

the influence of the cold Nova Scotian coastal current (Davis & Browne 1996).

During a survey made in Passamaquoddy Bay in 1954, about 300 crabs were caught per baited trap with a 24-h soak time. In 1958, the catch rate was recorded at 53 crabs per trap per day. It then dropped to 7.5 crabs in 1960 (Anonymous 1961). This decrease of crab abundance in the Bay of Fundy seems to coincide with a general cooling period, which was reported from 1953 to 1962 (Lauzier & Hull 1962). A significant diminution of crab abundance was also observed in Trenton (Maine, USA) where catches decreased from 271 crabs per trap per day in 1953 to a total absence in 1958 to 1965 (Welch 1968). Welch (1968) suggested that this rapid decrease in crab abundance might be caused by severe winter conditions along the New England coast between the late 1950s and mid 1960s. Similarly, on the other side of the Atlantic, winter was particularly cold in 1962 to 1963 around the British Isles and large adult crabs did not survive the cold weather, resulting in a major drop in density (Clay 1967). However, juveniles and smaller adult crabs survived and repopulated the British Isles. The recently established population in the Bay of Fundy was probably smaller than the one from the British Isles. Genetic variations may explain both populations' response to a cold environment. For instance, the Canadian green crabs may be less cold environment-adapted. It then took a longer period of time for the Maritime green crab populations to adapt to new environmental conditions through the cold period.

Collections and records of intertidal animals made by NSMNH (unpublished) in Halifax Harbor and at Lawrencetown in 1965 to 1966 did not include *C. maenas*. No record of the species was made during an extensive study in Petpeswick Inlet near Halifax Harbor in 1971 (Davis 1972, NSMNH unpublished records) or in the St. Marys River estuary in 1973 (Davis 1976).

No surveys were conducted during the period when the northern limit of the green crab distribution progressed toward the Canso area, CBI and the northern coast of NS. The green crab sampling program carried out by the NSMNH on the eastern shore of NS since the late 1970s was rather sporadic. No direct study has been conducted on *C. maenas* in this area until today. Green crabs were collected in Marie Joseph in 1982 and in Tor Bay (NS) in 1983, the species being most likely established at these localities before those dates. It was not observed in Guysborough Harbor in 1983. Green crab probably entered Chedabucto Bay around 1985, which potentially provided access to the Northumberland Strait through the Strait of Canso, and to the Bras d'Or Lakes through St. Peter's Canal. Anecdotal information suggested the presence of this species in the Bras d'Or Lakes before 1995 (Kara Paul & John M. Tremblay, pers. comm.). The species is widely distributed in the main lake since 2000.

Expansion of *C. maenas* from Cape Breton Island Toward the Southern Gulf of St. Lawrence

The westward expansion of this species within the last 20 years was rapid (Fig. 1). This species, considering that it was not reported frequently in northeastern CBI, may have invaded the SGSL through the Strait of Canso in the early 1990s. Squires (1990) misinterpretation of Bousfield and Laubitz's (1972) records led him to conclude that the species was present in Northumberland Strait in 1960. This result was due to the duplication of station number series (S-series) used for studies in the SGSL in 1960 and

southwestern NS in 1963. Bousfield and Laubitz (1972), however, did not record *C. maenas* in the Northumberland Strait during their studies. Eel fishermen interviewed from the western side of CBI caught green crabs in their nets for the first time in 1998. One fisherman from Margaree Harbor mentioned that he has been collecting green crabs since either 1994 or 1995. He latter stated that the abundance had increased in 1998. The earliest green crab report concerning St. Georges Bay was from an eel fisherman in Pomquet in 1997. The occurrence of green crabs in eel nets is directly related to the fishing effort during the eel fishing season. Most fishermen from Caribou up to Port Hastings have not encountered crabs within the years preceding the survey. In this area, the fishing effort increased when fyke nets were first used in 1993. As a decreasing trend in eel density and fishing effort was observed in 1995 (Chaput et al. 1997, Paulin 1997), the chance of encountering green crab might also have decreased after 1995. Crabs were observed in great numbers in Antigonish (St. Georges Bay) in 1999 (Jim Williams, pers. comm.).

A qualitative survey carried out along the coast of the SGSL (from NB to CBI and around PEI) from 1997 to 2001 (Table 1, Fig. 2) revealed that the green crab was present in estuaries along the northeastern shore of CBI and in the Bras d'Or Lakes in 1997. The survey also confirmed that *C. maenas* was present in Malignant Cove (NS) from at least 1997, which was the most advanced expansion in the SGSL at the time. In 2000, the abundance possibly became greater (the catching method used was greatly influenced by the abundance) and the distribution reached the eastern opening of the Northumberland Strait. The western limit of the green crab gradually moved from Merigomish in 1998 to Caribou River in 1999, indicating that the crab has been moving westward along the coast of NS. Shellfish aquaculturists started to express their concerns regarding green crabs off the northern coast of NS when crabs were reported near Sand Point in Tatamagouche Bay (J. Mark Hanson & Andrea Locke, pers. comm.) and in Wallace
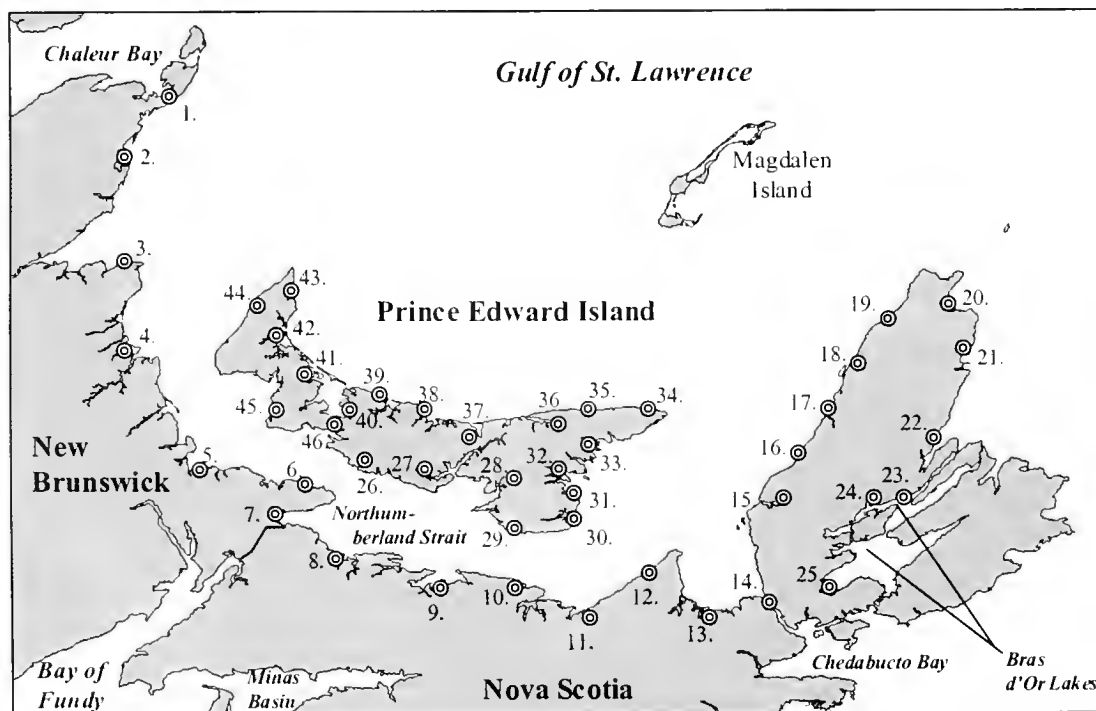
Figure 2. Sampling location for the survey of the occurrence of *Carcinus maenas* conducted in the southern Gulf of St. Lawrence and adjacent area during summer (June to September) between 1997 and 2001. Station number corresponds to the sampling sites described in Table 1.

Bay (Marc Ouellette, pers. comm.) in 2000 and 2001, respectively. The crab has recently (June 2002) been collected near Port Elgin in Baie Verte (NB) (J. Mark Hanson & Andrea Locke, pers. comm.).

Green crabs were present in Tor Bay in 1983 and probably invaded Chedabucto Bay around 1985. They then spread into St. Peters Bay to possibly reach the Bras d'Or Lakes before 1995 (D. Davis, unpublished). A lobster fisherman reported the presence of the species in Port Hastings, along the Strait of Canso in the early 1990s (John M. Tremblay, unpublished). It is difficult however, to trace the pathway of the species' expansion around CBI, as little information was collected in the late 1980s and early 1990s. The species did not seem to reach the SGSL through the Strait of Canso first because it was reported in St. Georges Bay only in 1997. The presence of *C. maenas* was rather first observed in the SGSL in 1994, on the western coast of CBI. Still, there is no evidence of invasion pathway into the western CBI.

Invasion of new habitats may be due to natural larval transport and migratory patterns, but may also be the result of transfer with other species (e.g., oysters, blue mussels, scallops [*Placopecten magellanicus*], American eels [*Anguilla rostrata*], and American lobsters) from already invaded regions. Roff et al. (1984) studied brachyuran larvae off the Scotian Shelf in 1977 to 1978 and reported that zoeae and megalopae of *C. maenas* were common, but restricted off the coast of southwestern NS. A blue mussel grower from Whitehead, 600 km northeastward (Fig. 1), collected green crabs in mid to late 1970s (John M. Tremblay, unpublished). This report is the only case of a simultaneous occurrence of the species at such distant locations throughout the northern geographic invasion history of this species in the western Atlantic. A low research effort on this species at that time may be the reason why we observe punctual invasions (i.e., not being observed in the White-head area for a long period after 1970). There is no reason to presume however, that the northeastward invasion of green crab along the NS coast is continuous and initiated by a single source from a southwestern area. The invasion of green crab could be the result of multiple invasions as suggested by Geller et al. (1997) for *C. aestuarii* in Japan and in South Africa. Further comparative studies have to be carried out on the genetic characteristics of the species along the coast of NS.

Invasion of Prince Edward Island

The geographic distribution of green crabs in PEI was limited to the Cardigan River system, in the summer of 1997 (Table 1, Fig. 2). In 1998, crabs were reported from Fortune Bay to Murray Harbor along the eastern coast of PEI. Our survey, held from 1997 to 2001, indicated that the geographical expansion from 1999 to present did not exceed Naufrage and Vernon Bridge on the north and south shores, respectively. Intensive surveys conducted by the PEI Department of Fisheries, Aquaculture and Environment (PEIDFAE), however, showed that *C. maenas* was mainly restricted to the southeastern coast in 1999, the distribution including North Lake on the north shore and Gascoigne Cove on the south shore. In 2000 crabs were detected in the Charlottetown Harbor area, and in 2001 the western limit of the distribution moved toward Victoria on the south shore and Savage Harbor on the northern shore. Ovigerous females were observed in samples collected in eastern PEI in the summer of 1999 (Gillis et al. 2000). This observation suggests that this species is locally self-reproductive. There have been isolated reports of green crabs in the blue mussel and American oyster culture sites in Cascumpec and

Malpeque Bays in the northwestern part of PEI in 2000 (Neil J. MacNair, pers. comm.). We consider that these crabs might have been accidentally introduced around the Island by way of aqua-culture activities, as no further report on the presence of green crab in this area was made since then.

According to an investigation carried out in 1998 and 1999 by the PEIDFAE, the green crab likely arrived as a result of natural larval transport from NS (Gillis et al. 2000). There is no factual data, however, to support the arrival of this species in eastern PEI by larval transportation. If true, the megalopal settlement would have occurred as early as the mid 1990s, shortly after the known introduction of the crab on the western coast of CBI. Zoea larvae can travel with currents in the open sea. Larvae from CBI could be the source that fed the southeastern shores of PEI. Oceanographic conditions between PEI and CBI appear to support this hypothesis. Lauzier 1965 and Koutitonsky & Bugden (1991) showed that a gyre is induced by wind and internal wave activity at the mouth of St. Georges Bay. Currently, the green crab is observed from Pleasant Bay on the northwest coast of CBI down to Baie Verte at the NS-NB border and from Savage Harbor to Victoria, PEI. The gradual westward progression of green crabs is taking place at a similar rate on both sides of the Northumberland Strait.

Potential Expansion in the Gulf of St. Lawrence

The coastal habitats of PEI are rich in estuaries and are surrounded by the warm summer waters of the Magdalen Shallows and the Northumberland Strait. The environmental characteristics in the SGSL are ideal for a rapid and effective proliferation of the green crab. Warmer coastal temperatures in the summer and shorter winters would allow the species to grow faster and to expand their habitats as observed in the last 10 years in NS. Lauzier and Hull (1962) showed that the Bay of Fundy area was under a general warming period in the 1940s and 1950s (the mean water temperature increased by 1.8°C from 1940 to 1953). According to Pocklington et al. (1994), the warmest years were observed from 1951 to 1953. This was followed by a cooler period from 1953 to the mid 1960s and 1970s. The sea surface temperature followed a similar trend. Temperatures were above normal from 1930 to 1960 and reached a maximum in the late 1950s. These investigators suggest, however, that there was a general cooling period from the 1960s to present. In fact, water temperatures between 1981 and 1990 in eastern Canada seem to be near the long-term average and significantly colder than recorded during the warm conditions of the 1950s (Pocklington et al. 1994). The green crab may have invaded the Bay of Fundy during the warming period and this species has now reached the SGSL. Good seasonal environmental conditions in this area may contribute to a northwestern geographical expansion in the SGSL. Cohen et al. (1995), for instance, predicted that *C. maenas* could establish itself from California to Alaska, considering the wide range of temperatures and salinities the species can tolerate in the Pacific Ocean.

At this point, the abundance of green crabs in PEI is lower than what is observed on the northern coast of NS. A mean catch rate of 10 ± 5 crabs per trap with a 24-h soak time was recorded in Basin Head between 2000 and 2002 on the eastern coast of PEI (Audet et al. in prep.). The same fishing gear captured hundreds of crabs in a few hours in Antigonish (NS) (Jim Williams, pers. comm.). As the green crab appears to be well established on the western coasts of CBI through the last decade, an increase in abundance and a westward expansion of the species are expected in PEI and NB in the near future. A close monitoring program is

needed to follow the progression of green crabs on a possible northwestward expansion from the edge of the Northumberland Strait to the Chaleur Bay, where coastal temperatures (Savoie & Lanteigne 2002) are favorable to the species.

Potential Impacts on the Aquaculture Industry

The green crab is an omnivorous species. Its diet includes polychaetes, crustaceans, mollusks, and green algae (Crother 1968, Ropes 1968). Juvenile crabs are considered, among all, as green algae grazers, using the sea lettuce (*Ulva lactuca*) beds as a refuge. Large adult males prey on various species including commercially exploited molluskan species (e.g., blue mussels and dogwhelks (*Nucella lapillus*), flat oysters (*Ostrea edulis*), Pacific oysters (*Crassostrea gigas*), the soft-shell clams and the northern quahog) (Glude 1955, Kaiser et al. 1993, Feare 1970, Marin et al. 1973, Mascaró & Seed 2000, Walton & Walton 2001). Naylor (1962) and Miron et al. (2002) observed that the feeding activity of the green crab varied considerably depending on water temperatures. They suggested that green crabs would cause certain damage to molluskan species during the summer period. Case studies from NS (MacPhail et al. 1955, Ropes 1968) and Maine (Smith 1954, Glude 1955) demonstrated a high vulnerability of molluskan species to green crab predation. Blue mussel and American oyster aquaculture are the most lucrative industries on PEI (Boghen 1995). Natural populations of soft-shell clams are currently heavily exploited and a trial production of northern quahogs is underway in PEI (Brown et al. 1995). The industry in the SGSL has an increasing interest in the cultivation of native shellfish. The rapid expansion of the green crab population in the same area may threaten the shellfish aquaculture industries. Some protective measures could be used, such as fencing aquaculture sites to prevent intrusion of the green crab as practiced in Norway to protect scallops (*Pecten maximus*) against the brown crab (*Cancer pagurus*) predation (Strand et al. 1999).

Physiological Adaptation and Limitation

The green crab has a high reproductive potential (e.g., 200,000 eggs per female) (Broekhuysen 1936). They are also known to be tolerant to extreme environmental conditions (Broekhuysen 1936, Wheatly 1981, Abelló et al. 1997). The green crab population established itself quickly in the North Pacific (Jamieson et al. 1998) by colonizing the intertidal habitat (0.7–1.4 m above mean lower low water) in sheltered areas. Green crab inhabits depths down to 10 m in the SGSL (Gillis et al. 2000). This is probably due to their physiological tolerance to low water temperature conditions during the winter period. Preliminary results obtained by Audet et al. (in prep.) revealed that key biological events (e.g., molting, mating, and egg bearing) occur later in the SGSL compared with similar events occurring in the southern Atlantic (Berrill 1982). Temperatures are warmer during summer periods, reaching 26°C in lagoons on the eastern coast of PEI and −2°C during the winter season. Water temperature remains <10°C for at least 8 months of the year. Although the embryonic stages are vulnerable to fluctuating water temperatures and salinities (Naga-

raj 1993, Anger et al. 1998), the species possibly adapted to a narrow breeding time frame during the warmer months. Zoeal larvae, which prefer high salinities, probably migrate offshore during ebb tides and re-invade the estuarine habitats as euryhaline megalopae (Queiroga 1998). Nagaraj (1993) reported that the four planktonic stages of *C. maenas* developed successfully in temperatures ranging from 10°C–25°C and salinities from 20 to 35‰. This may be the reason why the green crab has successfully established itself in the Bay of Fundy and off the southeastern coast of NS during the last 50 years despite low mean surface water temperatures (12°C–14°C) (Harding et al. 1983).

A threat to the ecological equilibrium is also possible. Long term effects are still difficult to identify at the moment, but may have great consequences. *Carcinus maenas*, with its high fecundity, high capability to tolerate a wide range of environmental conditions, and omnivorous feeding behavior, appears as an excellent invader and can certainly displace endemic species. Lagoons and estuaries around PEI that have been colonized by green crabs are also used by various commercial crustacean species such as the American lobster. Competition for space and food may be foreseen (Moody & Steneck 1993). The American lobsters, rock crabs (*Cancer irroratus*), and various mud crabs (*Rithropanopeus harrisii* and *Dyspanopeus sayi*) represent potential species that might have to compete with the green crab in the SGSL. On the North American Pacific coast, inter-specific competition forced juvenile Dungeness crab (*Cancer magister*) to emigrate from their natural oyster shell habitat. Green crabs also seem to be able to dominate equal size Dungeness crabs during altercations (McDonald et al. 2001). It is therefore important to closely monitor the ecology of non-indigenous species, as their ecological effects are not well known (e.g., alteration of food webs, displacement of other resident crustacean species).

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MINIMUM ENVIRONMENTAL POTASSIUM FOR SURVIVAL OF PACIFIC WHITE SHRIMP *LITOPENAEUS VANNAMEI* (BOONE) IN FRESHWATER

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ABSTRACT The effect of three essential osmoregulatory ions (Mg^{2+}, K^+, and SO_4^{2-}) on the short-term survival of Pacific white shrimp *Litopenaeus vannamei* in freshwater (<1 ppt total ion concentration) was examined in several experiments. Shrimp postlarvae (PL-18 and -28) were acclimated from seawater (32 ppt) to freshwater (700 ppm TDS; 280 ppm Cl^-) over 48 h and held for an additional 24 h before being placed in treatment solutions. Treatments consisted of various Mg^{2+}, K^+, and SO_4^{2-} concentrations in Na^+ and Ca^{2+} solutions that were all derived from chloride or sodium based chemicals added to distilled water. Ten shrimp were placed in triplicate 4-L plastic containers holding 2 L of treatment solutions for 24–48 h. Potassium was found to significantly ($P < 0.05$) increase shrimp survival, whereas Mg^{2+} and SO_4^{2-} had no effect. Solutions with K^+ exhibited an average increase in survival of 20% and 42% above solutions without K^+ at 24 and 48 h, respectively. This study demonstrates the necessity of K^+ in "freshwater" at a minimum concentration of 1 ppm for the survival of this euryhaline marine shrimp. The regulatory aspects involved in maintaining K^+ in crustaceans under hypo-osmotic conditions are discussed.

KEY WORDS: *Litopenaeus vannamei*, shrimp, osmoregulation, potassium, ions

INTRODUCTION

US seafood imports have steadily increased over the last several years. Shrimp imports alone for the year 2000 were valued at $3.8 billion (Harvey 2002), accounting for approximately 80% of the total shrimp consumed in this country. Although marine shrimp is the highest seafood import in terms of dollar value, the high cost of coastal land, user conflict, and strict requirements regarding effluent discharge have, at least in part, prevented the expansion of shrimp aquaculture in the U.S. (Hopkins et al. 1996). An opportunity exists to expand US shrimp culture through the use of inland well water with low concentrations of ions [700 –5000 ppm total dissolved solids (TDS)]. Saline well water exists under two thirds of the United States (Feth 1970) and some catfish farmers already use this water source for aquaculture (Teichert-Coddington, Green Prairie Aquafarm, personal communication, 2000) as it provides an added benefit of reducing the toxicity of nitrite in catfish blood (Boyd 1990).

The use of well water from inland locations for shrimp culture faces many challenges for development. The shrimp species best suited for low salinity or freshwater culture is the species most used for aquaculture in the western hemisphere: *Litopenaeus vannamei* (Ogle et al. 1992, Scarpa & Vaughan 1998). Information on essential environmental ions and minimum concentrations necessary for survival and growth of this shrimp is lacking, although salinity tolerance has been examined (Ogle et al. 1992, Scarpa & Vaughan 1998, Laramore et al. 2001, McGraw et al. 2002). *L. vannamei* is being grown successfully in freshwater (700–1000 ppm TDS) at Harbor Branch Oceanographic Institution (Van Wyk et al. 1999) and in low salinity water in other areas of the United States (Samocha et al. 1998, Ednoff 2001, Samocha et al. 2002).

Concentrations of the major ions involved in shrimp osmoregulation (Na^+, Ca^{2+}, Mg^{2+}, K^+, Cl^-, SO_4^{2-}; Schmidt-Nielsen 1990) and total salinities of ground waters vary widely in the United States (Saoud et al. 2002). A knowledge of which of the essential ions and their concentrations are necessary for survival and growth of marine shrimp in freshwater (<1000 ppm TDS) can help determine the suitability of well water sources for inland aquaculture of *L. vannamei*.

During investigations of the environmental ionic requirements of marine shrimp cultured in freshwater (McGraw & Scarpa 2002), it became apparent that K^+ had a significant effect on survival. The following work describes a series of experiments that examined the effect K^+ had on the short-term survival of the Pacific white shrimp *Litopenaeus vannamei* in freshwater.

MATERIALS AND METHODS

Experimental Design

Postlarval Pacific white shrimp (*Litopenaeus vannamei* Boone) (PL-9: 9 days after larval metamorphosis), were obtained from a commercial hatchery (Shrimp Improvement Systems, Islamorada, FL). Shrimp were cultured in seawater (local source, ~32 ppt) and fed a prepared diet (44% protein, Bonney, Laramore & Hopkins, Inc., Ft. Pierce, FL) *ad libitum* three times per day until the beginning of salinity acclimation (PL-15 and -25). Postlarval shrimp were acclimated from 32 ppt seawater to HBOI freshwater (~280 ppm chloride, ~700 ppm TDS) at a rate of 50% reduction in salinity per 8 h over a 48-h period (Van Wyk et al. 1999). Acclimation was stopped after 48 h. Shrimp were held in HBOI freshwater for another 24 h before being placed in triplicate 4-L plastic containers filled with 2 L of the various treatment solutions, which included HBOI freshwater (well water) as an outside control. Shrimp density was 5 PLs/L. Diffused air was used to aerate all treatment and control solutions.

Treatment solutions were prepared by adding reagent-grade chloride-based chemicals (NaCl, $CaCl_2*2H_2O$, $MgCl_2*6H_2O$ and KCl, Sigma Chemical, St. Louis, MO), except sulfate (Na_2SO_4), to distilled water. All ions in treatment and control solutions, except sodium and chloride, were measured using Hach DR/3 spectrophotometric methods (Hach Company, Loveland, CO). Chloride was measured using a titration kit (Lamotte Co., Chestertown, MD). Sodium concentrations were calculated. All measured ion concentrations were within 5% of the listed treatment values. Tem-

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perature and pH were measured using a standard mercury thermometer and pH meter (pH, Engineered Systems and Designs, Newark, DE), respectively. Survival of shrimp was checked at 24 and 48 h after placement into treatment solutions. Shrimp were not fed during the 48-h test period.

Statistical tests [general linear model (GLM), analysis of variance, Dunnets and Student–Newman–Keuls] were used to compare survival between ion treatments and control versus ion treatment waters (Lentner & Bishop 1993). All percentage survival data were transformed (arcsine*square root) before statistical analyses (SuperAnova, Abacus Concepts Inc., Berkeley, CA). Differences were considered significant if $P < 0.05$.

Effect of Mg^{2+} and K^+ Ions

The effect of different Mg^{2+} and K^+ concentrations on short-term survival of postlarval (PL-18) shrimp in Na^+ and Ca^{2+} solutions was examined. Sodium and calcium concentrations were held constant at 300 ppm (13 mM) and 60 ppm (1.5 mM), respectively. To this base solution, Mg^{2+} and K^+ were added at 75 ppm (3.1 mM) and 10 ppm (0.26 mM), respectively, alone, in combination, or not at all (Table 1).

Ion concentrations of treatment solutions were based on HBOI freshwater ion data taken from an alternate well water source that was different in ionic composition than the well water used in the present study as a control. Chloride concentrations of ion treatment solutions ranged from 568 to 796 ppm (16 to 22 mM) with calculated total ion concentrations varying from 928 to 1241 ppm. Temperature of all solutions was 25–27°C and pH values of treatment water ranged from 6.5 to 6.7, whereas the HBOI water pH was 8.2.

Effect of SO_4^{2-} Ions

The effect of sulfate ions on short-term survival of postlarval (PL-28) shrimp was examined because sulfate is: 1) present in HBOI freshwater; 2) considered to be an essential ion; and 3) was not tested in the previous experiment. Sodium, Ca^{2+}, and Mg^{2+} concentrations were held constant at 290 ppm (12.6 mM), 54 ppm (1.3 mM), and 53 ppm (2.1 mM), respectively (Table 2). To this base solution, K^+ and SO_4^{2-} were added at 15 ppm (0.39 mM) and 140 ppm (1.4 mM), respectively (Table 2). Sulfate was added to treatment solutions as Na_2SO_4 with all sodium ions accounted for. All treatment solutions were prepared as described previously with one exception; $NaHCO_3$ was added to produce 16 mg/L alkalinity as $CaCO_3$ (with all additional Na^+ ions accounted for, causing a decrease of 15 ppm of Cl^-). Chloride concentrations of treatment

solutions ranged from 592–682 ppm (17–19 mM) with calculated total ion concentrations ranging from 1079 to 1144 ppm (excluding bicarbonate ions). Temperature and pH of treatment solutions were 26–27°C and 7.3, respectively.

Effect of K^+ Ions

Results from the previous experiments indicated that K^+ had a major effect on short-term postlarval shrimp survival. Therefore, the effect of various K^+ concentrations on short-term survival of postlarval (PL-28) shrimp was examined. Sodium, Ca^{2+}, and Mg^{2+} concentrations were held constant at 290 ppm (12.6 mM), 54 ppm (1.3 mM), and 53 ppm (2.1 mM), respectively. To this base solution, K^+ (as KCl) was added at graded levels (1–50 ppm, 0.02–1.3 mM; Table 3). Sodium bicarbonate ($NaHCO_3$) was added to produce 80 mg/L alkalinity as $CaCO_3$. This increased pH values from 6.5 to 6.7 to between 7.4 and 7.6, closer to that of the control (8.2). Temperature was maintained at 26–27°C. Chloride concentrations of treatment solutions ranged from 626–694 ppm (17–20 mM) with calculated total ion concentrations ranging from 1023 to 1141 ppm (excluding bicarbonate ions).

RESULTS

Effect of Mg^{2+} and K^+ Ions

Mean survival of postlarval shrimp (PL-18) in treatment solutions ranged from 73–97% for 24 h and from 43–83% for 48-h survival periods (Table 1). Potassium had a significant effect ($P = 0.023$) on 24-h survival of *L. vannamei* postlarvae but not so on 48-h survival ($P = 0.075$). Magnesium did not significantly affect shrimp survival for either time-period (24 h: $P = 0.092$; 48 h: $P = 0.789$). There were no significant interactions for either time period (24 h: $P = 0.171$; 48 h: $P = 0.491$). Survival in the full complement ion solution (containing all five ions: Na^+, Cl^-, Mg^{2+}, Ca^{2+}, K^+) was not significantly different than HBOI water for the 24- and 48-h periods; (24 h: $P = 0.673$; 48 h: $P = 0.899$). The highest 24- and 48-h survivals were obtained with treatment 3 and the HBOI water, which contained all of the treatment ions.

Effect of SO_4^{2-} Ions

Mean survival of postlarval shrimp (PL-28) in treatment solutions ranged from 43–86% for 24 h and from 26–86% for 48 h (Table 2). Among the four individual treatments there was no statistical difference, however, there was significantly ($P < 0.01$) lower survival between treatments 1 and 2 (without K^+) compared with treatments 3 and 4 (with K^+). Survival for the treatment

TABLE 1.

Mean (± SE, $n = 3$) 24- and 48-h survival (%) of PL-18 *L. vannamei* in different ion solutions (ppm). Potassium had a significant effect ($P < 0.05$ level) only for the 24-h survival period.

Treatment Solution	ppm[a]						24-h % Survival	48-h % Survival
	Na^+	Ca^{2+}	Mg^{2+}	K^+	Cl^-	Total Ions		
1	300	60			568	928	73 (8.8)	60 (17.3)
2	300	60	75		786	1221	77 (6.7)	43 (13.3)
3	300	60	75	10	796	1241	97 (3.3)	83 (12.0)
4	300	60		10	577	947	83 (5.8)	77 (12.0)
Control	181	44	31	10	280	546	93 (5.8)	87 (8.8)

[a] Total ppm ion value for control does not include SO_4^{2-} (106 ppm) or trace elements.

TABLE 2.

Mean (±SE, n = 3) 24-and 48-h survival (%) of PL-28 *L. vannamei* in different ion solutions (ppm).

Treatment Solution	ppm							24-h % Survival	48-h % Survival
	Na⁺	Ca²⁺	Mg²⁺	K⁺	SO₄²⁻	Cl⁻	Total Ions		
1	290	54				542	886	43 (12.0)[a]	30 (15.3)[a]
2	290	54	53			682	1079	53 (13.3)[a]	26 (13.3)[a]
3	290	54	53	15		696	1108	86 (3.3)[a]	86 (6.7)[b]
4	290	54	53	15	140	592	1144	83 (12.0)[a]	76 (18.6)[b]
Control	181	44	31	10	106	280	652	90 (5.8)	90 (5.8)

Total ppm ion value for control does not include trace elements.
Survival values followed by a different superscript are significant at the $P < 0.05$ level.

containing all the major essential ions (#4, Table 2), although less, was not significantly different for either time period ($P = 0.92$, 0.78) compared with HBOI water.

Effect of K⁺ Ions

Mean survival of postlarval shrimp (PL-28) at different potassium concentrations ranged from 47–93% for 24 h and from 37–90% for 48 h (Table 3). At 24 h, there was no significant difference among treatments, but after 48 h, survival was significantly reduced at 0 ppm K⁺ ($P = 0.01$, Table 3). The HBOI water treatment showed intermediate survival compared with the other ion treatments.

DISCUSSION

There is a dearth of information regarding minimum environmental concentrations of individual ions necessary for the survival of marine shrimp species cultured in freshwater (<1000 ppm TDS). Preliminary experiments at HBOI have shown that Cl⁻ is necessary at concentrations >200 ppm for *L. vannamei* survival (Scarpa, unpublished data). Chloride and Na⁺ have been determined by Chen and Chen (1996) to be the major ions contributing (88.4%) to hemolymph osmolality in marine shrimp. The addition of Ca²⁺ to freshwater is thought to be necessary for the survival of shrimp because this ion is needed to form the exoskeleton, which is shed repeatedly during molting (Villalon 1991, Wyban & Sweeny 1991). Shrimp exuvia is composed mainly of CaCO₃ (99% of the inorganic portion; Richards 1951). *L. vannamei* does not possess internal Ca²⁺ reserves like some freshwater crustaceans (McWhinnie 1962). Therefore Ca²⁺ must be continually absorbed from the environmental medium (Robertson 1953, Greenaway 1983).

In the present study, Mg²⁺, SO₄²⁻, and K⁺ ions were examined for their effect on survival of postlarval *L. vannamei* in "artificial" freshwater (i.e., distilled water with sodium, chloride, calcium and carbonate). Magnesium and SO₄²⁻ were not found to have a critical effect on short-term survival. Magnesium and Ca²⁺ have been linked to membrane integrity (Douglas & Horne 1997) and Mg²⁺ concentrations in hemolymph have been correlated with crustacean activity (Mcfarland & Lee 1963). Sulfate is the third most prominent ion in seawater, but it has been shown to be nearly undetectable in shrimp hemolymph at low salinities (Dall & Smith 1981).

Treatment solutions without potassium in the present study had lower survivals compared with solutions with K⁺. Potassium was shown in all three experiments to be a significant factor contributing to the short-term survival of *L. vannamei*. The addition of 1 ppm of potassium doubled survivals over treatment waters with only Na⁺, Ca²⁺, and Mg²⁺.

Compared with the other essential ions, K⁺ is a minor constituent in brackish and fresh water (Horne 1969), but this ion plays a major role in metabolism of invertebrates (Schmidt-Nielsen 1990). Potassium was suggested by Robertson (1953) to be important in the maintenance of neromuscular efficiency in decapods, whereas other authors have discussed the importance of K⁺ in crustacean metabolism (Gross 1958, Bursey & Lane 1971, Dall & Smith 1981, Schmidt-Nielsen 1990). Enzyme activity is directly dependent on K⁺ concentration, which is maintained within narrow limits in the hemolymph of penaeids despite changing environmental salinity (Gross 1958, Bursey & Lane 1971, Dall & Smith 1981).

In the marine environment, K⁺ was constantly regulated in the hemolymph of *P. duorarum* as the salinity of the external medium

TABLE 3.

Mean (±SE, n = 3) 24- and 48-h survival (%) of PL-28 *L. vannamei* at different K⁺ concentrations (ppm).

Treatment Solution	ppm						24-h % Survival	48-h % Survival
	Na⁺	Ca²⁺	Mg²⁺	K⁺	Cl⁻	Total Ions		
1	290	54	53	0	626	1023	47 (14.5)[a]	37 (3.33)[a]
2	290	54	53	1	627	1025	83 (11.5)[a]	80 (6.7)[b]
3	290	54	53	10	635	1042	90 (5.8)[a]	77 (8.8)[b]
4	290	54	53	25	649	1071	93 (3.3)[a]	77 (3.3)[b]
5	290	54	53	50	694	1141	93 (3.3)[a]	90 (10.0)[b]
Control	181	44	31	10	280	546	80 (14.1)	60 (5.0)

Total ion value for control does not include trace elements.
Survival values followed by a different superscript are significant at the $P < 0.05$ level.

changed (Bursey & Lane 1971). Potassium concentrations of 9–10 meq/L in the hemolymph were maintained between salinities of 7 to 35 ppt, whereas Cl⁻ and Na⁺ concentrations were similar to that of the surrounding medium. Four Australian shrimp species studied by Dall and Smith (1981) showed hemolymph K^+ concentrations were maintained between 5 and 15 meq/L over a range of 10 to 50 ppt salinity, with a trend of K^+ accumulation with increasing salinity. Potassium ions in *P. monodon* hemolymph were strongly regulated during changing environmental salinity (Lin et al. 2000). Shrimp transferred from 45 to 15 ppt showed K^+ levels reached a steady state after 4 h. Euryhaline penaeids sampled from Gulf Coast waters showed higher K^+ concentrations in muscle tissue compared with stenohaline species taken from the same area (McFarland & Lee 1963).

The ability of K^+ to be stringently regulated in the hemolymph can be partly explained by the regulation process of this ion. Gross (1958) stated that hemolymph Na^+ and K^+ concentrations were maintained in an intertidal crab (*Pachygrapsus crassipes*) via intracellular pools as well as active uptake under hypotonic conditions. Changes in the Na^+ and K^+ concentrations between the hemolymph and surrounding medium were 84 and 68%, respectively, of the total hemolymph ion change while the additional 16 and 32% were assumed to come from internal salt pools. Hemolymph K^+ concentrations were maintained within narrower limits than Na^+ concentrations, despite the change in ion concentration of the surrounding medium. These salt pools were thought to be an ecological adaptation to buffer the ionic change between incoming and outgoing tides in an estuarine environment (Gross 1958). Gilles and Pequeux (1983) made a similar determination. A large decrease in the intracellular K^+ concentration of crustaceans appears to occur immediately following the application of hypoosmotic conditions. The decrease in intracellular K^+ concentration being inversely proportional to the extracellular K^+ concentration, with lower extracellular K^+ concentrations producing a greater release of K^+ from isolated cells.

The increase in pH between the first, second and third experiments of the present study did not appear to increase survival of shrimp. Optimum pH values of 6.6–8.5 for *L. vannamei* have been reported by Tsai (1990) and the pH values for all experiments listed here were within that range (within 0.1 pH value). Pillai and Diwan (1999) did not find any correlation of pH (7.04–7.84) with ion concentrations in the hemolymph of the shrimp *Metapenaeus*

monoceros taken from a tropical estuary over an 18-mo period. Wickens (1984) observed good growth and survival of *P. monodon* at pH values ranging from 6.7 to 7.9.

Although the present study used PLs of slightly different ages for each experiment (PL-18 to -28), it is unlikely that osmoregulatory ability differed between these age groups. McGraw et al. (2002) found that PL-10 *L. vannamei* had significantly lower survival than PL-15 and -20 when subjected to various acclimation rates, however, survival of PL-15 and -20 age groups were not different from each other. This is probably caused by the full development of gills and osmoregulatory capacity of postlarval *L. vannamei*, which occurs at approximately PL-12 (Lucu 1990, Pequeux 1995,Van Wyk et al. 1999). Similar results have been observed for other penaeid species postlarvae (Olin & Fast 1992, Tsuzuki et al., 2000).

It is also unlikely that major environmental ion deficiencies may be compensated through dietary supplementation and, therefore, feed supplementation during the trial would have had little impact. Dietary calcium supplementation for catfish cultured in calcium-free water had little effect on body calcium levels or spinal deformities (Scarpa & Gatlin 1993).

The present study establishes the importance of potassium to *L. vannamei* survival in freshwater solutions. Potassium addition to ponds as potash (K_2O) has been recommended for pond fertilization (Boyd 1990) and ion supplementation (Boyd 2002). Potash has been used as a source to increase potassium concentrations in ponds for growing shrimp (Teichert-Coddington, Green Prairie Aquafarm, personal communication, 2000); however, the economical feasibility of this practice for shrimp culture in inland freshwater locations has yet to be determined. Because of this, decisions regarding potential inland sources of saline well water for growing *L. vannamei* should focus in part on the presence and concentration of potassium in water sources.

ACKNOWLEDGMENTS

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PHYSIOLOGICAL AND GENETIC VARIATIONS IN DOMESTICATED AND WILD POPULATIONS OF *LITOPENAEUS VANNAMEI* FED WITH DIFFERENT CARBOHYDRATE LEVELS

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ABSTRACT The relationship between polymorphism of α-amylase and physiologic and biochemical behavior of *L. vannamei* was used to determine whether artificial selection based on body weight and body size affect the adaptation ability of shrimp to use dietary carbohydrates as a source of energy. Shrimp fitness was addressed by measurement of energy balance using growth (P), oxygen consumption (R), and ammonia excretion (U) of juveniles from wild, 7th, and 25th generations of cultured shrimp. Hemolymph glucose, digestive gland glycogen, amylase activity, and amylase polymorphism was also evaluated in the three shrimp populations. Heterozygosity, amylase activity, and starch metabolism were affected by artificial selection of *L. vannamei*. Shrimp from a 25th-cultured generation had less heterozygosity and physiologic alteration than did wild shrimp. Shrimp from a 7th-generation cultured shrimp population showed an intermediate state of genetic and physiologic alteration. Although a statistical comparisons cannot be made between the three studied populations, it is evident that there is a reduction in amylase activity related to shrimp domestication, with high values in wild shrimp (between 24 to 39 IU mg^{-1} protein), intermediate in 7th-generation cultured shrimp (between 16 to 25 IU mg^{-1} protein), and low in 25th-generation cultured shrimp (between 3.6 to 15.8 IU mg^{-1} protein). A reduction in the frequency of alleles of amylase genes possibly related to domestication of shrimp was also demonstrated. It appears that the reduction of allele frequency of amylase genes affected the adaptative ability of shrimp to use dietary carbohydrates as a source of energy and molecules and caused farmed populations to be protein dependent. Results of energy balance studies indicate that there are differences in production efficiency (P/AS) between populations: a reduction in P/AS as a function of generations of farmed shrimp suggests that efficiency with which shrimp transform energy into biomass is reduced with artificial selection.

KEY WORDS: Litopenaeus vannamei, physiology, genetics, populations, domestication, bioenergetics, blood parameters

INTRODUCTION

The Pacific white shrimp *L. vannamei* (Boone) is the most important shrimp species cultivated in the Americas and the second in word production (Benzie 2000). More than 90% of the shrimp cultivated in 1998 on the American continent were *L. vannamei* (132 000 t; Rosenberry 1998). For that reason, shrimp farmers are establishing selective breeding programs for *L. vannamei* throughout the natural range of the species, as well as the US Atlantic coast and Brazil (Sunden & Davis 1991, Paiva-Rocha 2001, García-Calleja 2000). These programs are motivated in part by the serious disease problems caused by uncontrolled farmed population movements (Wyban et al. 1993, Bedier et al. 1998) and are focused to obtain better profitability through the selection of body weight or body size for optimal harvest. Although a better growth rate has been observed in breeding programs with *L. vannamei*, the impact of reported reduction of genetic diversity (Sunden & Davis 1991) on the general physiology of shrimp is not known (Benzie 1998). In a recent study Xu et al. (2001) showed a reduction in genetic diversity in cultured *P. monodon* compared with wild populations. That genetic differentiation pattern among populations was related to the prevalence of IHHNV viral disease in the same populations, indicating that the change in genetic diversity of shrimp could change the disease susceptibility of cultured or wild shrimp, affecting their fitness.

Assimilation (As) is the key characteristic of living organisms because it is a direct index of the energy allocated to body weight or gametes or to maintain homeostasis. According to Lucas (1993),

As = P + R, where P is the energy allocated to production of biomass or gametes and R is the metabolizable energy. Although the fitness of a population has reproductive consequences, in a practical sense many researchers have been using the energy balance on juvenile forms to determine how the environmental fluctuations or types of food affect the energy allocation in Crustacea trying to predict the environmental or nutritional consequences in energy partitioning (Mayzaud & Conover 1988, Stickle et al. 1989, Du-Preez et al. 1992, Koshio et al. 1992, Hopkins et al. 1993, Rosas et al. 1993, Rosas et al. 1995, Guerin & Stickle 1997, Rosas et al. 1998, Rosas et al. 2001).

The energy derived from food depends on mechanisms of transformation of dietary components that, in turn, depends on the ability of organisms to hydrolyze, absorb, and assimilate those dietary nutrients (Ceccaldi, 1998). In a series of recent articles, we have demonstrated that energy allocation derived from dietary carbohydrates (CHO) has been found to be a limiting factor in *L. stylirostris, L. vannamei*, and *L. setiferus* (Rosas et al. 2000a, Rosas et al. 2000b, Rosas et al. 2001). In these works, we reported that glucose uptake in metabolism was limited because of saturation of α-amylase when shrimp are fed with diets above 33% CHO. At the same time, the digestive gland was saturated with glycogen in shrimp fed with diets >33% CHO, affecting nutrient absorption and in consequence growth rate and biomass production. Shrimp fed without dietary CHO can produce their own CHO using the gluconeogenic pathway, demonstrating that shrimp protein metabolism is well adapted to produce its own metabolic energy despite energy lost through ammonia excretion.

Shrimp α-amylase is one of the best-studied polymorphic digestive enzymes in shrimp. Two allelic forms were measured in *Asellus aquaticus*, four isoforms in *Palaemonetes varians*, seven

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isoforms in *P. elegans* three isoforms in *P. serratus* and *L. vannamei*, and three in *Farfantepenaeus notialis*, in *L. schmitti*, and in *L. setiferus* (Lomholt & Christensen 1970, Christensen & Lomholt 1972, Van Wormhoudt 1983, Van Wormhoudt & Favrel 1988, Diaz et al. 1995, Le Moullac et al. 1996, Ball et al. 1998, Arena 1999, García-Machado et al. 2001). This enzyme can be induced or repressed by dietary CHO, protein levels, or by circadian, annual, or moult cycles (van Wormhoudt 1974, van Wormhoudt 1977). Van Wormhoudt et al. (1980) reported a reduction in amylase activity in *Palaemon serratus* as a function of the increase in dietary glucides. Rosas et al. (2000a) showed an increase in α-amylase of *L. stylirostris* as a function of an increase in dietary CHO levels. Lovett and Felder (1990) stated that a significant increase in amylase activity of *L. setiferus* postlarvae might be a response to low levels of CHO in the postlarval diet. Le Moullac et al. (1996) reported a reduction of amylase activity in *L. vannamei* when the amount of this protein increased in diets, showing that α-amylase gene expression could be repressed by casein, reflecting the control that diet has on activity of amylase isoforms. In the present research, a relation between polymorphism of α-amylase and physiologic and biochemical behavior of *L. vannamei* was used to study whether artificial selection based on body weight and body size affected the ability of shrimp to use dietary CHO as a source of energy. Shrimp fitness was addressed through measurement of energy balance using growth, oxygen consumption, and ammonia excretion of juveniles from wild, 7th, and 25th generations of cultured shrimps. Hemolymph glucose, digestive gland glycogen, amylase activity, and amylase polymorphism was also evaluated in the three shrimp populations.

MATERIAL AND METHODS

The study was divided between two experiments. The first was conducted in Mexico where comparisons were made wild and 7th-generation specimens of *L. vannamei*. The second experiment was conducted at the French Marine Research Institute (IFREMER) Tahiti facilities with 25th-generation specimens of *L. vannamei*. Both experiments were conducted under the same basic conditions and with the same experimental diets.

Experimental Conditions

For experiment 1, live wild *L. vannamei* ($n = 200$; 0.8 ± 0.1 g wet weight) were collected from Huizache and Caimanero Lagoon on the Pacific Coast of México. Shrimp were transported by plane in plastic bags with cool sea water (30‰ salinity; 20°C) to the Experimental Marine Biology Laboratory of National Autonomous University of Mexico in Cd. del Carmen, Campeche, México. Shrimp were acclimated to laboratory conditions for 2 wk before any experimental procedure was initiated. During this period shrimp were maintained in a circular external pond (20 m²) with aerated (O₂ > 5.0 mg/L) natural seawater (32‰; 29 ± 2°C). During acclimation, shrimp were fed twice each day on a commercial shrimp diet containing 45% protein (Api Aba camarón ultra, Malta Clayton SA⁽ᵂ⁾). At the same time, a sample of 7th-generation cultured shrimp ($n = 200$; 0.03 ± 0.03 g live weight) from a farm located in Sisal, Yucatán, was transported to the laboratory in cool sea water (35‰ salinity, 24°C) and acclimated under identical conditions to those described above.

After 2 wk of acclimation, a sample of each population was removed and distributed in 90-l plastic tanks. For experiment one, shrimp were reared for 55 to 58 days in a flow-through sea water system (32‰ salinity) at a density of 10 shrimp per tank. For experiment 2, we used 1600 postlarvae (0.009 ± 0.001 wet weight) of 25th-generation *L. vannamei* obtained in the IFREMER hatchery facilities. In IFREMER shrimp were reared in 800-L tanks for 36 days in a flow-through sea water system (36‰ salinity) at a density of 100 shrimp per tank.

In both experiments shrimp were fed three times a day (0800, 1400, and 2000 h), uneaten food particles were removed twice a day (0730 and 1700 h) and water quality variables were maintained as temperature 28 ± 1°C, dissolved oxygen >5.0 mg/L, and pH >8.2 ± 0.3. In both locations the photoperiod was set at 12h/12h. Samples of digestive gland for biochemical and genetic analysis from experiment 1 were stored at −80°C and then freeze-dried until analysis. Digestive glands from 25th-generation cultivated shrimp were freeze-dried at the IFREMER facilities in Tahiti before analysis.

Diets

L. vannamei juveniles were fed practical diets, formulated with two levels of carbohydrate (CHO); 3% and 44%. Experimental diets were prepared by thoroughly mixing dry ingredients with oil and then adding water until a stiff dough resulted. The dough was passed through a mincer with a 2-mm die, and the resulting spaghetti-like strings were air dried at 60°C. After drying, the strings were broken up and sieved to a convenient pellet size and stored at −4°C.

Growth and Survival

The growth rate was evaluated as the difference between wet weight at the beginning and end of the experiment and calculated as daily growth coefficient (DGC,%; Cho 1992):

$$DGC = 100 \times \{[\text{final weight (g)}]^{1/3} - [\text{initial weight (g)}^{1/3}]\}/\text{time (days)}$$

The DGC measure was chosen to make comparisons in growth tests because initial weights were different between treatments (Bureau et al. 2000, Cho 1992). The survival rate was calculated as the difference between the number of live animals at the beginning and end of the experiment.

Amylase Activity

At the end of growth trials, digestive glands from fasting (12 h) shrimp (40 per treatment) were dissected immediately, quickly frozen in liquid nitrogen, and then kept at −80°C for subsequent analysis. Frozen samples were homogenized in 500 μL of ice-cold, deionized water. Homogenates were centrifuged at 16000 g for 6 min at 8°C. Part of the supernatant was diluted in 10 volumes of ice-cold deionized water. Homogenates (crude or diluted) were immediately used for enzyme analysis (Brito et al. 2001). The soluble-protein content was measured in diluted homogenates by the Bradford (1976) method using the Sigma Micro Protein Determination Kit (Procedure No. 610). Samples were read in a Bio-Rad model 550 microplate reader at 495 nm. Duplicate assays for each sample were made. Amylase activity was assayed in diluted homogenates according to the method Bernfeld (1955) with 1% oyster glycogen (Sigma G8751) as substrate in 10 mM phosphate buffer, pH 7. One unit of amylase activity was defined as 1 mg of maltose liberated in 1 min at 30°C. Each sample was assayed in duplicate. Activity was expressed in units of μM substrate cleaved

per minute, based on an extinction coefficient $\varepsilon_{410} = 18000$ L mol^{-1} cm^{-1}. Each sample was assayed in duplicate.

Hemolymph Glucose

Blood glucose measurements were made in the same shrimp sampled for amylase activity. Before sampling, shrimp were placed in chilled (18°C) and aerated seawater for 5 min to reduce the effect of manipulation before the hemolymph extraction (Rosas et al. 2000a). Only shrimp in intermolt stage (C stage) were used. Hemolymph (approximately 200–300 µL per shrimp) was individually sampled through a chilled syringe needle inserted at the base of the fifth pereiopod after the shrimp had been dried with a paper towel. The individual weight (±0.05 g) was measured. Molting stages were identified by uropod examination (Drach & Tchernigovtzeff 1967). Commercial kits were used for glucose (GH; GOD-PAD, Merck-740393) determinations and were read with a microplate using 20 µL of plasma (obtained after 8000 g centrifugation) and 200 µL of enzyme chromogen reagent. Absorbance was recorded in a microplate reader (Bio-Rad model 550) and concentrations were calculated from a standard solution of substrate.

Glycogen Concentration in Digestive Gland (DGG)

Glycogen was extracted in the presence of sulfuric acid and phenol (Dubois et al. 1965). The digestive gland was first homogenized in trichloroacetic acid (5%) for 2 min at 6,000 rpm. After centrifugation (7000 g), the supernatant was quantified. This procedure was done twice. One milliliter of trichloroacetic acid was pipetted into a tube and mixed with 5 volumes of 95% ethanol. The tubes were placed in an oven at 37–40°C for 3 h. After precipitation, the tubes were centrifuged at 7000 g for 15 min. The glycogen (pellet) was dissolved by addition of 0.5 mL of boiling water and then 5 mL of concentrated sulfuric acid and phenol (5%) were added and mixed. The content of the tubes was transferred to a cuvette and read at 490 nm in a spectrophotometer.

Amylase Allozyme Analysis

Digestive glands from each population were homogenized in 500 µL of TRIS-phosphoric acid buffer (0.06 Mol /L, pH7) and centrifuged at 12000 rpm (4°C, 20 min). We used conventional 10% vertical polyacrylamide gel electrophoresis with TRIS-glycine as the running buffer (Davis 1964). Polyacrylamide gels were run at 250 V for 4 h. Band staining was done using an agar gel (1%) with 1% amylose, 1 M Ca, and 1 M Mg, phosphate buffer (pH 7), and 250 mM NaCl. The acrylamide–agar gel matrix was incubated at 37°C for 20 min and then stained with lugol solution (1:5) to obtain the bands. Alleles were coded by letters according their relative migration on gels (García-Machado et al. 2001). A locus was considered polymorphic when the frequency of the most common allele in the population did not exceed 95% and rare when the frequency was <0.005%. Genetic variability deviations of Hardy-Weinberg (H_e) expectations were determined using Wright's F statistics (Wright 1965).

Energy Balance

Energy balance was estimated using the equation of Lucas (1993):

$$Ab = R + U + P$$

where Ab is the absorbed energy (joules day^{-1} gww^{-1}), R is routine respiration, U is the energy lost through ammonia excretion, and P is the energy invested in production of biomass. Assimilated energy (AS) was estimated using the equation (Rosas et al., 1998):

$$AS = P + R$$

Production (P) was obtained from the growth rate of the shrimp. The mean value of 4900 ± 147 J gdw^{-1} was used to transform the growth data into production units (P; J g^{-1}dw d^{-1}). This value was obtained from analyzing the energy content of the muscle of 25 shrimp by means of a calorimeter (Parr), previously calibrated with benzoic acid.

Respiration (Rrout) or basal metabolism (Hem) was obtained through oxygen consumption measurements in nine fasting (12 h) shrimp on each dietary regimen. Oxygen consumption was measured on individual shrimp in a continuous flow respirometer (Rosas et al. 1998). Oxygen consumption was calculated as follows:

$$VO_2 = O_{2e} - O_{2ex} \times Fr$$

where VO_2 is oxygen consumption (mg O_2 h^{-1} animal^{-1}), O_{2e} indicates oxygen concentration at the entrance to the metabolic chamber (mg L^{-1}), O_{2ex} is oxygen concentration at the exit (mg L^{-1}), and Fr is the flow rate (L h^{-1}). Oxygen concentration was measured using a digital oximeter (YSI 50B digital, Dayton, OH) with a polarographic sensor (±0.01 mg L^{-1}), previously calibrated with oxygen-saturated seawater at 28°C. The shrimp were then fed food pellet fragments of 0.06 ± 0.002 g each in the respirometric chambers. The same amount of food was placed in a control chamber without an organism to estimate the oxygen lost by food decomposition. Oxygen consumption of fed shrimp was measured every hour for 4–6 h between 0800 and 1300–1500 h. Once the experiment was concluded, the shrimp were weighed. Specific routine oxygen consumption rate (mg g^{-1} h^{-1}) was estimated from the VO_2 of the unfed shrimp. The specific rate of the apparent heat increase (AHI), mg g^{-1} h^{-1}, was estimated from the difference between VO_2 of the unfed shrimp and the maximum value attained after feeding. A 14.3 J mg^{-1} conversion factor of oxygen consumption was used to transform the unfed and fed VO_2 to J g^{-1} dry weight (dw; Lucas 1993).

Along with the oxygen consumption measurements, water samples for ammonia excretion were obtained. Ammonia excretion was determined as the difference between the ammonia concentration at the entrance and the exit of each respirometric chamber and multiplied by the rate of water flow. The concentration of ammonia (total ammonia; $NH_4^+ + NH_3$) was measured using a flow injection-gas diffusion system (Hunter and Uglow, 1993). This technique consists of a carrier stream of NaOH (0.01 M) separated from an indicator solution (bromothymol blue 0.5 g L^{-1}) by a gas permeable membrane (PTFE). All ammonia in the sample is converted to gaseous NH_3, which diffuses across the membrane and reacts with the indicator to produce a pH-dependent color change that is detected by a photometer. A calibration curve was made using different concentrations of $(NH_4)_2SO_4$. The ammonia excretion of unfed and fed shrimp (postprandial nitrogen excretion; PPNE) was converted to energy units using the value of 20.5 J per mg N-NH$_3$ excreted (Lucas 1993) and defined as U_{rout} for the energy lost before feeding and U_{ppne} the energy lost after feeding. Total ammonia excretion was defined as U_{Total}.

R_{AHI} and U_{ppne} (J g^{-1} ww day^{-1}) were estimated considering the time needed for peak oxygen consumption after feeding and the number of rations fed to the shrimp per day ($Rt = 3$),

TABLE 1.

Daily growth coefficient of *L. vannamei* juveniles from wild and cultivated populations; experiment 1: wild vs. 7th-cultured generation comparisons.

	Wild		7th Generation	
	HCHO	LCHO	HCHO	LCHO
Initial weight, g	1.31 ± 0.02	1.39 ± 0.02	0.06 ± 0.05	0.07 ± 0.01
Final weight, g	8.42 ± 0.19	8.54 ± 0.38	4.10 ± 0.34	6.0 ± 0.22
Survival, %	78 ± 6[a]	60 ± 6[a]	68 ± 10[a]	68 ± 6[a]
Time, days	55	57	55	58
DGC, %	1.71 ± 0.4[a]	1.63 ± 0.4[a]	2.20 ± 0.2[b]	2.42 ± 0.2[c]
N	6	6	6	6

Different letter means statistical differences, *P* < 0.05.
Values are mean ± SE.
HCHO, high dietary carbohydrates; LCHO, low dietary carbohydrates.

$$R_{AHI} = [(VO_2 \text{ af} - VO_2 \text{ bf} \times 14.3 \text{ J mg}^{-1}) \times (T \times Rt)]$$
$$U_{ppne} = [(N - NH_3 \text{ af} - N - NH_3 \text{ bf} \times 20.5 \text{ J mg}^{-1}) \times (T \times Rt)]$$

where VO_2 or $N-NH_3$ are the oxygen consumption after (af) and before (bf) feeding, 14.3 J mg^{-1} and 20.5 J mg^{-1} are the constant to convert VO_2 or $N-NH_3$ in energy units. T is time (h) to reach the peak after feeding.

R_{rout} and U_{rout} ($J \text{ g}^{-1}$ ww day^{-1}) were estimated as:

$$R_{rout} = [(VO_2 \text{ bf} \times 14.3 \text{ J mg}^{-1}) \times (T_{wf} \times Rt)]$$
$$U_{rout} = [(N - NH_3 \text{ bf} \times 20.5 \text{ J mg}^{-1}) \times (T_{wf} \times Rt)]$$

where T_{wf} is the difference between time of one day (24 h) and $(T \times Rt)$.

Statistical Analysis

Statistical analyses were used separately in each experiment. Analysis of growth rates was performed independently for each population to emphasize dietary influence. Student *t*-tests were used on final average weight gains. The effect of dietary carbohydrate was analyzed for physiologic and genetic data using 2-way analysis of variance in Experiment 1, and one-way analysis of variance in Experiment 2. Arc sine transformation was used prior to analysis of survival data expressed in percentages. Homogeneity of variances of all distributions was verified with Cochran's test. Means obtained during the treatment were compared by using Duncan's multiple range test (Zar 1974).

RESULTS

Growth and Survival

Experiment 1

The daily growth coefficient (DGC%) was affected by dietary CHO and was higher in shrimp from the 7th generation than in wild shrimp (Table 1; *P* < 0.05). The DGC of 7th-generation shrimp was higher in shrimp fed with low dietary CHO than that in shrimp fed with high dietary CHO (*P* < 0.05). No differences were observed between wild shrimp fed with high or low dietary CHO levels (Table 1; *P* > 0.05). Survival was not affected by dietary CHO in either of the shrimp populations. A mean value of 69% survival was obtained in all treatments (Table 1).

Experiment 2

L. vannamei juveniles from Tahiti population (25th generation) were not affected by dietary CHO (Table 2; *P* > 0.05).

Amylase Activity

Experiment 1

The amylase activity was affected by dietary CHO and was higher in wild shrimp than in 7th-generation shrimp (Fig. 1A; *P* < 0.05). A higher amylase activity was observed in wild shrimp fed with high dietary CHO (35 0.1 IU/mg protein) than in wild shrimp fed with low dietary CHO (26.8 IU/mg protein). A significantly lower mean value of amylase activity was obtained in shrimp from the 7th-generation population (21 IU/mg protein) than in wild shrimp (Fig. 1A; *P* < 0.05).

Experiment 2

Dietary CHO levels significantly affected the amylase activity with high values in shrimp fed with low dietary CHO (13.5 IU/mg protein) and low values in shrimp fed with high dietary CHO (4.1 IU/mg protein) (*P* < 0.05; Fig. 1B).

Hemolymph Glucose

Experiment 1

A lower glucose hemolymph level was measured in wild shrimp fed with low dietary CHO (0.13 mg/mL) compared with that measured in wild and 7th-generation cultured shrimp (mean value of 0.28 mg/mL; Fig. 2A, *P* < 0.05).

TABLE 2.

Daily growth coefficient for juveniles of *L. vannamei* from 25th-cultured generation: Experiment 2.

	HCHO	LCHO
Initial weight, g	0.009 ± 0.001[a]	0.009 ± 0.001[a]
Final weight, g	0.72 ± 0.04	1.02 ± 0.05
Survival, %	85 ± 5	88 ± 6
Time, days	36	36
DGC, %	1.91 ± 0.7[a]	2.21 ± 0.78[a]
N	8	8

Different letter means statistical differences, *P* < 0.05.
Values are mean ± SE.
HCHO, high dietary carbohydrates; LCHO, low dietary carbohydrates.
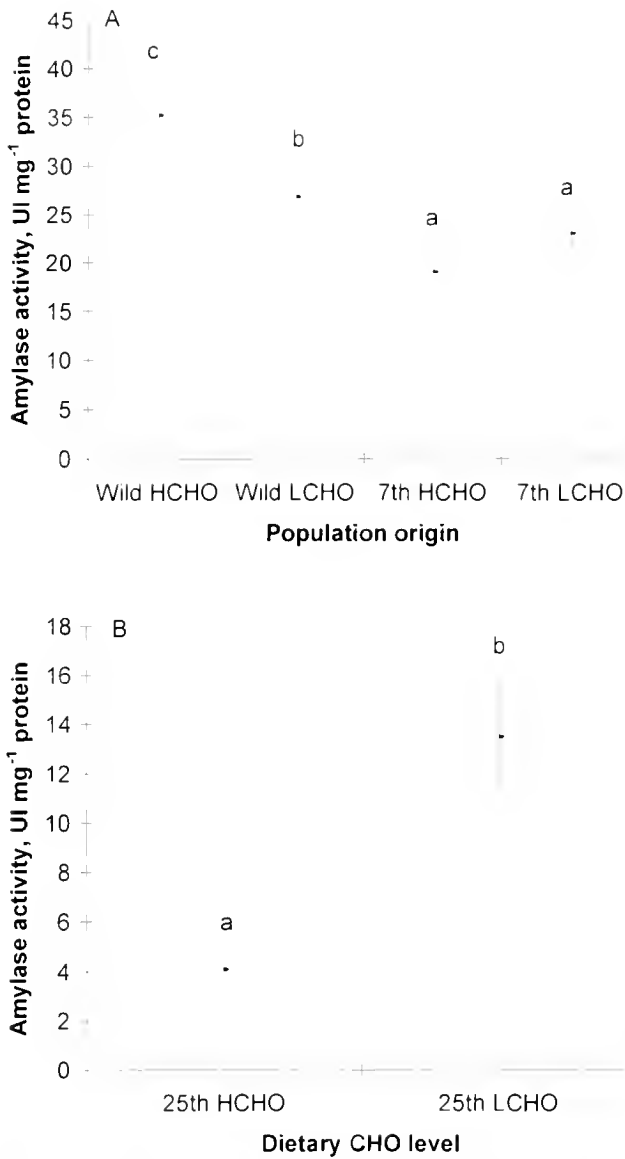
Figure 1. Amylase activity by wild and 7th-generation cultured *L. vannamei* (A) and 25th-generation cultured *L. vannamei* (B). Mean ± SE. Different letter means statistical differences at $P < 0.05$ level.

Experiment 2

A significantly high glucose hemolymph level was measured in 25th-generation shrimp fed with high CHO (1 mg/mL) that was 2.6 times the value in shrimp fed with low CHO (0.39 mg/mL) (Fig. 2B; $P < 0.05$).

Digestive Gland Glycogen

Experiment 1

Digestive gland glycogen concentration was affected by dietary CHO and the origin of shrimp (Fig. 3A). In wild and 7th-generation cultured shrimp, a high glycogen concentration was measured in shrimp fed with low dietary CHO ($P < 0.05$).

Experiment 2

In 25th-generation cultured shrimp, the high dietary glycogen level was measured in shrimp fed with high dietary CHO (2.0

mg/g) that was 30% higher than that in shrimp fed with low dietary CHO (1.4 mg/g; $P < 0.05$; Fig. 3B).

Pattern of Allozyme Variation

An eight-band pattern was observed in the electrophoretic analysis of amylase. These patterns were classified into two systems; system 1 with alleles a, b, and c and system two with five alleles: a, b, c, d, and e (Fig. 4). Both systems were polymorphic (Table 3). System 1 was more conservative than system 2. In such a system, alleles a, b, and d were rare with an allelic frequency <0.05. A reduction in H in system 2 was observed into domesticated populations, with high values in wild shrimp (H = 0.29) and low values in 25th-generation cultured shrimp populations (H = 0.08), reflecting a high percentage of homozygosity. Amylase loci from wild and 7th-generation cultured shrimp were in equilibrium. Locus from the 25th-generation of cultured shrimp showed significant deviation from Hardy–Weinberg proportions (heterozygosis deficit; $P < 0.05$) (Table 4).

Energy Balance

Experiment 1

Oxygen consumption of 12-h fasting shrimp was affected by dietary CHO in both wild and cultured populations (Table 5). The
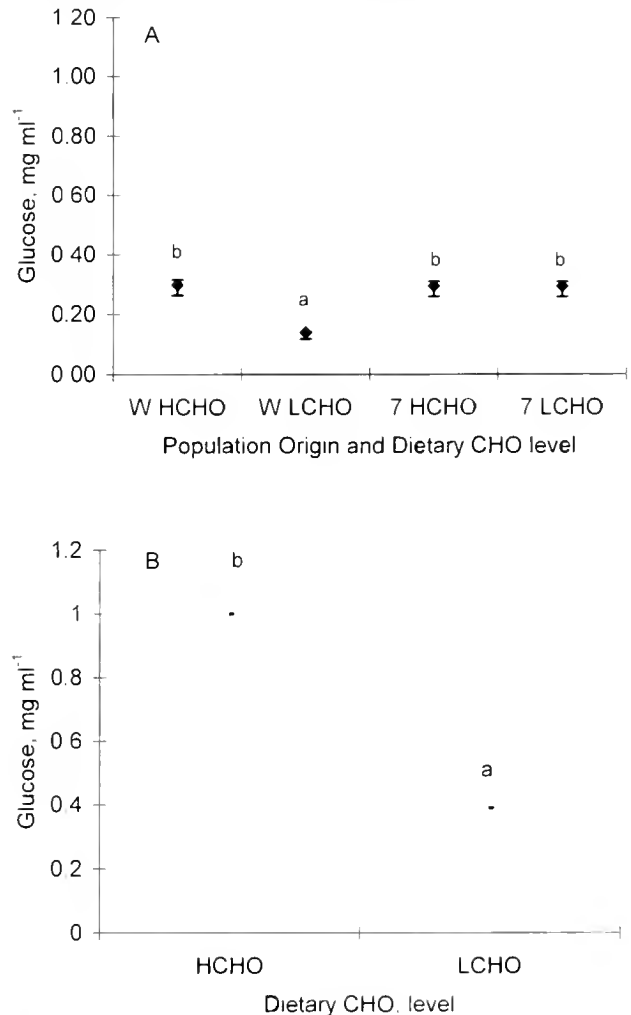
Figure 2. Glucose hemolymph level of wild and 7th-generation cultured *L. vannamei* (A) and 25th-generation cultured shrimp (B). Mean ± SE. Different letter means statistical differences at $P < 0.05$ level.
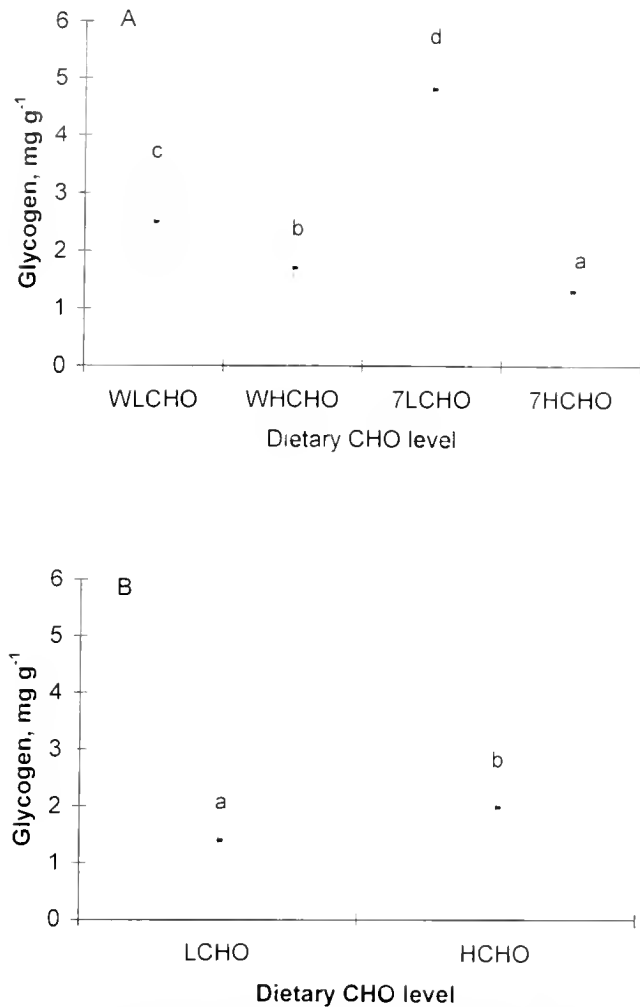
Figure 3. Digestive gland glycogen of wild and 7th-generation cultured *L. vannamei* (A) and 25th-generation cultured shrimp (B). Mean ± SE. Different letter means statistical differences at *P* < 0.05 level.

highest oxygen consumption was measured in 7th-generation cultured shrimp (0.65 mg O_2/h/g wet weight) fed with high dietary CHO ($P < 0.05$). The lowest oxygen consumption value was in wild shrimp fed with low dietary CHO (0.19 mg O_2/h/g wet weigh; $P < 0.05$). The oxygen consumption rate increased after feeding in each treatment (Table 5). Oxygen consumption of shrimp during feeding followed either of two patterns; one for wild shrimp fed with low dietary CHO and the other for the remaining shrimp groups. During feeding, oxygen consumption of wild shrimp fed with low dietary CHO was significantly lower than in wild shrimp fed high dietary CHO shrimp and 7th-generation shrimp fed with high or low dietary CHO. In each, oxygen consumption increased rapidly after feeding and decreased afterwards until reaching levels similar to those at the start of experiment. The time required to achieve oxygen consumption peak was higher in 7th-generation shrimp fed with high dietary CHO (2 h) than in all remaining shrimp groups (0.5 to 1 h).

Ammonia Excretion

Ammonia excretion in fasting wild shrimp (mean value of 0.06 mg N-NH₃/h/g wet weight) was significantly lower than in 7th-generation shrimp (mean value of 0.15 mg N-NH₃/h/g wet

TABLE 3.

Genetic diversity (H_e) in wild and cultured populations of *L. vannamei*.

Population	System 1 He	System 2 He
Wild	0.66	0.29
7th generation	0.51	0.27
25th generation	0.51	0.08

weight). Ammonia excretion increased after feeding, reaching a maximum value between 0.5 to 3 h after feeding depending on shrimp group (Table 6). The highest postprandial ammonia excretion value was recorded in 7th-generation shrimp fed with low dietary CHO and the lowest in wild shrimp fed with high dietary
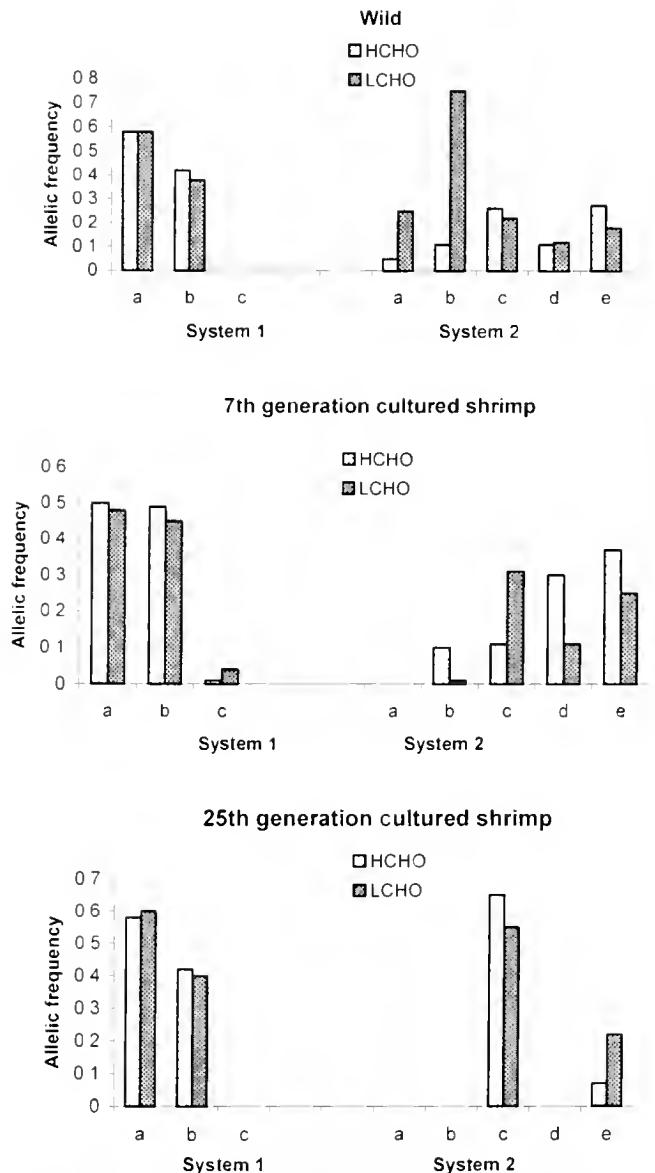
Figure 4. Allele frequencies of wild, 7th-, and 25th-generation cultured *L. vannamei* fed with high dietary CHO (HCHO) and low dietary CHO (LCHO).

TABLE 4.

Allelic frequencies comparison among different populations of *L. vannamei* from wild (México), 7th-generation cultivated shrimp and 25th-generation cultivated shrimp.

Population	Wild		7th Generation		25th Generation	
	S1	S2	S1	S2	S1	S2
Wild	—		NS	*	*	*
7th generation	—			—	NS	*
25th generation	—			—		—

NS, without significant statistical difference. *Statistical differences at $P < 0.05$ level.

CHO. Intermediate values were recorded in the remaining shrimp groups ($P < 0.05$).

The respiratory energy (R_{Total}) varied between populations and was affected by dietary CHO (Table 7). Of the R_{Total}, 17% was wasted in R_{AHI} in wild shrimp fed with low dietary CHO in comparison with the 3.4–4% waste as R_{AHI} in the remaining shrimp groups (Table 7). R_{rout} was observed between 83–97% of R_{Total} with the lowest value in wild shrimp fed with low dietary CHO. There were statistical differences between U_{Total} between populations and between treatments in 7th-generation cultured shrimp (Table 7; $P < 0.05$).

The percentage of U_{Total} that was U_{rout} varied between shrimp populations with the lowest value in wild shrimp fed with low dietary CHO (37%) and the highest (82%) in 7th-generation shrimp fed with low dietary CHO. The energy wasted after feeding (U_{pp}) was higher in wild shrimp fed with low dietary CHO (63% of U_{Total}) than that in 7th-generation cultured shrimp fed with the same diet (18% of U_{Total}). Absorbed energy ($Ab = P + R + U$) showed differences between shrimp groups and was affected by dietary CHO with high values in wild shrimp fed with high dietary CHO ($824 J^{-1} g^{-1}$ ww day^{-1}) and low values in 7th-generation cultured shrimp fed with same diet ($598 J^{-1} g^{-1}$ ww day^{-1}; Table 7). U_{Total} varied between 5–11% of Ab with low values in wild shrimp fed with high dietary CHO and high values in 7th-generation cultured shrimp fed with high and low dietary CHO (11% and 10%). Between 89 and 95% of Ab was assimilated. The energy assimilated (AS) was the result of adding R to P. The As value was affected differently in each shrimp population. In wild shrimp the highest value was observed in shrimp fed with high dietary CHO whereas in 7th-generation cultured shrimp the highest value was observed in shrimp fed with low dietary CHO (Table

7). Respiratory efficiency (R/AS) was lower in wild than in 7th-generation cultured shrimp and was affected by dietary CHO in each shrimp group (Table 6). Inversely, growth efficiency (P/AS) was higher in wild than in 7th-generation shrimp and highest in shrimp fed with low dietary CHO in both shrimp groups.

Experiment 2

Oxygen Consumption

No difference was measured in 12-h fasting oxygen consumption values between treatments (mean value of 0.23 mg O_2/h/g wet weight; Table 8; $P > 0.05$). A similar maximum oxygen consumption value was observed in both dietary shrimp groups (0.32 mg O_2/h/g wet weight). The time to reach the peak was different between treatments with 1 h for shrimp fed with high dietary CHO and 2 h for shrimp fed with low dietary CHO (Table 8).

Ammonia Excretion

In 25th-generation shrimp, 12-h fasting shrimp had similar values of ammonia excretion between treatments (mean value of 0.022 mg $N-NH_3$/h/g wet weight; $P > 0.05$; Table 9). After feeding, the ammonia excretion increased. The time to reach the peak was similar in both treatments with high values in shrimp fed with high dietary CHO (0.040 mg $N-NH_3$/h/g wet weight) and low values in shrimp fed with low dietary CHO (0.035 mg $N-NH_3$/h/g wet weight; $P < 0.05$).

Dietary CHO affected R_{Total} (Table 10). Shrimp fed with high dietary CHO had the higher proportion of energy from R_{Total} that was channeled to R_{rout} (96%) and at the same time the lower proportion of R_{Total} that was used in R_{AHI} (4%). In contrast the higher proportion of energy of U_{Total} that was lost as U_{rout} was in

TABLE 5.

Oxygen consumption (mg O_2/h/gww) of *L. vannamei* after 12-h fasting (time = 0) and at time increments after feeding: Experiment 1.

Time After Feeding, h	Wild		7th Generation	
	HCHO	LCHO	HCHO	LCHO
0	0.49 ± 0.09^d	0.19 ± 0.05^a	0.65 ± 0.06^b	0.54 ± 0.05^a
0.5	0.59 ± 0.11^b	0.44 ± 0.04^c	0.61 ± 0.09^b	0.57 ± 0.08^a
1	0.61 ± 0.11^b	0.46 ± 0.07^c	0.65 ± 0.09^b	0.70 ± 0.09^b
2	0.58 ± 0.07^b	0.28 ± 0.05^b	0.72 ± 0.04^c	0.59 ± 0.11^a
3	0.52 ± 0.08^a	0.39 ± 0.04^c	0.48 ± 0.05^a	0.59 ± 0.06^d
4	0.46 ± 0.06^d	0.22 ± 0.03^d	0.35 ± 0.04^d	0.61 ± 0.14^d

Different letter means statistical differences. $P < 0.05$.

Values are mean ± SE.

HCHO, high dietary carbohydrates; LCHO, low dietary carbohydrates.

TABLE 6.

Ammonia excretion (mg $N-NH_3$/h/gww) of *L. vannamei* after 12-h fasting (time = 0) and at time increments after feeding: Experiment 1.

Time After Feeding, h	Wild		7th Generation	
	HCHO	LCHO	HCHO	LCHO
0	0.067 ± 0.002^a	0.05 ± 0.008^a	0.15 ± 0.02^b	0.14 ± 0.02^a
0.5	0.18 ± 0.004^b	0.15 ± 0.02^b	0.15 ± 0.05^b	0.34 ± 0.03^c
1	0.12 ± 0.003^b	0.19 ± 0.03^{bc}	0.16 ± 0.007^b	0.35 ± 0.04^c
2	0.19 ± 0.003^b	0.21 ± 0.03^c	0.16 ± 0.03^b	0.24 ± 0.03^b
3	0.08 ± 0.003^a	0.22 ± 0.06^c	0.25 ± 0.03^c	0.33 ± 0.05^c
4	0.11 ± 0.003^a	0.07 ± 0.009^d	0.06 ± 0.01^d	0.14 ± 0.04^d

Different letter means statistical differences, $P < 0.05$.
Values are mean $\pm$ SE.
HCHO, high dietary carbohydrates; LCHO, low dietary carbohydrates.

shrimp fed with low dietary CHO (87%) in comparison to 75% lost in U_{rout} in shrimp fed with high dietary CHO. Inversely the proportion of U_{Total} that was lost as U_{pp} was higher in shrimp fed with high dietary CHO (25%) than in shrimp fed with low dietary CHO (13%; Table 10). In both treatments 95% of energy absorbed (*Ab*) was assimilated (*AS*). Dietary CHO affected *AS* and growth and respiratory efficiencies. Shrimp fed with low dietary CHO showed the higher *AS* and growth efficiency (72%) compared with shrimp fed with high dietary CHO (61%; Table 10).

DISCUSSION

In *L. vannamei* shrimp from the 25th-cultured generation exhibited less heterozygosity than did wild shrimp. From results obtained, the 7th-generation cultured shrimp showed an intermediate genetic and physiologic alteration. Although results demonstrate significant genetic differentiation among cultured and wild populations when based upon only an amylase allozyme marker, we acknowledge the necessity to confirm such differences at the mtDNA level through sequence variation of the amylase gene as recommended by Xu et al. (2001) and García-Machado et al. (2001). A more detailed study involving molecular biology and genetic alterations by domestication of *L. vannamei* is in process. As a consequence of selection in cultured populations, carbohy-

drate metabolism routes (hydrolysis, absorption, and synthesis) in shrimp fed with different dietary CHO was affected. A different enzyme activity-dietary CHO relation was observed depending on population characteristics; wild shrimp amylase activity was induced by high dietary CHO whereas low dietary CHO induced a high amylase activity in cultured shrimp. If reduction of heterozygosis means a reduction in amylase genes, then amylase activity induction was a compensatory response to obtain the highest possible glucose from the diet, increasing enzyme synthesis when shrimp are fed with low dietary CHO. On the contrary, in wild shrimp an excess of dietary CHO induced amylase activity because those shrimp have all the isoforms of the amylase enzyme to respond directly to the dietary starch. If amylase production in domesticated shrimp is efficient enough to process dietary CHO, it can be analyzed in a general context. Although a statistical comparison cannot be done among the three studied populations, it is evident there is a reduction in amylase activity as a function of domestication, with high values in wild shrimp (between 24 to 39 IU mg^{-1} protein), intermediate in 7th-generation cultured shrimp (between 16 to 25 IU mg^{-1} protein), and low in 25th-generation cultured shrimp (between 3.6 to 15.8 IU mg^{-1} protein; Fig. 1). Such reduction indicates that the reduction of allel frequency of amylase genes affected the adaptative ability of shrimp to use

TABLE 7.

Energy balance in juveniles of *L. vannamei*: Experiment 1.

	Wild		7th Generation	
	HCHO	LCHO	HCHO	LCHO
R_{rut}, J/day/gww	147.1 ± 1.9	57.1 ± 10.7	167.3 ± 18.8	162.1 ± 26.7
R_{AHI}, J/day/gww	5.2 ± 0.8	11.6 ± 1.1	6.0 ± 0.8	6.8 ± 0.8
R_{Total}, J/day/gww	152.3	68.7	173.3	168.9
U_{rut}, J/day/gww	24.7 ± 0.73	18.5 ± 2.70	46.1 ± 6.1	60.3 ± 8.6
U_{PPNE}, J/day/gww	13.8 ± 1.9	31.4 ± 4.7	18.5 ± 2.4	12.9 ± 1.6
U_{Total}, J/day/gww	38.5	49.9	64.6	73.2
P, J/day/gww	633.4 ± 70	614.6 ± 73	359.9 ± 46	500.9 ± 65
Absorption (*Ab*) J/day/gww	842.2	732.6	597.8	743.0
Assimilation (As) J/day/gww	785.7	683.3	533.3	669.8
Ef assimilation, As/Ab	95.3	93.3	89.2	90.1
Respiratory efficiency, % R/As	19.4	10.1	32.5	25.2
Production efficiency, % P/As	80.6	89.9	67.5	74.8

Mean $\pm$ SE.
HCHO, high dietary carbohydrates; LCHO, low dietary carbohydrates.

TABLE 8.

Oxygen consumption (mg O$_2$/h/gww) of *L. vannamei* (25th generation) 12 h fasting (time = 0) and at time increments after feeding: Experiment 2.

Time After Feeding, h	HCHO	LCHO
0	0.24 ± 0.02[a]	0.21 ± 0.01[a]
1	0.31 ± 0.02[b]	0.31 ± 0.02[b]
2	0.29 ± 0.02[b]	0.33 ± 0.02[c]
3	0.28 ± 0.02[b]	0.27 ± 0.02[c]
4	0.26 ± 0.02[ab]	0.27 ± 0.02[c]
5	0.25 ± 0.02[a]	0.28 ± 0.02[c]
6	0.27 ± 0.02[b]	0.25 ± 0.02[b]

Different letter means statistical differences, $P < 0.05$.
Values are mean ± SE.
HCHO, high dietary carbohydrates; LCHO, low dietary carbohydrates.

dietary CHO as a source of energy and molecules, which could cause farmed populations to be protein dependent.

Juveniles of *Litopenaeus vannamei* can synthesize their own glucose from protein through a gluconeogenic pathway (Rosas et al. 2001). Shrimp fed with low dietary CHO had digestive gland glycogen levels that were higher than when fed with high dietary CHO because the enzymatic system is induced to synthesize CHO from protein (Cuzon et al. 2001). In the present study, an increase in digestive gland glycogen was measured in wild and 7th-generation shrimp fed with low dietary CHO indicating that an induction mechanism is working. In contrast, in the 25th-generation farmed shrimp, that mechanism appears to be working in the opposite direction, producing more digestive gland glycogen in shrimp fed with high dietary CHO than in shrimp fed with low dietary CHO. If Amylase genes are repressed after 25th generations of selection then a high probability exists that other genes could be repressed also, producing changes and reducing the gluconeogenic route in shrimp.

This indicates that artificial selection of shrimp favored more than size and harvest weight, as it also favored protein metabolism by acting on shrimp digestive capacity. The use of high levels of animal protein in shrimp feeds in all phases of shrimp culture, from larvae to broodstock (including *Artemia*, krill, *Cyclops*, high qual-

Figure 5. Growth efficiency (P/AS,%) of wild (W) 7th (7)-, and 25th (25)-generation cultured *L. vannamei* fed with different carbohydrates levels. HCHO, high dietary carbohydrates; LCHO, low dietary carbohydrates.

ity fish meal, and squid) is responsible for activation and repression of genes. For amylase, Le Moullac et al. (1996) reported a reduction of enzyme activity in *L. vannamei* after an increase in dietary protein, which was related to a regulating role of amino acids on amylase expression. They observed a disappearance of one amylase mRNA associated with a high protein level suggesting that a regulation of amino acids would take place at the transcriptional level. Because, in selected shrimp, protein metabolism was favored and growth rate depended on dietary protein (Andrews et al. 1972), one can explain why 7th and 25th-generation farmed shrimp possess a higher growth rate than wild shrimp (Tables 1 and 2).

There are several costs that are necessary to take into account with the breeding programs that only take into account the size of shrimp at harvest, which is also related to growth efficiency. From results on energy balance, there are differences in production ef-

TABLE 9.

Ammonia excretion (mg N-NH$_3$/h/gww) of *L. vannamei* (25th generation) 12-h fasting (time = 0) and at time increments after feeding: Experiment 2.

Time After Feeding, h	HCHO	LCHO
0	0.020 ± 0.002[a]	0.024 ± 0.0004[a]
1	0.030 ± 0.002[b]	0.028 ± 0.001[b]
2	0.040 ± 0.002[c]	0.035 ± 0.001[c]
3	0.030 ± 0.001[b]	0.029 ± 0.001[b]
4	0.037 ± 0.002[bc]	0.026 ± 0.001[b]
5	0.029 ± 0.001[b]	0.030 ± 0.0009[b]
6	0.03 ± 0.001[b]	0.027 ± 0.0008[b]

Different letter means statistical differences, $P < 0.05$.
Values are mean ± SE.
HCHO, high dietary carbohydrates; LCHO, low dietary carbohydrates.

TABLE 10.

Energy balance of *L. vannamei* (25th generation): Experiment 2.

	Dietary CHO	
	HCHO	LCHO
R_{rut}, J/day/gww	72.07 ± 6.0[a]	54.1 ± 2.6[b]
R_{AHI}, J/day/gww	3.0 ± 0.85[a]	10.3 ± 1.7[b]
R_{Total}, J/day/gww	75.1	64.4
U_{rut}, J/day/gww	7.4 ± 0.7[a]	8.9 ± 0.2[b]
U_{PPNE}, J/day/gww	2.5 ± 0.3[a]	1.4 ± 0.1[b]
U_{Total}, J/day/gww	9.84	10.2
P, J/day/gww	96.78 ± 14[a]	137.6 ± 20.6[b]
Absorption (Ab), J/day/gww	181.7	212.2
Assimilation (As), J/day/gww	171.8	202.0
Ef assimilation As/Ab	94.6	95.2
Respiratory efficiency, % R/As	43.7	31.9
Production efficiency, % P/As	56.3	68.1

Different letter means statistical differences, $P < 0.05$.
Values are mean ± SE.
HCHO, high dietary carbohydrates; LCHO, low dietary carbohydrates.

ficiency between populations (Fig. 5); a reduction of the P/AS ratio depending on the generations of farmed selected shrimp indicate that efficiency with which shrimp transform energy into biomass is reduced with artificial selection. That situation has several implications on coastal ecology. When selected shrimp are lost by pond break caused by floods or hurricanes they could be liberated to surroundings environment. If those shrimp are from a breeding program based on size only, they shrimp could growth faster and consume more protein than wild shrimp, wasting energy due to its reduced assimilation efficiency and wasting other nutrients offered by the natural environment in the form of CHO and in consequence changing the relation between nutrients and consumers. In this same sense a reduction in *P/AS* ratio could have implications on the shrimp industry if is considered that a reduction in production efficiency could means the use of foods with more and more fish meal to satisfy the protein requirement of shrimp provoking that the shrimp industry to compete with other industry that use fish meal to produce meat for human consumption.

On the other hand, selection shrimp programs could have relevance for the health of farmed shrimp. Recently, Xu et al. (2001) showed that there is a relation between genetic diversity and IHHNV sensitivity of *P. monodon* from Philippines. Although such relation is not understanding at all it could means that at the same time that shrimp are selected for size some other genes related with virus tolerance could be selected as well, provoking a segregation of the genes involved in virus resistance. If such concepts are applied to *L. vannamei* from breeding programs we could help to develop an industry based on rapid growth, low efficiency and vulnerable shrimp. It will necessary change looking for an shrimp based in the conception of breeding program that try to select shrimp that have wider adaptative ability to respond demands including all that are related to feed composition, productivity, and sustainability (Fenucci et al. 1982, Boureau et al. 2000), and biosecurity (Xu et al. 2001).

ACKNOWLEDGMENTS

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EFFECT OF TEMPERATURE ON POST-PRANDIAL METABOLISM OF BROWN SHRIMP *FARFANTEPENAEUS CALIFORNIENSIS*

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ABSTRACT The effect of three temperatures (19, 23, and 27°C) on the postprandial metabolism (apparent heat increment) of juvenile Farfantepenaeus californiensis was evaluated. The unfed metabolic rate and post-prandial metabolic rates were determined with an intermittent-flow respirometer during 5 h. A peak in oxygen consumption was found 2 h after feeding at 19 and 27°C whereas at 23°C the peak was found after 1 h. The unfed metabolic rate at 23°C was not different from that at 27°C. The maximum metabolic rates of fed animals were 2.1, 1.6, and 1.7 times that of unfed animals in order of increasing exposure. The highest apparent heat increment was found at 27°C. Energy loss varied from 4.11 to 11.43 J. Calculated Q_{10} thermal coefficients indicate metabolic overcompensation for temperature changes between 19 and 27°C, and between 19 and 23°C, except at the maximum metabolic rate. In contrast, Q_{10}s for temperature changes between 23 and 27°C indicate compensation.

KEY WORDS: energy loss, *Farfantepenaeus californiensis*, oxygen consumption, postprandial metabolism, temperature

INTRODUCTION

Rubner (1902) defined the heat increment resulting from biochemical reactions to ingestion of a meal as specific dynamic effect. Since then, various terms, such as specific dynamic action, heat of nutrient metabolism, thermogenic action, calorigenic effect of food, postprandial respiration, and heat increment, have been used widely to represent energy losses associated with feeding in ectotherms (Jobling & Davies 1980, Beamish & Trippel 1990). The physiologic basis of this increased heat production includes postabsorptive processes related to ingestion, particularly of protein-rich food, the metabolic work required for formation of excretory nitrogen products, and the synthesis in the tissues of proteins and fats from the newly absorbed food derived substrates like amino and fatty acids. The energies required for grasping, chewing, and swallowing food are technically distinct from the heat increment but are difficult to separate experimentally (Beamish & Trippel 1990). The apparent heat increment (AHI) is the energy required for the mechanical processes of feeding and the ingestion and digestion of food (Hewitt & Irving 1990). In homeotherms, heat increment has multiple influences, including time spent in eating, muscular work, secretion of saliva, fermentation heat, transport of the absorbed nutrients, hormonal effects, and pharmacological effects of food constituents, and is related to the enthalpy change associated with the generation of ATP (Blaxter 1989). In fish, there is ample evidence that AHI is influenced by meal size and feeding frequency, temperature (Bret 1976); size of the animal (Beamish 1974); quantity, quality, and proportions of the dietary energy components (Smith et al. 1978); and the nutritional status (Hart 1980). Despite the amount of information published on AHI, experimental techniques have varied greatly among the studies, and observations of the effects of temperature on AHI have not been consistent. In addition, there is little information on AHI in Penaeids (Hewitt & Irving 1990, DuPreez et al. 1992, Rosas et al. 1996). DuPreez et al. (1992) reported that the AHI for *P. monodon*

Fabricus ranged from 2–17% when fed commercial pellets and from 2.4 to 19.5% when fed shrimp flesh. Rosas et al. (1996) reported that the highest AHIs were found for *P. duorarum* Burkenroad and *P. notialis* Pérez Farfante feeding on a 65% diet, whereas the lowest were found for *P. setiferus* Linnaeus and *P. schmitti* Burkenroad fed a 40% protein diet. The authors concluded that AHI varied with diet protein content for all these species.

Brown shrimp *Farfantepenaeus californiensis* Holmes is currently being evaluated as a cold-tolerant species with potential for aquaculture at our center. Studies of nutritional and metabolic aspects that are influenced directly by factors such as temperature are important to better understand the physiology of this species. This study presents information about the effect of temperature on the AHI of juvenile *F. californiensis*. Some physiologic responses and possible mechanisms of adaptation are discussed.

MATERIALS AND METHODS

Juvenile *F. californiensis* from the Centro de Investigaciones Biologicas del Noroeste experimental shrimp farm were selected randomly, fed a commercial diet containing 35% crude protein (RANGEN®) with filtered seawater at a salinity of 37 ppt. A photoperiod of 12-hL:12-hD was maintained throughout the study. Shrimp were acclimated (1°C/day) at three different temperatures (19, 23, and 27°C) for a period of 5 days. After a 24-h starvation period, 12 animals of each temperature treatment were placed individually in an intermittent flow respirometer system similar to the one described by Villarreal (1989) 2 h before commencing the test to minimize the effect of handling and previously calibrated at each experimental temperature. The fasting metabolic rate was determined for 2 to 3 h thereafter. Next, shrimp were allowed to feed on commercial pellets for 1 h. Uneaten food was siphoned out completely and collected, and water was replaced completely. Oxygen intake was recorded hourly for 5 h after ingestion of the meal with an oxygen electrode (Yellow Spring Instruments, Model 58). Water was replaced completely after each record to prevent accumulation of ammonia. At the end of the experiment, shrimp were weighed on a digital balance after blotting. Data were cor-

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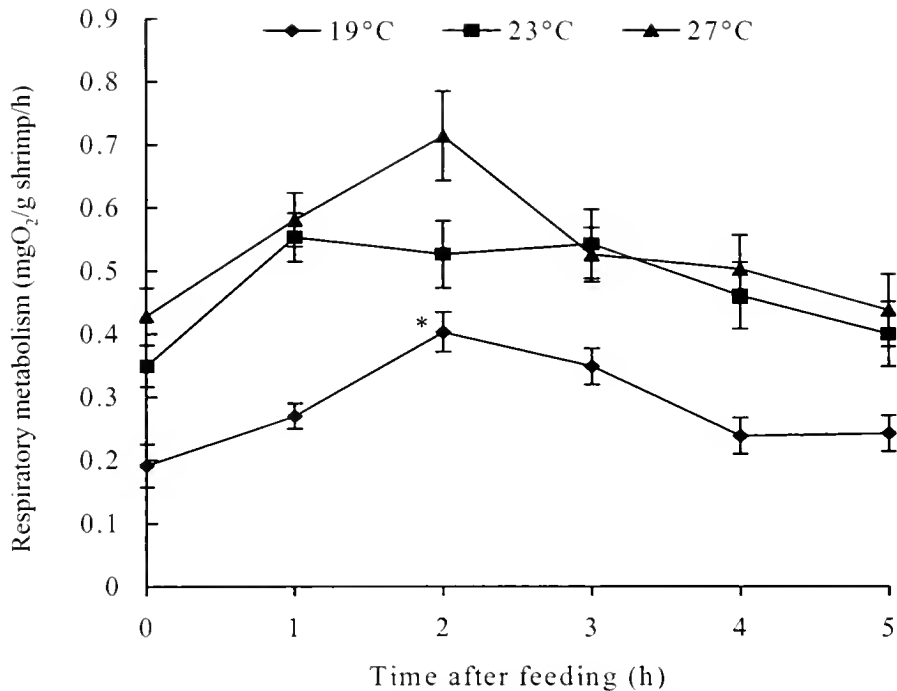
Figure 1. Respiratory metabolism (mgO_2/g shrimp/h) of juvenile *Farfantepenaeus californiensis* after feeding on a 35% crude protein diet (RANGEN®) at different temperatures (°C). Unfed respiration is shown as the respiration at time 0. n = 12 shrimp/temperature. *Significant differences.

rected for oxygen consumption with a control respirometer with no shrimp. AHI (J) at each temperature was calculated as:

$$AHI = (maximun\ postprandial\ rate - unfed\ rate)(20.06)$$

over the period studied (Rosas et al. 1996, Lucas 1993). Differences between treatments were defined by one-way ANOVA and the Tukey multiple range test.

RESULTS

Respiration (mg O_2/g shrimp/h), as a function of time, is shown in Figure 1. Time 0 was defined as the end of the 24-h starvation period. The highest unfed metabolic rate occurred at 27°C, but it was not significantly different from that at 23°C ($P > 0.05$). The lowest unfed metabolic rate was at 19°C and represented approximately 55% of the value at 23 and 27°C.

A tendency to increase metabolic rates at all temperatures after feeding was observed, but this increase was significant only at 19°C. At 19 and 27°C, the highest rate was reached after 2 h. At 23°C, the maximum was observed after 1 h, and was sustained over 2 h. The highest overall increase in metabolic rate after feed-

ing of 67% occurred at 27°C, whereas at 23 and 19°C, the metabolic rate increased 59% and 110%, respectively (Table 1). The time after commencement of feeding until the appearance of the first feces varied from 30 to 60 min at 27 and 23°C, whereas at 19°C the time was approximately 90 min.

AHIs are shown in Table 1. The highest AHI was at 27°C and the lowest AHI was at 23°C. When AHI was expressed as energy lost, values varied from 4.11 to 11.43 J. These values were corrected for the time needed to reach the peak and represent the metabolic efficiency of heat loss. The highest value was at 27°C and the lowest was at 23°C.

Q_{10} coefficients were calculated for temperature increments between 19 and 23°C, and 19 and 27°C, and are shown in Table 2. A Q_{10} value of 2 indicates a doubling of the metabolic rate with an increase in temperature of 10°C. Q_{10} for 23–27 for the unfed period showed adaptation, whereas Q_{10}s for 19–23 and 19–27 showed overcompensation. Q_{10} for 23–27 for the feeding period showed compensation for almost the entire trial except during the second hour. Q_{10} for19–23 showed overcompensation, except for the second hour when there was adaptation. Little compensation was observed between 19 and 27°C in this experiment.

TABLE 1.

Mean effect of temperature on unfed and postprandial metabolism (mgO_2/g shrimp/h), apparent heat increment (AHI, J), and energy lost (J) in juvenile *Farfantepenaeus californiensis*.

Temp. (°C)	Unfed Rate (mgO_2/g Shrimp/h) ± SD	Maximum Postprandial Rate (mgO_2/g Shrimp/h) ± SD	Increase (%)	AHI (J)	Time to Reach Peak (h)	Energy Lost (J)
19	0.192 ± 0.068^d	0.403 ± 0.063^d	110	4.23	2	8.46
23	0.349 ± 0.066^b	0.554 ± 0.077^b	59	4.11	1	4.11
27	0.428 ± 0.091^b	0.713 ± 0.142^c	67	5.72	2	11.43

N = 12 shrimp/temperature. Entries with the same letter are not statistically different ($P > 0.05$).

TABLE 2.

Calculated Q_{10} values for unfed and postprandial metabolic rates in juvenile *Farfantepenaeus californiensis*.

Time After Feeding (h)	Q_{10} (19–23 C)	Q_{10} (19–27°C)	Q_{10} (23–27 C)
0	4.49	2.73	1.66
1	6.02	2.61	1.13
2	1.95	2.04	2.14
3	3.02	1.67	0.92
4	5.19	2.54	1.24
5	3.5	2.09	1.25

Values for Q_{10} were calculated using the formula $Q_{10} = (R_2/R_1) \exp^{[10/(t_2-t_1)]}$, where R_2 and R_1 are the metabolic rates at temperatures t_2 and t_1, respectively.

DISCUSSION

DuPreez et al. (1992) concluded that the magnitude and duration of oxygen consumption peaks could be influenced by digestion rate, environmental temperature, and activity of the animal. In our study, a peak was seen 2 h after feeding at 19 and 27°C, and decreased thereafter, showing that oxygen consumption of F. californiensis was affected only by food in the experimental device used. Perhaps the amount of food consumed or the digestion rate were responsible for sustaining maximum post-prandial rate over 2 h at 23°C.

The induction in oxygen consumption for *F. californiensis* was approximately 43% higher at 19°C than at 27°C (Table 1). Furthermore, the maximum metabolic rate at 19°C equaled the prefeeding rate at 23°C. Juveniles at 19°C appeared lethargic, and we observed a lower ingestion rate at this temperature. Although preliminary trials indicated that 5 h was adequate for complete digestion of food, we noticed that the digestive tract of some shrimp at 19°C still contained food at the end of the experiment. The slower appearance of feces showed that more time is needed to finish digestion at 19°C, and could be related to a decrease in the appetite and the general movement of shrimp. DuPreez et al. (1992) found two peaks for *P. monodon*, 30 min and 6 h after feeding. Perhaps we would have found a second peak if the trial had continued past 5 h at 19°C, when the shrimp reinitiated digestion.

Villarreal and Ocampo (1993) concluded that *F. californiensis* postlarvae and juveniles exposed to a temperature range from 19 to 31°C could adjust their normal metabolic rate with gradual, short-term temperature modifications. Similar results were obtained in this study, in which the unfed metabolic rate at 23°C was close to that at 27°C. It seems that organisms at 23°C have a mechanism for metabolic compensation or adaptation to temperature variations at this stage of life (Q_{10} for 23–27 = 1.66). This equivalence in metabolic rate could be explained as a modification in enzyme kinetics. Ocampo and Ezquerra (2002) found that the effect of temperature on total *in vivo* protease activity of *F. californiensis* at 23 C was 58% higher than that at 27°C, for shrimp that had been acclimated for 50 days to the temperatures, and suggested that different digestive protease enzymes arise as an adaptation mechanism to temperature and dissolved oxygen variations.

In general, homeostatic regulation of enzymatic catalysis in animals can be accomplished in two ways: by modifying enzyme concentration, or by modifying catalytic efficiency (Hochachka & Somero 1973). When enzymatic concentration is increased, the rate of reaction increases. At 23°C, juveniles might increase their reaction rate as a quantitative strategy for temperature compensation (see Q_{10}s). However, this quantitative strategy might be less efficient during cold adaptation since less time is required for changing enzyme concentration via synthesis of new protein. This metabolic reduction enhances the resistance of *F. californiensis* to low temperature stress. The process seems to involve controlled decreases in metabolism and organelle function, coupled with simultaneous controlled stabilization of macromolecule and organelle structures (Hochachka 1990).

DuPreez et al. (1986) calculated AHI as the increase in oxygen consumption over the time until oxygen consumption decreased to the prefeeding level. In our study, AHI was expressed as the difference in oxygen consumption between unfed and fed animals (Rosas et al. 1996). AHI is best expressed as percent metabolizable energy (Blaxter 1989). Taylor et al. (1987) stated that most animals displayed maximum metabolic rates that were 5 to 20 times the normal rates. However, some experiments did not take into account stress caused by handling, and the need for a "resting" period in the chamber before initiating the trial. Rosas et al. (1996) reported the maximum metabolic rate of fed *P. schmitti* was 2.6 to 3.6 times that of unfed. In our study, maximum metabolic rates found were 2.1, 1.6, and 1.7 times that of unfed animals, with increasing temperature.

We emphasize that the heat increment AHI corresponds to the production of ATP (maintenance heat increment) and tissue energy deposition (production heat increment). Cho and Kaushik (1990) estimated the heat increment of feeding for a maintenance ration is approximately one third of the total heat increment, and the rest is used for productive gain. In general, AHI might range from 11–24% of digestible energy (6–19% of gross energy intake; Beanish & MacMahon 1988), and is a more or less constant fraction of dietary energy (Brody 1964). Further research is needed to relate protein intake with AHI in *F. californiensis*. However, the results of this experiment show that *F. californiensis* juveniles present a metabolic strategy to digest food efficiently at 23°C, leaving more time to consume food and saving heat energy loss. This strategy is not related to their optimum aquaculture temperature, but is related to their physiologic optimum, which would indeed be a good temperature to maintain the animals.

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ABSTRACTS OF TECHNICAL PAPERS

Presented at The 23rd Annual

MILFORD AQUACULTURE SEMINAR

Milford, Connecticut

February 24–26, 2003

CONTENTS

OVERVIEW, 23rd MILFORD AQUACULTURE SEMINAR.
Walter J. Blogoslawski, United States Department of Commerce, National Oceanic & Atmospheric Administration, National Marine Fisheries Service, Northeast Fisheries Science Center, Milford Laboratory, 212 Rogers Ave., Milford, CT 06460.

There were 162 registrants for the 23rd Milford Aquaculture Seminar, a 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.

Forty-two formal papers and posters were presented by attendees from eleven US states, the District of Columbia and Canada. Meeting attendees represented three vocational aquaculture high schools, 13 universities, five 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 aquaculture research activities at the NMFS Milford lab in algae and fish culture, fish feeds, scallop culture, and the role of genetics in culture and enhancement of aquacultured products. Other papers covered crop insurance, fish farming in cranberry bogs and how pollutants can bioaccumulate in culture feeds. Mr. Tim Keeney, NOAA Deputy Assistant Secretary for Oceans and Atmosphere, described NOAA's position on aquaculture during a luncheon address.

The Seminar has developed a tradition of offering the latest information available in the field in an informal atmosphere. This has succeeded in promoting a free exchange among all with an interest in the success and future of aquaculture. This Seminar continued that approach 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 thirty-three separate aquaculture companies met in an evening session for their annual industry group meeting of the East Coast Shellfish Growers Association. The Association's goals are to promote and protect shellfish members' needs in state and regional contexts and involve all stakeholders in the task of enhancing the shellfish aquaculture industry. In addition, federal and state agencies involved in regulation of offshore aquaculture described the new permitting system and how it might affect the industry's development.

The meeting was sponsored by the National Marine Fisheries Service, Northeast Fisheries Science Center, Milford Laboratory, Milford, CT. Abstract printing was courtesy of the U. S. Department of Agriculture, Northeastern Regional Aquaculture Center, N. Dartmouth, MA.

PHOTO DOCUMENTATION AS A VITAL ELEMENT IN COMMUNITY BASED SHELLFISH RESTORATION PROGRAMS.
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Community involvement in local programs dedicated to various aspects of shellfish restoration has grown dramatically in recent years. Documentation is increasingly important and expected and can be used as a powerful tool to benefit any program. The Special Projects in Aquaculture Training (SPAT) program has, from its onset in January of 2001, compiled an extensive library of photographic images as one part of its documentation process.

The photo documentation provides an ongoing chronology of the program's projects and growth. It is being used to document scientific data collection and community involvement in restoration and stewardship activities. It is useful in the grant application and subsequent reporting process. As a visual aid and information sharing tool, it is being used to educate and to communicate to a broader public through posters, marketing and Power Point® presentations.

Photographic recognition of individual volunteer participation in restoration activities highlights the grass roots efforts and helps illustrate the social dynamics of a program.

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INSURING YOUR AQUACULTURE CROP.
David Berry, Hartford Company, 2625 S. 158th Plaza, Omaha, NE 68130.

As an aquaculturist, you face various inherent financial risks. Among them are the loss of the initial investment in the crop, loss of the investment in any growing facilities, and loss of income associated with the finished crop. This session will help you better understand these risks, and ways to minimize them. It will explain how insurance can diminish your financial losses, and some of the other functions it performs. An overview of some risks for which insurance can be purchased will be given, such as power failure, disease, and windstorm. Attendees will also be given an outline of the underwriting and claims process involved with an insurance policy, and a brief review of the Federal Clam Insurance Program.

ECONOMICS, MARKETING AND HOW THEY RELATE TO GROWERS HUSBANDRY METHODS.
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The current Shellfish production in the United States and Canada has a wholesale trade of approximately 243 million US$. There is a substantial amount of imported shellfish that when added to this further creates a serious economic sector of the seafood industry. It is estimated that with an increased supply of safe, quality, branded product that the market place could be in excess of 325 million US$ over the next decade.

Consumer taste and consumption patterns are in constant change in our brand conscious society, the understanding of this and the relationship to social class structure and the buying habits

present evidence and opportunity for the shellfish industry to grow very profitably.

To address and take advantage of these factors, shellfish growers have to deliver what the customer wants and not just what the shellfish grower can supply. Technology and strategies have been developed from the larval stage though husbandry practices to point of sale marketing that will attract and develop new and repeat customers.

The challenge the industry will face will be the supply of a "Safe, Quality, Branded product" that can be sold at a premium price; this means that farm yield and efficiency is an equally important part of the equation.

The information presented will allow growers and industry specialists in attendance to learn what is available in production technology and marketing initiatives as well as the direction that they may take to develop a more profitable shellfish business for themselves or their specific regional area now and in the future.

PRELIMINARY INVESTIGATIONS OF SHELTER COMPETITION AMONG THE ASIAN SHORE CRAB AND NATIVE MUD CRABS. Diane J. Brousseau, Sara Brady, and Allison Schaffer, Biology Department, Fairfield University, Fairfield, CT 06824.

This study examined the potential impact of the recently introduced Asian shore crab, *Hemigrapsus sanguineus*, on shelter utilization by two native species of mud crabs, *Eurypanopeus depressus* and *Panopeus herbstii*, using laboratory experiments and field sampling at two sites in western Long Island Sound (Black Rock Harbor, BRH; Milford Harbor, MH). Abundance and distribution patterns of these species differed at the two sites. Similar numbers of mud and Asian crabs were found under rocks at BRH, but Asian crabs outnumbered mud crabs 15:1 at MH. Asian crabs were most abundant at mid-tide level, whereas 90% of the mud crabs occurred low in the intertidal. This is likely due to the low tolerance for desiccation exhibited by xanthid crabs (Grant & McDonald 1979). At low tidal elevation, where most of the overlap occurred, between-site differences in under-rock microhabitat utilization were present. Only mud crabs were found beneath 75% of the rocks sampled at BRH, but at MH, mud crab species alone were found under only 5% of the rocks. Relative crab densities likely affect competitive outcomes and ultimately space utilization patterns. Results of shelter competition experiments conducted in the laboratory did not support the hypothesis that *H. sanguineus* affects shelter utilization by native mud crabs. The percentage of mud crabs occupying shell shelters remained unchanged when Asian crabs were present, but the percentage of Asian crabs occupying shell shelters decreased relative to controls in trials where mud crabs were present. These findings suggest that *E. depressus* and *P. herbstii* may affect patterns of habitat use by *H. sanguineus*, especially in the lower intertidal, where these species occur together. However, direct experimental manipulations in the field

coupled with long-term monitoring are needed to fully understand the role of competitive interactions in determining the local distribution of these species.

GOVERNING OFFSHORE AQUACULTURE: PROGRESS AND CHALLENGES. Susan M. Bunsick, Marine Policy Consultant, 3114 Wisconsin Ave., NW, #702, Washington, DC 20016.

Six key components of a governing framework for offshore aquaculture are identified, and used as benchmarks in assessing progress toward the development of offshore aquaculture policy for the U.S. Exclusive Economic Zone. From an aquaculturist's perspective, the most important components of a governing system for offshore aquaculture are mechanisms for (1) granting a range of rights to the aquaculturist and (2) protecting those rights. From the broader perspective of a national government, there is a need for mechanisms that (3) protect the rights of other legitimate users of public waters and (4) consider a range of other important national interests and policy priorities. There is also a need to (5) develop administrative systems that are fair, effective, and efficient. This may include a requirement that (6) the aquaculturist provide some form of compensation in exchange for the right to locate and operate an aquaculture operation in public waters. Federal agencies, the research community, and others have begun to address the development of a governing framework for offshore aquaculture in the United States. While these initiatives have resulted in some progress, challenges remain.

AUGMENTING THE LOBSTER CATCH: OYSTER AQUACULTURE IN MODIFIED LOBSTER TRAPS. Joe Buttner, Northeastern Massachusetts Aquaculture Center and Department of Biology, Salem State College, Salem, MA 01970; Dale Leavitt, Roger Williams University, One Olde Ferry Rd., Bristol, RI 02809.

Traps used by commercial fishers to capture the American lobster (*Homarus americanus*) are constructed and fished in ways that approximate technologies commonly employed to culture the eastern oyster (*Crassostrea virginica*). By modifying traditional lobster traps to incorporate trays for oysters it was hypothesized that oysters would survive, grow, and augment the income of lobstermen while promoting acceptance of aquaculture among commercial fishers, local communities, and regulatory agencies. To explore the biological feasibility and practical integration of oyster aquaculture in modified lobster traps a 2-y. cooperative study involving commercial lobstermen, regulatory agencies, and research/extension personnel was initiated in 2001.

Ten lobstermen, six from Massachusetts' Northshore and four from Massachusetts' Southshore/Cape Cod/Islands were identified, trained, and provided with modified traps and oysters from an approved source. Modified traps were fished adjacent to or in the same line as unmodified traps between May/June and October/

November in 2001 and 2002. Lobstermen recorded information (date, depth fished, capture rate, and handling effort) in standardized journals.

Results indicated that oysters in modified lobster traps survived, grew, and can be managed without excessive handling or interfering with lobster removal. Lobster capture rates varied widely and it is unclear whether the modified lobster traps fish differently from traditional traps.

Oyster growth was temperature dependent and integration of oyster culture in lobster traps seems most appropriate on the Southshore, Cape Cod, and the Islands. It is likely that the technology can be transferred to other areas and applied to other bivalves, providing supplemental income to lobsterfishers while nurturing acceptance of aquaculture and perpetuating an important New England tradition, commercial fishing.

VARIATION IN QPX SUSCEPTIBILITY WITH HOST GENETIC ORIGIN.

Lisa M. Ragone Calvo, and **Eugene M. Burreson,** Virginia Institute of Marine Science, College of William and Mary, Gloucester Point, VA 23062; **Susan E. Ford** and **John N. Kraeuter,** Haskin Shellfish Research Laboratory, Rutgers University, 6959 Miller Ave., Port Norris, NJ 08349; **Dale F. Leavitt,** Roger Williams University, One Olde Ferry Rd., Bristol, RI 02809; and **Roxanna Smolowitz,** Marine Biological Laboratory, Woods Hole, MA 02543.

In recent years epizootics of quahog parasite unknown (QPX), a protistan pathogen of hard clams, *Mercenaria mercenaria,* have occurred in Massachusetts, New York, New Jersey, and Virginia. Although it has been found in wild hard clam populations, this parasite has most seriously affected cultured hard clams suggesting that aquaculture practices may promote or predispose clams to the disease. In this investigation we examined the influence of host genetic origin and geographic location on QPX disease susceptibility. Five clam strains, originating from Massachusetts, New Jersey, Virginia, South Carolina, and Florida were produced at a single hatchery and evaluated during a 3-year period for growth, survival, and QPX susceptibility at three QPX endemic sites (Massachusetts, New Jersey, and Virginia). Severe winter-associated clam losses occurred at the Massachusetts site precluding completion of the study at that location. At the Virginia site, mortality at the termination of the experiment was 79% in Florida clams and 52% in South Carolina clams, as compared to 36% in Virginia, 33% in Massachusetts, and 20% in New Jersey clams. Differences between stocks were significant with mortality in the Florida and South Carolina clams being significantly higher than in the northern clams. QPX prevalence in the South Carolina and Florida stocks ranged from 19–21% and 27–29% respectively in the second and third year of the study, while QPX prevalence in the Virginia, New Jersey, and Massachusetts stocks was 10% or less. Mortality was significantly correlated with QPX prevalence during the second and third years of the investigation.

A similar trend was observed at the New Jersey site. Mortality at the termination of the experiment was estimated to be respectively 53%, 40%, 20%, 6%, and 4% in the Florida, South Carolina, Virginia, Massachusetts, and New Jersey clam stocks respectively. QPX was first detected in the clams 14 mo after planting. At 17 and 22 mo after planting, prevalences ranged from 13–18% in the Florida stock, 20–38% in the South Carolina stock, 0–18% in the Virginia clams, and 0–5% in the New Jersey and Massachusetts clam stocks. These results suggest that genotype-environment interactions are important determinants of QPX disease. As such, hard clam culturists should consider the geographic origin of clam seed an important component of their QPX disease avoidance/management strategies.

THE POTENTIAL OF HEAT SHOCK TREATMENT FOR IMPROVED SALINITY TOLERANCE OF *SALMO TRUTTA*.

Julie Cominsky, Maureen Mikos, and **Katie Sicona,** Bridgeport Regional Vocational Aquaculture School, 60 Saint Stephens Road, Bridgeport, CT 06605.

Heat shock treatment has been applied to cross protection studies, including salinity, ammonia, and nitrogen compounds. Brown trout, *Salmo trutta,* were selected for this study to determine the potential of heat shock treatment for improved tolerance of salinity stresses. The heat shock was conducted in 10-gallon freshwater tanks for 10 minutes. Visual observations were conducted at 30-sec intervals. These visual observations included swim patterns and orientation, mucus excretion, respiratory motion rate, and scale loss. The fish were then removed from the heat shock treatment tanks and transported to the post shock recovery tank system. The post shock recovery tank system consisted of four 10-gallon tanks in a cold-water bath. The heat shock treatment range was 23–29°C set at 2°C intervals. As the shock temperatures increased, negative behavior patterns were observed, with mortalities occurring at 29°C. Based on these observations 27°C was determined the optimal temperature to perform the heat shock treatment in the salinity applications of 5 ppt to 20 ppt at 5 ppt intervals. Noticeable disparities between the control set and the heat shock set were not realized until the 20 ppt concentration was conducted. At 20 ppt the heat shock data showed a 100% survival rate over 96 h of salinity exposure, while, the control set showed a 70% survival rate over 96 h of salinity exposure.

DEEP WATER, LONGLINE SHELLFISH FARMING IN NARRAGANSETT BAY.

Todd Corayer, Salt Water Farms LLC, 30 George St., Wakefield, RI 02879.

Salt Water Farms is developing a multi-species aquaculture business specifically sited to make use of an underutilized water column, and excellent environmental factors in an effort to establish responsible, large-scale shellfish aquaculture in waters with many historical users.

Anchored with two different custom mooring configurations for this dynamic, open water site and serviced by our 36′ vessel, the New Hope, we have deployed both vertical cage assays for *Crassostrea virginica* culture and blue mussel drop-socking, in conjunction with spat collectors. Approximately 300,000 oysters, at an average size of 25 mm were confined in cages at densities that reflected market size spatial requirements. *Mytilus edulis* spat collectors, both synthetic and recycled pot warp, were set to identify spawning patterns and preferences. Blue mussels were also set into socking and have been examined throughout their grow-out. In cooperation with the University of Rhode Island, seasonal grab samples are being conducted, to determine any effects the farm may have on the benthic environment.

Despite the usual learning curve, we experienced sufficient growth rates to enable a reasonable percentage of oysters to reach market size by the end of the growing season. Positioned midwater, the design is an effort to establish a prototype large-scale farm that can operate successfully in the midst of other recreational and commercial users. Our main goal is to build a farm business where both animal and gear stocking densities have been thoroughly tested and analyzed to have minimum environmental and social impact, while operating profitably.

THE RHODE ISLAND AQUACULTURE INITIATIVE.

Barry A. Costa-Pierce, Rhode Island Sea Grant College Program, Graduate School of Oceanography, University of Rhode Island, Narragansett, RI 02882.

In an attempt to elevate Rhode Island from last place among the 50 states in aquaculture production, Senator Jack Reed obtained 1.5 million US$ for developing aquaculture in the Ocean State. The Rhode Island Aquaculture Initiative (RIAI) is a unique collaboration that unites federal and state interests as well as academic, regulatory, and industry resources.

Funding from the National Oceanic and Atmospheric Administration was awarded to the Rhode Island Coastal Resources Management Council (CRMC), the states lead regulatory agency for aquaculture. CRMC, in turn, enlisted the Rhode Island Sea Grant College Program to administer the project. In 2002, the RIAI directed 600,000 US$ of that money toward aquaculture research and development in the state through a series of multi-year research grants and 1-y "mini-grants", awarding funding for projects that seek to improve the health and longevity of farmed shellfish, educate students and communities about aquaculture, address concerns about aquaculture's effects on the environment, help researchers and aquaculturists access aquaculture data, and reduce conflicts between aquaculturists and traditional capture fishermen. Funding for new capacity-building projects and industry-relevant aquaculture research has been made to help jump-start a new era of aquaculture development in Rhode Island—a place where everyone says our collective challenges are among the greatest any-

where—and help Rhode Island become a world-class aquaculture research and development center.

EMBRYONIC BLOOD CELL FORMATION IN *LIMULUS POLYPHEMUS* (HORSESHOE CRAB). Yvonne Coursey, Nina Ahmad, Barbara McGee, Nancy Steimel, and Mary Kimble, Department of Biology, University of South Florida, 4202 E. Fowler Ave., SCA 110, Tampa, FL 33620.

Invertebrates produce blood cells, but unlike vertebrates where blood cell production (hemopoiesis) takes place primarily in the bone marrow, hemopoietic sites in invertebrates vary from species to species. The blood cells (amebocytes) of *Limulus polyphemus* Linnaeus are among the most widely studied of any invertebrate. Despite having received an enormous amount of attention the site(s) of blood cell formation in *Limulus* have remained elusive. The primary goals of this research were to determine where horseshoe crabs (*Limulus polyphemus*) produce their blood cells, and when during embryogenesis blood cell production begins.

To distinguish *Limulus* amebocytes from other tissue, a polyclonal antibody was raised against purified coagulogen protein, the major protein found in the amebocyte granules. The anti-coagulogen antibody allowed the identification of maturing embryonic blood cells from all other embryonic cells. Blood cell production begins in the developing embryo at stage 18, approximately half way through embryonic development. Embryonic blood cells are located in body cavities. Blood cells mature in circulation, as seen by the increase in granulation of blood cells comparing stage 18 to stage 20 embryos. The presence of coagulogen in the granules was confirmed using the anti-coagulogen antibody.

PRELIMINARY FINDINGS ON THE EFFECT OF MANIPULATING PHOTOPERIOD ON GONADAL INDEX OF THE BAY SCALLOP (*ARGOPECTEN IRRADIANS IRRADIANS*). Peter N. DeSanctis, and Kim Tetrault, Cornell Cooperative Extension of Suffolk County–Marine Program, Marine Environmental Learning Center, Southold, NY 11971.

Using gonadal index as a measure of fecundity, a preliminary experiment was performed in an attempt to demonstrate the effect of light on the reproductive capacity and rate of conditioning of the bay scallop. Populations of scallops were exposed to a regime of ambient light, continuous light, or continuous dark. All other variables, such as water temperature and feed were held constant for the three test populations. In this initial and abbreviated study, it was observed that differences in gonadal index varied with photoperiod. It was found that scallops subjected to continuous light showed in a higher gonadal index throughout the test period as compared to the other treatments. Subsequent investigations will address fecundity, egg size and quality and minimizing conditiontime to spawning.

ROTIFER PRODUCTION ON MICROALGAL DIETS: DEFINING PARAMETERS FOR OPTIMAL PRODUCTION.

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Production of live feeds can be a bottleneck in finfish aquaculture. Producing sufficient numbers of rotifers on live microalgae can be an especially problematic step in this process. A balance of producing large volumes of suitable quality algae, maintaining appropriate growth parameters for rotifers, and timing is required for success. Rotifer production at the Milford National Marine Fisheries Service Lab has had varied levels of success and failure during finfish aquaculture projects. These experiences, and a review of literature, suggest that a narrow set of parameters in both microalgae culture and rotifer culture must be met to assure consistent live feed production. Previous work at the Milford lab identified the *Tetraselmis* strain, PLY429, of microalgae as the best food for rotifer production of ten algae tested. It was also found that maintaining algal densities of six million cells per milliliter yielded the highest conversion efficiency of algal biomass to rotifer biomass. This study focuses on identifying the specific parameters required to rear rotifers successfully on a moderate scale.

Six, 30-liter, round-bottom drain vessels were used to test a single parameter at two different levels (each level in triplicate) at a time. Parameters tested included: ammonia level, light level, algal cell density, and mixing method. Rotifers were stocked at 50/ml to begin each trial. Total rotifers produced, percent of rotifers with eggs, and various water/culture quality parameters were measured during each trial. Maintaining ammonia levels below 1 ppm in the algae and rotifer cultures was essential to rotifer growth. Illuminating the "green" rotifer cultures to levels of 1000 microeinsteins/square meter/second PAR at the surface led to higher rotifer production and reduced ammonia levels compared to room light alone. Maintaining algae densities at a constant high level (2–3 million cells/ml) produced more rotifers than letting rotifers graze down the algae population. Adequate bubbling to keep dissolved oxygen levels over 5 mg/l throughout the "green water" culture was also essential to rotifer production. When all tested parameters were optimized, and with full 30-liter vessels, it was possible to consistently produce 500 rotifers/ml (15 million per tank) from 50/ml in 5–7 days.

RARITAN BAY HARD CLAM FISHERY MANAGEMENT: GETTING THE DATA TO MAKE DECISIONS.

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The hard clam fishery in the Raritan and Sandy Hook Bays was a viable entity until a hepatitis outbreak in the early 1960s closed the fishery. In 1983, a state-sponsored relay program and private depuration allowed the fishery to re-open in Monmouth County. At this time there are about 200 full and part-time clammers working those areas. They supply two depuration plants or move their catch to relay beds in approved water in Ocean County for a 30-days purging process. They harvest about 35–40 million clams a year that have a dockside value of over 5 million US$. Some clammers can gross over $100,000 in this operation.

Although a stock assessment was done by the State in 1983 a lack of funds prevented more surveys until 2000 when the New Jersey Department of Fish and Wildlife Bureau of Shellfisheries surveyed the same area again. At the same time, Rutgers Haskin Shellfish Lab, Rutgers Institute of Marine and Coastal Sciences, and Rutgers Cooperative Extension along with Brookdale Community College conducted studies to determine the age and growth of the shellfish and a natural mortality study. Since the fishery appears to be very lucrative, there is increased interest by others to open depuration plants. The information from these three studies can allow the industry and the state to work better together to manage the harvest pressure and the participation in the area. Fortunately, the data show that the stocks are at higher levels than when harvest restarted in 1983, possibly allowing for further exploitation.

AQUACULTURE POLICY IN CONNECTICUT—CONSTRUCTING A PERMITTING ROADMAP FOR STAKEHOLDERS.

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The permitting system for marine-based aquaculture in the State of Connecticut has had a complete overhaul in the past 2 y. As floating and submerged shellfish structures (longlines, cages, bags, racks, etc.) have been shown to be an efficient and productive method for growing shellfish, their use has grown dramatically. The implementation of these types of gear has raised a number of permitting issues concerning: navigation, boater safety, aesthetics, environmental effects, liability, etc.

A new aquaculture permitting policy was set up in Connecticut in October of 2001. The Connecticut Department of Agriculture, Bureau of Aquaculture (DA/BA) has collaborated with the United States Army Corps of Engineers (USACE) and the Connecticut Department of Environmental Protection (DEP) to develop the Connecticut General Programmatic Permit for Aquaculture. The extensive new permitting process requires review of the above listed issues and others by a number of state (DA/BA, DEP), federal (USACE, National Marine Fisheries Service, United States Fish & Wildlife Service, United States Environmental Protection Agency), and in some cases, local officials.

The Connecticut Sea Grant Extension Program (SGEP) has sponsored a workshop series on aquaculture policy and the permitting process. SGEP's partners include USACE, DA/BA, CT DEP, and municipal shellfish and harbor management commissions that aid in workshop development. The series includes workshops specialized for various stakeholders including growers, policy-makers, extension services, researchers, educators, and the general public. The intent of these workshops is to provide stakeholders with information on Connecticut's aquaculture permitting process from local, federal and state perspectives, and to address the questions or concerns of these stakeholders.

The goal of this workshop series is to facilitate communication and information transfer among stakeholders in the aquaculture permitting process. A list of objectives or "needs" was developed at the first planning meeting. The immediate needs from the policy makers' standpoint were:

(1) To develop a roadmap for aquaculture permitting in Connecticut.
(2) To develop an online "Guide to Aquaculture in Connecticut."
(3) To develop a new strategic plan for aquaculture in Connecticut.

COMMUNITY EFFORTS TO RESTORE LOCAL CLAM FLATS. Jack Grundstrom, Shellfish Constable, Rowley, MA 01969; **Bonnie McAneney, Scott Weston, Mark Fregeau,** and **Joe Buttner,** Northeastern Massachusetts Aquaculture Center and Department of Biology, Salem State College, Salem, MA 01970.

Since May 1999, officials and volunteers (primarily shellfishers) have released or redistributed millions of wild-caught and hatchery-reared softshell clams (*Mya arenaria*) onto approved tidal flats in Rowley, Massachusetts. Initially, 6 capture nets (35' × 8' nets with a 1/4″ × 1/8″ mesh) were installed on flats in the Rowley River. Only two nets successfully collected wild clam seed. In 2000, 20 capture nets were set and all nets retained seed; some nets collected thousands of clams per square foot. Most clams caught in 2000 were distributed among local flats, ~200,000 were transferred to the Northeastern Massachusetts Aquaculture Center's (NEMAC) Cat Cove Marine Laboratory and overwintered. Concurrently, the same number was held using spat bags in the Rowley River. These clams were seeded in the spring of

2001 and covered with predator exclusion netting (35' × 14' or 50' × 14' with a 1/4″ × 1/4″ mesh). In 2001, over 60 capture nets were deployed and all collected softshell clam seed with maximum density reaching a few hundred per square foot. High densities were reduced by replacing the capture nets (35' × 8') with larger predator exclusion nets (50' × 14').

Between 1999 to 2001 natural recruitment yielded large numbers of clam seed; however, in 2002 almost no seed was collected under capture nets in Rowley (and nearby towns such as Gloucester and Ipswich). Poor recruitment was partially mitigated by hatchery production. The town of Rowley received over 800,000 hatchery-reared clams from NEMAC. Clams were cultured in a Floating Upwelling System (FLUPSY). In the fall, clams were planted and covered by predator exclusion nets, to be harvested when they attain market size. To restore and maintain healthy clam flats requires broad community support that includes monitoring and record keeping, facilitating wild recruitment, possibly a hatchery, creative networking, and a lot of work!

GROWTH OF RHODE ISLAND QUAHOGS, *MERCENARIA MERCENARIA,* IN EXPERIMENTAL UPWELLERS AS A PART OF THE NORTH CAPE OIL SPILL RESTORATION PROJECT. Edward Jaskolski and **Michael A. Rice,** Department of Fisheries, Animal and Veterinary Science, University of Rhode Island, Kingston, Rhode Island 02881; **Karin Tammi,** Department of Environmental Management Coastal Fisheries Laboratory, 1231 Succotash Rd. Wakefield, RI 02879.

The growth of northern quahogs, *Mercenaria mercenaria,* spawned from native Rhode Island non-*notata* broodstock was evaluated in experiment upwellers for the purpose of evaluating seed production methods for shellfish restoration projects. Downwellers were constructed to accommodate 1.2 million small (400 μm to 1 mm) hatchery-reared seed. Seed were moved to upwellers once they reached an average valve length of ~2 mm. Upwellers were purchased from commercial sources and deployed at two sites. The primary location for the study was the Rhode Island Department of Environmental Management Coastal Fisheries Lab, Jerusalem, Rhode Island, with a secondary location at Roger Williams University, Bristol, Rhode Island. Growth of the quahog seed was determined as a function of location, stocking density, levels of biofouling, and water flow through the upweller silos. The quahog seed reached a maximum size of 13 mm at the end of the 2002-growing season. To minimize overwintering and predation loss the quahogs were overwintered in benthic cages. The seed will be field planted in designated shellfish restoration sites in the 2003 season when they reach an average valve length of 20 mm. This is publication number 3972 of the College of the Environment and Life Sciences, University of Rhode Island.

IN SEARCH OF LABOR SAVING CULTURE STRATE-GIES FOR THE BAY SCALLOP, *ARGOPECTEN IRRADI-ANS IRRADIANS*. Richard C. Karney, Martha's Vineyard Shellfish Group, Inc., P. O. Box 1552, Oak Bluffs, MA 02557; Enid K. Sichel, Woods Hole Oceanographic Institution, Woods Hole, MA 02543.

Farming the bay scallop, *Argopecten irradians irradians*, is a labor-intensive proposition, primarily due to biofouling control on the netting of culture structures. Attempts to field culture small, early juveniles (2 mm) requires the use of small-mesh nettings (1.5 mm) that require almost daily brushings to maintain adequate water flow to support survival and growth. Larger mesh nettings used to grow older scallops require less frequent cleaning, however, the number of cages required increases dramatically as the scallops grow. Several culture strategies including, reduced densities, cage-less culture methods using artificial eelgrass, biodegradable burlap nurseries, and adhesives were investigated as possible means of avoiding the labor costs associated with net cleaning.

Juvenile scallops were cultured in spat bag nurseries at four densities (~3,000, 5,000, 7,000, and 11,000/bag) to determine if simply lowering the density could reduce the requirement for frequent bag brushing. Although growth correlated inversely with density, growth at even the lowest density was poor.

"C-weed®", an artificial polyethylene (HDPE) eelgrass attached to a weighted aerated pipe, was investigated for its potential as both a spat substrate for setting scallops and a cageless field nursery system. Seed scallops that had set on the C-weed® grew well in the field but the initial set on the artificial eelgrass in the hatchery was poor. The use of biodegradable burlap to set and field-culture juvenile scallops remains a superior method.

Twenty commercially available adhesives were tested for possible application in a cageless culture methodology that involves attaching juvenile scallops to polyethylene netting with the adhesives. Several promising adhesives have been identified for further investigation.

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THERE IS SOMETHING FISHY ABOUT THAT CRAN-BERRY BOG! Dale Leavitt, Roger Williams University, One Olde Ferry Rd., Bristol, RI 02809; Brad Morse, DoubleM Cranberry Company, 980 Walnut Plain Rd., Rochester, MA 02770; Scott Soares, Mass Department Food & Agriculture, 251 Causeway St., Boston, MA 02114; Keith Wilda, Western Mass Center for Sustainable Aquaculture, University of Massachusetts, Amherst, MA 01002.

Over-production and limited market development have decreased the farmgate value of cranberries to the point where it is not covering production costs. Cranberry farming in southern New England is an economically important industry that also controls vast amounts of undeveloped land in an area that is rapidly ap-proaching build-out. It is imperative to develop alternate crops to permit cranberry farmers to stay in business thereby protecting an important sector of the local economy and protecting the land. We have been involved during the past 2 y in modifying a new fish farming technology, the partitioned aquaculture system (PAS), to allow its use within a cranberry bog system while not changing the overall physical structure of the cranberry bog. A demonstration bog/PAS fish farm has been operating for part of one summer growing largemouth bass, yellow perch, and brown bullheads. Although a full grow-out season has not been realized, preliminary growth data suggest that the bog/PAS fish farm has potential to allow the cranberry farmer to produce an alternate crop within the bog system. While some water quality parameters were not completely controlled (i.e., pH and dissolved oxygen) we were able to grow fish at a rate comparable to other fish farms. At this time we are planning to further develop the farm next summer to enhance phytoplankton production, the means of removing soluble nitrogen from the fish waste, by way of better control of water quality. In this presentation, we plan to introduce the concept of the parti-tioned aquaculture system and demonstrate its application within a cranberry bog.

THE SPREAD OF SEA LETTUCE IN ESTUARIES OF NORTH AMERICA AND EUROPE AND ITS POTENTIAL EFFECTS ON SHELLFISH CULTURE. Clyde L. MacKenzie, Jr., USDOC, NOAA, Northeast Fisheries Science Center, James J. Howard Marine Sciences Laboratory, 74 Magruder Road, Highlands, NJ 07732.

In recent decades, the distribution of sea lettuce, *Ulva* sp., has spread due to increasing loads of nutrients in estuaries in North America and Europe. The sea lettuce covers vast areas of shallow flats in some years. A 2000 study in New Jersey and a 2001 study in Italy show that sea lettuce has a detrimental effect on macro-fauna. In New Jersey, small invertebrates were 2% as abundant on the surface of sea lettuce, *U. lactuca*, sheets and 25% as abundant under the sheets as they were on unvegetated sand bottoms nearby. In the Venice Lagoon in Italy the presence of sea lettuce *U. rigida* substantially changed the species composition of macrofauna and lowered their density from what it was 30 y earlier. The results suggest that the presence of sea lettuce substantially decreases abundance of small invertebrates and changes their species com-position. Sea lettuce crowds out eelgrass, *Zostera marina*, softshell clams, *Mya arenaria*, and forces northern quahogs, *Mercenaria mercenaria*, to emerge from the bottom. Aquaculturists who grow softshell clams and quahogs should remove sea lettuce from their planted beds. This can be done with a haul seine; a 50- or 100-foot minnow seine is suitable. Removal needs to be done twice a summer, initially about the first of June and again in late July or early August. Controlling sea lettuce also improves the condition of the overall ecosystem in estuaries.

CRYPTHECODINIUM COHNII, HETEROTROPHIC MA-RINE DINOFLAGELLATE: IS IT A GOOD ALTERNATE SOURCE OF ESSENTIAL FATTY ACIDS FOR FIRST-FEEDING LARVAL FINFISH? **Christopher Martin, Dean Perry, David Nelson,** and **Robin Katersky,** USDOC, NOAA, National Marine Fisheries Service, Northeast Fisheries Science Center, Milford Laboratory, Milford, CT 06460; **Stephen Metzler,** End to End, 415 Port Centre Parkway, Portsmouth, VA 23704; **Fu-Lin Chu** and **Eric Lund,** Virginia Institute of Marine Science, College of William and Mary, Gloucester Point, VA 23062.

Traditionally, fish culturists have turned to autotrophic microalgae for enrichment of larval prey (i.e., rotifers and brine shrimp nauplii). For this purpose, algal strains have been selected for their essential fatty acid composition. Two long-chain polyunsaturated fatty acids (PUFAs) have received special attention since they have been shown to improve growth and survival of larval fish. These are docosahexaenoic acid (DHA, 22:6n-3) and eicosapentaenoic acid (EPA, 20:5n-3). Their role in the normal development of the brain and visual system of young fish is of particular interest. The amount of DHA and EPA varies widely among microalgae with some strains containing more of one than of the other. Consequently, it is usually necessary to enrich with a mixture of two or more different algal strains in order to achieve the desired fatty acid level in larval prey. Our objective is to demonstrate that a commonly occurring heterotrophic dinoflagellate, *Crypthecodinium cohnii* Biechler, could serve as a source of DHA in this application.

C. cohnii is a small, colorless dinoflagellate commonly found in association with decaying macroalgae (especially *Fucus* spp.) in the littoral zone of temperate and tropical waters. It is readily isolated from its favored substrate and cultured on seawater enriched with simple sugars and yeast extract. Having a short generation time (8–10 hrs), cultures attaining a density of ca.10^6 cells/mL may be achieved in 3–4 days. It grows best in the absence of light and, while it is not fussy, seems to produce more fatty acids at 30°C than at lower temperatures. *C. cohnii* is an excellent source of DHA. Purification of DHA from this organism has led to commercial production of enrichment products for use specifically in aquaculture.

We chose American Type Culture Collection strain 30772 [*Crypthecodinium cohnii* Biechler] for this work based on its reported fatty acid composition. We demonstrated the ease with which it could be grown in axenic batch culture on simple media. We showed that live cells of this heterotrophic dinoflagellate are acceptable to both rotifers and brine shrimp. Moreover, we documented the transfer of PUFAs from *C. cohnii* to larval fish via enriched rotifers. Finally, we confirmed that larval tautog fed twelve days on rotifers enriched with live cells of *C. cohnii* accumulated DHA and EPA in the near-optimum ratio 3.31 ± 0.02. In contrast, larvae fed rotifers enriched with a mixture of *Isochrysis*

sp. and *Tetraselmis* sp. accumulated DHA and EPA in the ratio 1.42 ± 0.07.

URBAN AQUACULTURE IN CONNECTICUT. **Paul D. Maugle,** Mohegan Aquaculture LLC., 5 Crow Hill Rd., Uncasville, CT 06382.

Aquaculture in Connecticut has for the last 150 years traditionally harvested native-set shellfish from the bottom. Connecticut's oysters are among the most valued oysters reared in the United States. Knowing that bottom harvesting of native-set shellfish is not inherently sustainable in Eastern Connecticut waters, Mohegan Aquaculture LLC has chosen to have, at its core, a shellfish hatchery, coupled with mid-water and surface rearing of shellfish. The hatchery will be based on the systems installed by the Garbo Lobster Company in the village of Stonington, Connecticut. This hatchery when complete is projected to have the productive capability of more than 200 million shellfish seed each year. This would make it one of the most productive on the east coast of the United States.

The company's goal is to become a leading North American aquaculture producer of premium marine shellfish. With that goal in mind Mohegan Aquaculture will look to control the entire production process from culturing the microalgae that form the live feed for the larval shellfish to packing the finished product. This ensures that the company can deliver a superior quality eating experience to both ethnic and white tablecloth markets.

When completed, the Stonington facility will house several profit and cost centers including a commercial scale shellfish hatchery, a wet storage facility, a commercial scale nursery, upwellers, support space for near-shore longline and tray operations, and mooring facilities for shellfish harvest and long-line tender boats. The company has adapted several proven aquaculture technologies to create its own proprietary production systems.

Mohegan Aquaculture's production model utilizes a three species portfolio approach—bottom seeding of quahog clams, scallop, and oyster production in various types of floating midwater and corral structures. The enterprise will also strive to augment its production capabilities by working with third party contract growing partners. This hybrid production approach allows the enterprise to focus on its core business competencies including production techniques, management expertise, and shellfish-value adding while utilizing available outside production capacity.

THE FIRST 18 MONTHS OF A COMMUNITY-BASED SHELLFISH RESTORATION PROJECT FOR EASTERN LONG ISLAND, NY. **Mary F. Morgan, Kathleen K. Becker, Marion Maino,** and **Kim Tetrault,** Cornell Cooperative Extension of Suffolk County Marine Program, Marine Environmental Learning Center, Southold, NY 11971.

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 Peconic Estuary. Special Projects in Aquaculture Training (SPAT) is based on the understanding that enhancement of shellfish beds contribute 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, which currently supports only 1% of its historic stocks.

Nationally published data have indicated that hands-on opportunities in the environment help people become good caretakers of the environment. From the beginning, it has been a goal of the project to involve community members in a long-term effort both to restore locally important marine resources and to develop a stewardship ethic. While capturing the interest and dedication of community members is a labor-intensive, year-round undertaking, the project has motivated many members of our Long Island community. As of August 2002, 209 marine shellfish gardens are being maintained by individuals or families totaling 284 individuals. The community is involved in varying degrees in everything from tending their own aquaculture gardens, attending monthly seminars, building and operating a community hatchery, developing an all-volunteer creek water quality testing team, public education efforts such as speaking at local civic groups, donating materials and supplies, and to creating and selling a cookbook to raise funds for the continuance of the project.

EFFECTS OF WEANING STRATEGIES ON GROWTH AND SURVIVAL OF JUVENILE SUMMER FLOUNDER, *PARALICHTHYS DENTATUS*. Jessica Müsche and David A. Bengtson, Department of Fisheries, Animal and Veterinary Science, University of Rhode Island, Kingston, RI 02881.

The transition from live feed to formulated diets, which aquaculturists call weaning, is a difficult period in the hatchery rearing of some marine fish. Elevated mortality during this period is often due to cannibalism as some fish adapt to the new diet and grow while others do not adapt. Certain strategies have been developed with other species to optimize the weaning process. We conducted four experiments on the effects of weaning strategies on the growth and survival of newly metamorphosed juvenile summer flounder, *Paralichthys dentatus*. The goal of these experiments was to reduce growth variability and increase survival.

Each experiment consisted of three treatment groups and one control group, with three replicates (75-L aquaria) each. The control group in each experiment consisted of fish that were fed live brine shrimp nauplii throughout the course of the experiment. In each experiment, the actual weaning period lasted for two weeks, followed by several weeks of feeding with the new diet. All aquaria were on a flow-through system, receiving Narragansett Bay water at 20°C.

In experiment I, we attempted to determine the optimum age at which to wean the fish. Fish were weaned onto a dry commercial pellet at 2, 4, or 6 wk post-metamorphosis. Fish that were weaned at 6 wk post-metamorphosis had the smallest mean lengths, but they had the least variability in growth and the highest survival. We, therefore, conducted each of the remaining experiments beginning at six weeks post-metamorphosis for that group of fish.

In experiment II, we studied the timing of weaning diet presentation. Fish were given either a dry diet in the morning and brine shrimp nauplii in the evening, both dry diet and brine shrimp simultaneously in the morning and evening, or a dry diet and brine shrimp on alternate days. There were no significant differences in survival or growth among the treatments; however, the fish given brine shrimp and dry diet simultaneously had the lowest variability in growth.

In experiment III, we explored the use of intermediate weaning diets. Fish were weaned directly to a dry pellet, weaned to frozen adult brine shrimp and then a dry pellet, or weaned to a semi-moist pellet and then a dry pellet. While there were no significant differences in growth between the fish fed dry pellet only and those fed frozen brine shrimp, the fish fed the semi-moist diet had a significantly lower growth rate. The fish fed frozen brine shrimp had the lowest variability in growth of the treatments. There were no significant differences in survival among treatments.

In experiment IV, we attempted to use already-weaned fish to teach unweaned fish to accept a pelleted diet. Aquaria in each treatment were provided no already-weaned fish, one already-weaned fish, or five already-weaned fish. Clear barriers that allowed water to flow through were placed in the tanks to separate already weaned from unweaned fish. At the end of the experiment there were no significant differences in survival or growth among treatments, and very little difference in growth variability. In each experiment, the control groups had the highest survival. The control groups also had the lowest variability in size, with the exception of the first experiment in which those fish weaned at 6 weeks post-metamorphosis had the lowest variability in size.

NATURAL SPAWNING OF BLACK SEA BASS, *CENTROPRISTIS STRIATA*, AT THE NMFS MILFORD LABORATORY AND THE UMASS DARTMOUTH LABORATORY WITH OBSERVATIONS ON SPAWNING BEHAVIOR. David A. Nelson and Dean Perry, USDOC, NOAA, National Marine Fisheries Service, Northeast Fisheries Science Center, Milford Laboratory, Milford, CT 06460; Edward Baker, 136 Beechwood Hill Tr, Exeter, RI 02882.

The black sea bass, *Centropristis striata*, is an important sport and commercial fishery along the United States Atlantic coast. Black sea bass are managed under the Magnuson-Stevens Fishery Conservation and Management Act and by the Atlantic States Marine Fisheries Commission. Because the black sea bass is a temperate reef species and is unavailable to bottom trawlers, capture is limited to anglers and pot fisheries. The demand for black

sea bass exceeds supply and the high market value has prompted researchers to evaluate its potential for commercial aquaculture.

Reproductively, black sea bass are protogynous hermaphrodites, developing first as females and later, at 3–4 y of age, transforming into males. Early attempts at spawning black sea bass centered around artificial spawning, collecting adult black sea bass in spawning condition and hand-stripping both males and females. Later attempts focused on inducing ovulation by intramuscular injection of two hormones; human chorionic gonadotropin or luteinizing hormone releasing hormone analog (LHRHa) and hand stripping.

Milford Laboratory and UMASS Dartmouth Laboratory, have used photothermal manipulation to induce spawning. Black sea bass were placed in tanks of ambient, flowing seawater (10°C). The day/night cycle was controlled by a timer that turned fluorescent lighting on and off. Lighting was adjusted every three days to simulate the day/night cycle that was occurring in nature until 15 h of light and 9 h of darkness was reached. When ambient temperature reached 18–20°C and the day/night cycle was 15 h of light and 9 h of darkness the black sea bass spawned. Fish were allowed to spawn in the tanks and embryos were collected on a 500-µm screen or in a 800-µm net. Fish were spawned under these conditions from mid-April to the middle of July at the Dartmouth Laboratory and from the end of May until the beginning of July at the Milford Laboratory. Percent viable embryos ranged from 0% (first eggs produced) to 100% (in the middle of the spawning season).

We have also made observations on the spawning behavior of black sea bass in the course of our conditioning procedures. One dominant male (alpha) appeared to control spawning. The dominant male segregated other males and females in the tank (10 females to 5 males at Milford and 9 females to 5 males at UMASS). This one male prevented other males from mingling with the females. When spawning occurred a female would swim up to the alpha male and present herself. Both fish would move to a separate portion of the tank where the female would release eggs and the male would release milt. When spawning was complete the female returned to the other females and the alpha male positioned himself between the females and the other males.

GROWTH OF JUVENILE BLACK SEA BASS, *CENTROPRISTIS STRIATA*, IN A RECIRCULATING SEAWATER SYSTEM.

David A. Nelson, Dean M. Perry, and Robin Katersky, USDOC, NOAA, National Marine Fisheries Service, Northeast Fisheries Science Center, Milford Laboratory, Milford, CT 06460; Stephen Metzler, End to End Technical Services Inc., Suite 102, 415 Port Centre Parkway, Portsmouth, VA 23704.

The black sea bass, *Centropristis striata,* is currently being investigated as a potential aquaculture species. Work to date has focused on spawning and larval development, conditions for culture of larvae, stocking densities of larvae and juveniles, feeding

trials of juveniles and sub-adults, cannibalism in juveniles, habitat preferences in juveniles, and the effect of water velocity on the juveniles position and movement. Many of the studies on juveniles have been conducted with wild-caught black sea bass. Although black sea bass show great potential for aquaculture, studies have not demonstrated the time required to produce a market-size fish.

Our goal is to grow black sea bass from larvae to market size adults (454–680 grams) in 24 months. Black sea bass are spawned naturally by photothermal manipulation. Embryos are collected on a 500-µm stainless steel screen. Viable embryos float and are separated from dead embryos in an Imhoff cone. The embryos are placed in 1,140 L cone-bottom tanks filled to 1,100 L with 20°C seawater. These tanks are part of a closed, recirculating seawater system with a biofilter and U.V. light. Embryos are allowed to hatch (48 h) and grow into juveniles in these tanks. Fish remain in this system for 3–4 mo and are culled by size before being transferred to two 1,067 L half-round tanks (120.5 cm diameter × 60.2 cm depth × 180.7 cm length). Filtered seawater (10-µm) is recirculated in these tanks with 10% water replacement/day. These tanks have biofilters and U.V. lights associated with them. Flow rate is 113.6–151.4 L/min. Temperature in these tanks is maintained at 20 ± 1°C. Fish are weighed and measured on the day of transfer and once every two weeks thereafter. After 477 days in this recirculating seawater system, fish have grown from an initial mean length of 91 mm and a mean weight of 15.6 g to mean lengths of 232.3 mm and 197.7 mm and mean weights of 242.2 g and 177.9 g in the two tanks. Juvenile black sea bass that were produced from fish spawned in 2002 have grown from mean lengths of 80 and 106.9 mm at transfer to 117.2 and 146.5 mm in 83 days. Mean weights have increased from 10.9 and 23.6 g to 32.8 and 65.5 g in 83 days. Fish spawned in 2001 had specific growth rates of 0.49% and 0.56% per day. Black sea bass spawned in 2002 had specific growth rates of 0.6% and 0.8% per day.

THE POTENTIAL OF POLYCHLORINATED BIPHENYLS CONTAMINATION OF AQUACULTURE PRODUCTS THROUGH FEED.

Christopher Parkins, Bridgeport Regional Vocational Aquaculture School, 60 Saint Stephens Road, Bridgeport, CT 06605.

Polychlorinated Biphenyls (PCBs) are a group of industrial organochlorine chemicals that are a major environmental concern. They are used commercially because they are chemically inert liquids, have low vapor pressures, are inexpensive to produce and are excellent electrical insulators. Due to the fact that PCBs are inert chemicals and soluble in fatty tissues, PCBs undergo biomagnification.

Most aquaculture products rely on commercially processed feeds. These feeds are based on wild-stock fishmeal, which may be contaminated with PCBs found in the natural environment. Through the consumption of these aquaculture products PCBs pose numerous health risks to humans. These include birth defects,

carcinogenic potential and negative impacts to the immune system. The feed types, which are being tested, include Zeigler Trout Feed®, Silver Cup Floating and Sinking Trout Feed® and Hartz Turtle Feed®. The PCBs are extracted from the feed samples using a microwave extraction system following EPA method 3546. A temperature programmable gas chromatograph with a dry electrolytic conductivity detector (DELCD) was used following EPA method 8082 to determine the qualitative level of Aroclor® 1260 in the samples. Two trials were performed which showed the absence of Aroclor® 1260 in all tested samples.

® The use of trade names is to identify products and does not imply endorsement by the National Marine Fisheries Service.

EFFECTS OF HIGH LEVELS OF AMMONIA, PH, AND SALINITY IN ALGAL FEEDS ON THE MASS PRODUCTION OF ROTIFERS. Dean M. Perry, David A. Nelson, Robin Katersky, and Mark Dixon, USDOC, NOAA, National Marine Fisheries Service, Northeast Fisheries Science Center, Milford Laboratory, Milford, CT. 06460; Stephen Metzler, End to End Technical Services Inc., Suite 102, 415 Port Centre Parkway, Portsmouth, VA 23704.

The rotifer, *Brachionus plicatilis*, has been widely used as a live food for feeding the larval stage of marine fishes. Successful aquaculture of marine fish requires adequate and reliable production of high-quality, nutritious rotifers. One method of culturing rotifers is to feed them microalgal diets that promote rapid growth and reproduction. The rotifers used in our aquaculture studies of the tautog and black sea bass were fed the algal strain *Tetraselmis* sp. (PLY 429). This alga not only promotes rapid reproduction of the rotifers, but also contains the n-3 and n-6 polyunsaturated fatty acids that have been shown to promote growth and survival in larval marine fish. *Tetraselmis* was cultured under semicontinuous conditions in three large, open rectangular fiberglass tanks that received constant fluorescent lighting. These tanks were maintained between 200–300 L. Rotifers were fed *Tetraselmis* from two of the three tanks on a rotating basis. Initially, for about 1 week, rotifers showed an increase from 4 to 16 million. After that time, the rotifer population declined to five million and remained at that level for 2 wk. During that time, and for the next 3 mo, sporadic measurements of ammonia, salinity, and pH were taken in each of the three algal tanks. High levels of unionized ammonia (>1 mg/l), and abrupt changes in salinity (±5 ppt) and pH (±1 pH unit) in the algal tanks coincided with decreases in the rotifer population. Those measurements indicated that either individual fluctuations in salinity, pH and ammonia, or a combination of two or more of these factors adversely affected rotifer production. We conclude that changes in salinity, pH, and ammonia levels, as well as increased numbers of bacteria and ciliates in algal cultures can be counterproductive to maintaining high rotifer populations. It is recommended that algal tanks be monitored daily during high rotifer production times for salinity, pH, and ammonia levels. Also, large open algal tanks should be monitored on a regular schedule for bacteria (*Vibrio*) and ciliates. Some alternatives to using live algae include concentrated algal pastes, baker's yeast and commercial products.

EVALUATION FACTORS FOR AQUACULTURE GEAR APPLICATIONS. Cori Rose, Senior Project Manager, United States Army Corps of Engineers, New England District, Regulatory Division, 696 Virginia Rd., Concord, MA 01742; Peter Francis and Robin Bray, Connecticut Department of Environmental Protection, Office of Long Island Sound Programs, 79 Elm Street, Hartford, CT 06106; Tessa S. Getchis, Connecticut Sea Grant, University of Connecticut, 1080 Shennecossett Road, Groton, CT 06340.

In response to the expansion of aquaculture activities and utilization of developing rearing techniques, there is an increasing need for review and evaluation of aquaculture proposals to ensure adequate protection of the environment, wild populations and their habitat, and the compatibility of such enterprises with existing users of the public resource. Regulatory agencies (federal, state, and local) are mandated to review applications for foreseeable future impacts, which a grower may not consider or be aware of. It is the charge of such agencies to achieve a balance between sometimes competing interests while ensuring appropriate regulation of the industry with due regard to the environment and its many users. For example, it is some or all of these agencies' responsibility to ensure that granting of a permit, lease or other authorization will not adversely impact marine resources or pose unacceptable disease, ecological, health, safety, or welfare risks to persons, the environment, or aquatic resources. In addition, agency determinations must also ensure that an authorized activity does not conflict with or negatively impact any recreational, commercial or other use of the proposed project area, or adversely impact the value or use of private property in and around the area.

The charge to an applicant proposing an aquaculture project, especially for a project that entails innovative technologies not currently used in a geographical area or for the culture of nonindigenous stock, is to provide enough information for regulators to make a reasoned decision. However, this can be a daunting task and the various parties' differing expectations regarding the amount and type of information needed may result in costly delays or protracted regulatory reviews. The purpose of this talk is to impart the type of information that should be submitted along with an application for aquaculture in Connecticut in order to facilitate the state/federal joint regulatory review process; and also to discuss regional guidance that currently exists to aid aquaculture applicants, convey expectations of the standard level, and provide the quality of information that may be solicited from regulatory agencies when seeking authorization of aquaculture projects.

A COMPARISON OF MORTALITY IN THE AMERICAN LOBSTER, *HOMARUS AMERICANUS*, USING TWO METHODS OF TAGGING. Anthony Rossomando, Ryan Kilmartin, and **John Roy,** The Sound School, 60 South Water St., New Haven, CT 06519; **Richard Cooper,** UCONN, 1084 Shennecossett Road, Groton, CT 06340.

The American lobster, *Homarus americanus,* has been the subject of tagging studies for the past several decades. The benthic life cycle of the lobster and the ease with which they are trapped make them a species that lends itself readily to recapture studies. Population declines in southern New England during the past decade have made investigations into the recruitment methods of this species a priority for several studies. The means by which the species propagate makes the female lobster the preferred sex for many studies. A large percentage of the female animals that survive to maturity will bear eggs annually. The impact of tagging female lobsters in Southern New England, where the population is declining, warrants the investigation of the stress caused by the tagging procedure.

Outcomes from catch and release studies that depend on the capture of tagged animals to produce data are influenced by recapture percentages. While many factors influence the success of the recapture rate, mortalities that result from the capture, tagging, and subsequent release of aquatic animals adversely affect all study outcomes. Investigators and scientific researchers have used many methods of marking animals that have been taken in this type of study. Students from the Sound School Regional Aquaculture Center conducted a study to compare the effects of tagging adult female lobsters with both Floy tags and Back tags.

The results from this study indicate that mortalities associated with the stresses caused by tagging increased in tagged specimens. Mortalities of 19.1% and 14.3% were recorded in Back and Floy tagged lobsters respectively while the lobsters held as controls had mortalities of 9.5%. The students at the Sound School have had first hand experience with the dramatic declines in the lobster populations in western Long Island Sound during the late 1990s. We believe that it is becoming increasingly important to monitor accurately the existing stocks of lobsters at all levels of the fishery. However, it has become increasingly apparent through our studies that tagging efforts, which employ either the Floy or Back tag to study *Homarus americanus,* may be inflicting substantial mortalities among the sampled portion of the population.

IT TAKES A COMMUNITY TO BUILD A HATCHERY. Otto Schmid, Armand DeLuca, and **Kim Tetrault,** Cornell Cooperative Extension of Suffolk County Marine Program, Marine Environmental Learning Center, Southold, NY 11971.

The program Special Projects in Aquaculture Training (SPAT) at Cornell Cooperative Extension, in Southold, New York, has just completed its second year of operation, having attracted over 200 families volunteering over 11,000 h. Construction of a community

hatchery began in the fall of 2001 and was made operational in the spring of 2002. SPAT members supplied all of the labor necessary to do the carpentry, plumbing and electrical work. This became a valuable learning experience for 12 core workers, augmented by numerous additional SPAT members on an "as available" basis. Utilizing many recycled materials, donations of supplies and equipment and volunteer labor, the cost of the hatchery was minimized. During the 2002 winter layover following its initial growing season, the layout and exterior were revised and improvements made to make the hatchery more efficient, in addition to adding a maintenance annex for tools and equipment. The hatchery at this time houses six 400 L larval rearing conicals along with the necessary aquaculture equipment needed to produce approximately 6–9 million larvae per spawn.

Selected species of bivalves are spawned in the hatchery and the larvae are raised through metamorphosis, at which time they are moved to downwellers in the Marine Center nursery. All conicals are maintained three times weekly at which time the equipment is cleaned and the larvae are culled and restocked to a desired density. Larvae are fed a mixed diet of algae produced at the Marine Center. Larvae are set using a variety of techniques.

The community hatchery is the product of the diverse talents of many individuals. It serves as an invaluable tool for practicing the concepts learned during the training initiatives of the program in a hands-on and productive manner. As with many of the components of the SPAT program, the hatchery is a work in progress and is unique in many ways. The individuality and commitment of the SPAT members have allowed the hatchery to perform effectively in its first year of operation and is anticipated to greatly increase production in the 2003-growing season. The emphasis will be placed on the production of bay scallops (*Argopecten irradians irradians*) with a target goal of 10–15 million post-set.

EFFECTS OF CONTAINER SIZE ON GROWTH AND METAMORPHOSIS OF LARVAL SUMMER FLOUNDER, *PARALICHTHYS DENTATUS*. Laurie Stafford, Jessica Müsche, and **David A. Bengtson,** Department of Fisheries, Animal and Veterinary Science, University of Rhode Island, Kingston, RI 02881.

Commercial aquaculture of the summer flounder, *Paralichthys dentatus,* began in the 1990s. Although research on optimum conditions and methods to rear the larvae has been conducted for years, many factors remain to be studied. Growth rates of summer flounder begin to vary greatly in the late larval period. Because metamorphosis is size-dependent, the fastest growing fish settle first and often cannibalize their slower-growing siblings who settle later. Many variables may affect growth and metamorphosis. We examined effects of container size by conducting an experiment of 49 days duration in which larvae were raised from age 12 days (after hatch) through metamorphosis. Fish were obtained from GreatBay Aquaculture in Portsmouth, NH and were the result of

their first purely F1 male × F1 female crosses. The experiment consisted of three treatments: 2-L, 20-L, and 150-L containers with four replicates of each treatment and stocking densities in all containers of 10 fish/L. Three specific variables were examined: survival, growth (as measured by total length), and the rate of metamorphosis (as measured by settlement times of the fish; settled fish were removed from each container every 3 days). Although there were no significant differences in survival among the three treatments, container size did affect growth and metamorphosis. The length of the fish in the 20-L containers was significantly greater than in the other two treatments until shortly before metamorphosis, after which the fish in the 150-L containers surpassed the other treatments (ANOVA, $P < 0.05$). Analysis of the distributions of settlement over time indicated that fish in the 20-L containers metamorphosed earlier than fish in the 150-L containers (Kolmogorov-Smirnov test, $P < 0.05$), but metamorphosis of fish in the 2-L containers was not significantly different from that of fish in either 20-L or 150-L aquaria. Because commercial aquaculture of summer flounder larvae is conducted in volumes of 1,000 L or greater, our results may have more significance for the research community than for the industry. Nevertheless, container size can affect summer flounder larval growth and metamorphosis.

GENETIC STRATEGIES FOR CULTURE AND STOCK ENHANCEMENT OF BIVALVES. Sheila Stiles, Joseph Choromanski, and Dorothy Jeffress, USDOC, NOAA, National Marine Fisheries Service, Northeast Fisheries Science Center, Milford Laboratory, Milford, CT 06460.

Genetic selection, which exploits the heritable component of variation through breeding, has enhanced significantly the efficiency of livestock and crop production in agriculture. Aquacultured species lag behind, but have provided some successes. However, inadvertent selection and, therefore, narrowing of the gene pool can occur with standard hatchery practices, such as spawning small numbers of broodstock and screening or culling larvae and juveniles for size. This inbreeding effect can be minimized by developing appropriate strategies such as introducing new broodstock to increase genetic diversity. In addition, in some hatcheries, there may be certain characteristics that are desired to improve or increase production. Selecting animals for growth, disease resistance or shell color could increase the frequency of these traits. For example, some scallops have obvious shell markings, such as stripes, which could be used in stock identification as *notata* clams are used in the clam industry, and oysters with disease resistance.

Selective breeding studies are underway employing scallops with striped shells as markers for stock enhancement, as one major limitation to previously conducted stock enhancement programs has been a lack of identification of stocks. The objective of this project is to investigate the feasibility of producing, through selective breeding, increased numbers of bay scallops with distinctive phenotypes for field identification. These naturally-occurring scallops with distinctively visible markers at low frequencies of 1–5% are being developed to determine the reproductive success or genetic contributions of transplanted populations to stock enhancement efforts possibly in sanctuaries. Preliminary laboratory results indicate a positive response to selective breeding with an increased frequency of at least 50% of scallops with striped shells, and favorable growth and survival.

Other components of a breeding program, which could include marker-assisted selection (MAS) and quantitative trait loci (QTLs), should consider the following points: the status of the population, the goals to be attained (i.e., harvesting, stock restoration or enhancement), facility and personnel needs, mating schemes, genetic monitoring (for genetic diversity), and periodic assessments. Genetic monitoring methods include cytogenetics, allozyme, mt and nuclear DNA analyses, and PCR technology. Alternative biotechnological and supplemental approaches to breeding encompass technology of gene transfer and chromosome engineering, such as induced polyploidy (triploidy and tetraploidy). Genetic applications additionally could involve DNA-based probes and assays to detect disease agents as in MSX and Dermo studies with oysters, as well as the generation of molecular tags to identify stocks of shellfish. All of these diverse aspects are applications of genetics to aquaculture and fisheries management that should be considered in strategies to maximize production.

OYSTER TRIPLOIDY TRIALS ON MARTHA'S VINEYARD. Amandine Surier and Richard C. Karney, Martha's Vineyard Shellfish Group, P. O. Box 1552, Oak Bluffs, MA 02557.

Triploidy is the condition of possessing three times the haploid number of chromosomes in the cell nucleus. Because triploid bivalves are sterile, their meat quality remains constant throughout the year and the energy usually used for reproduction is diverted towards somatic growth and disease resistance. Because of those unique qualities, their production has attracted worldwide attention since the early 1980s. At this time triploidy has been successfully applied to the economic benefit of the Pacific oyster industry on the west coast of the US and also in France.

Under funding from the Sailors' Snug Harbor foundation of Boston, triploidy was induced in the American oyster *Crassostrea virginica* in an attempt to locally produce triploid strains of oysters for the growers of the island of Martha's Vineyard. Triploidy was induced with a low risk chemical, 6-DMAP that has been shown to be slightly less efficient than Cytochalasin B but much safer to handle and is water-soluble. The success of induction was measured by flow cytometry at the Virginia Institute of Marine Science. By the third attempt, a 12-min treatment at a concentration of 400 μM yielded 94% triploidy. After 9 days of development, differential mortality led to a percentage triploidy of over 96% in that same batch.

Although we were successful in producing the triploid oysters,

the late production date coincided with deteriorating water quality prevalent later in the summer. Due to a toxic algae bloom (*Prorocentrum sp.*), high bacteria (*Pseudomonas sp.*) and a minor oil spill, only a couple of thousand triploid and diploid control survived and their growth was altered by the exposure. On October 2nd the surviving animals were transferred to one of the growers' high flow, tidal upweller nurseries. However the oysters did not grow and only time will tell if they were hardy enough to survive overwintering.

RAZOR CLAM, *ENSIS DIRECTUS*, GROWTH RATES IN NIANTIC RIVER, CONNECTICUT. John Wadsworth, Niantic Bay Shellfish, LLC, 15 First Street, Waterford, CT 06385, USA; **Tessa S. Getchis** and **Nancy Balcom,** Connecticut Sea Grant, University of Connecticut, 1080 Shennecossett Road, Groton, CT 06340.

In 2001, Niantic Bay Shellfish, LLC partnered with the Northeastern Regional Aquaculture Center as part of a regional project to develop growout culture methods for the razor clam, *Ensis directus*.

Approximately 10,000 seed (20 mm) were distributed evenly (one clam per 6.45 square cm) into felt-lined wire cages (0.6m:length × 0.6 m:width × 0.3 m:height) and were filled to a height of 15 cm of sediment. The cages were set and buoyed on leased ground in the Niantic River in Waterford, Connecticut. MLW was 0.6–0.9 m (site-dependent) with a tidal height of 0.85 m. Monthly inventories to determine clam density and growth (length and width to ± 0.01 mm) were performed beginning in September 2001. The clams increased in length from 18.84 ± 2.22 mm to 74.25 ± 6.54 mm in the first 2 y of the project. Grow-out trials have continued with limited success, as surviving clams are slow growing and have been increasingly susceptible to predation by green crabs.

THE LONG AND WINDING ROAD: TOWARDS SUSTAINABLE FISHERIES MANAGEMENT AND MEANINGFUL SHELLFISH RESTORATION (WELLFLEET, MA). Bill Walton, Wellfleet Shellfish Department, 300 Main Street, Wellfleet, MA 02667.

Over the last year, the Town of Wellfleet (Cape Cod, MA, USA) has begun the long and often contentious process of developing a long-term shellfish management plan. Here I describe the evolution of this plan from a traditional fisheries management approach (e.g., gear limitations, increased fees, etc.) to a community-driven document that relies on input from the shellfishing community while promoting sustainability. Topics will include spawning sanctuaries, cultching, predator control, disease management, and monitoring efforts. In addition, I will review several steps we have taken toward increasing the efficiency of local shellfish restoration efforts.

MOVING TOWARDS COMMERCIALIZATION OF SOFT-SHELL CLAM CULTURE ON MASSACHUSETTS' NORTHSHORE. Scott Weston, Bonnie McAneney, Mark Fregeau, and **Joe Buttner,** Northeastern Massachusetts Aquaculture Center and Department of Biology, Salem State College, Salem, MA 01970.

To support community-initiated enhancement and aquaculture efforts on Massachusetts' Northshore that target the softshell clam (*Mya arenaria*), the Northeastern Massachusetts Aquaculture Center (NEMAC) produced nearly 2 million juvenile clams in 2002. Beyond serving as a regional hatchery and nursery, NEMAC expanded outreach efforts that include technical assistance, educational activities and networking with shellfishers and regulators.

Survival of clams spawned by NEMAC personnel in 2002, to 2.0 mm, exceeded 80%. Resultant juvenile clams were distributed in July to Massachusett's sites: 650,000 (ave. ln. = 2.5 mm) to Rowley and 170,000 (ave. ln. = 3.5 mm) to Martha's Vineyard. Another 220,000 clams (ave. ln. = 14.0 mm) over-wintered in Smith Pool at NEMAC's Cat Cove Marine Laboratory (CCML) were also transferred to Rowley. About 800,000 juvenile clams were retained in spat bags placed in protective plastic cages and floated in Smith Pool. By the end of the growing season, clams had grown to 6.0 mm (ave. ln.) with survival rates approaching 95%. Clams are being over-wintered in submerged cages for release onto approved flats in the spring/early summer 2003.

To facilitate and expand clam production, a dual use Dock/Floating Upwelling System (FLUPSY) was acquired by the Town of Rowley, through NEMAC's small grants program. Clams (650,000) were cultured in the FLUPSY as a cooperative effort involving Rowley shellfishers (maintenance and coordination), the Boy Scouts (maintenance and data collection) and NEMAC (technical support, environmental monitoring and supplies). Surviving clams were released onto the Rowley tidal flats and covered with predator exclusion netting (6.4 mm mesh). NEMAC also advanced private seed collection and grow-out projects in Ipswich and Gloucester, Massachusetts by providing materials and training. It is anticipated that clams spawned at the CCML will attain market size in 2003, the beginning of a sustainable, shellfish aquaculture industry on Massachusetts' Northshore.

DEMAND FEEDING OF BAY SCALLOPS, *ARGOPECTEN IRRADIANS IRRADIANS* USING AN AUTOMATED CONTROL SYSTEM. James C. Widman Jr. and **David J. Veilleux,** USDOC, NOAA, National Marine Fisheries Service, Northeast Fisheries Science Center, Milford Laboratory, Milford, CT 06460.

We have developed a system that allows juvenile bay scallops, *Argopecten irradians irradians*, to be exposed to near-constant concentrations of phytoplankton, even as scallops consume it. Chlorophyll-a fluorescence levels are used to monitor phytoplankton cell concentration in the juvenile scallop culture system. Seawater from the scallop culture is continuously pumped through a

WET® labs submersible fluorometer using a Pondmaster® magnetic drive pump. The fluorometer outputs an analog signal (voltage) proportional to the fluorescence. The analog signal is measured by an ADAC® model 5516 DMA data acquisition board installed in a personal computer. An algorithm reads the voltage/fluorescence and switches a relay on or off depending on the value. When the fluorescence drops below a preset value, the relay turns on and starts adding phytoplankton to the scallop culture with a peristaltic pump. On reaching the desired fluorescence (algal cell concentration), the algorithm switches the relay off which in turn stops the addition of algae to the culture system. By continuously monitoring the fluorescence level of the culture water, the algal cell concentration can be maintained and scallops are fed on demand.

Scallops with an initial mean shell height of 7.2 mm grew to a mean shell height of 18.4 mm in 78 days using our prototype system. This growth was achieved while testing the mechanics and logic of the system. Additional monitoring systems are being built so we can analyze how algal cell concentration affects scallop growth. Our goal is to maximize scallop growth while minimizing phytoplankton consumption. This system would be amenable to feeding oysters, clams, mussels, brine shrimp, rotifers, and other phytoplankton grazers.

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A DECISION TREE FOR DESIGNING A PROCESS TO PRODUCE MICROALGAL FEEDS FOR AQUACULTURED ANIMALS.

Gary H. Wikfors, Barry C. Smith, Shannon L. Meseck, Mark S. Dixon, and **Jennifer H. Alix,** USDOC, NOAA, National Marine Fisheries Service, Northeast Fisheries Science Center, Milford Laboratory, Milford, CT 06460.

Research, commercial, and publicly-funded mariculture facilities generally have a need to produce microalgal cultures to feed molluscan broodstock, larvae, and post-set, or to rear zooplankton as live feed for larval finfish or crustaceans. Too often, facilities and procedures for microalgal feed production are based on inappropriate, previously-existing examples, leading to production processes that are under-scaled, expensive to operate, and undependable. A production process based on quantitative and qualitative needs would be preferable; a decision tree seems to be a useful tool for designing a microalgal feed-production process in a new aquaculture operation or improving an existing facility.

Main considerations for process-design decisions are defined by the nutritional and water-quality needs of the animals being fed—What? How Much? And How Often? Choices of "What" microalgae to grow will, to some extent, constrain the shapes and configuration of containers, and culture management options (i.e., batch or some form of continuous or semi-continuous culture management). In some applications, especially very intensive, recirculating systems, microbiological, and chemical aspects of water-quality become critical in defining feed-culture quality. Once ac-

ceptable qualitative food requirements are identified, then the quantitative characteristics of the process—"How Much and How Often"—must be addressed. Daily harvest volumes can be calculated by dividing algal biomass quantities required to feed animals by estimated (conservatively!) biomass densities per unit volume of algal culture using the selected culture management. Options to replicate small production units many times or to monitor and manage several large cultures intensively can be considered in the context of dependability of the process. Rationale for choices made in Milford Laboratory microalgal feed-production processes—and surprises and problems encountered during operation of these processes—will be discussed.

INITIAL INVESTIGATION OF AN ANNUAL *PROROCENTRUM* BLOOM IN LAGOON POND, MARTHA'S VINEYARD.

William M. Wilcox, Martha's Vineyard Commission, PO Box 1447, Oak Bluffs, MA 02557, USA; **David W. Grunden,** Oak Bluffs Shellfish Department, PO Box 1327, Oak Bluffs, MA 02557.

For 5 out of the last 6 years blooms of *Prorocentrum* have been observed in Lagoon Pond on Martha's Vineyard. This salt-water embayment supports various uses including recreational boating and shellfishing. It has two hatcheries located on its shores: the Martha's Vineyard Shellfish Group's shellfish hatchery and the Massachusetts Division of Marine Fisheries Lobster Hatchery. This project is the first to investigate the possible causes of this almost annual *Prorocentrum* bloom.

This report summarizes the lab and field data collected from five sample locations in Lagoon Pond during the period from mid-May through mid-September 2002. The data reported include vertical profiles of temperature and dissolved oxygen; dissolved and particulate nutrient analyses from both surface and deep sample sites; and chlorophyll, bacterial and phytoplankton analyses, and transparency.

July and August rainfall was 5.5 inches less than the historic average for these two months. In addition, water table levels throughout the outwash plain set new records for monthly low stands as measured since 1991 (Wilcox 2003). This produced a lower than usual amount of fresh water input from rainfall, groundwater, runoff and fresh water surface inflow. As these sources are major contributors of nitrogen, there was less input of this nutrient to the system.

During the course of study, the Lagoon maintained good water column transparency with Secchi depth averaging 3 to 3.4 meters and never falling below 2.1 meters. The dissolved oxygen saturation was typically above 80 percent in the surface water but fell briefly to a low of 12 percent at 5 to 7 meters depth at one station. The pond is always limited by the availability of nitrogen but cycles between times when silica is and is not limiting to the growth of phytoplankton.

In July, the Shellfish Hatchery quahogs became heavily fouled with vorticella, hydrozoans and bryozoans and showed symptoms

of lack of food or poor quality food. There were some indications of mild bacterial infection but the usual die-off did not materialize in July as it has typically in previous years.

A groundwater survey was conducted and, at the seeps sampled, nitrogen was 20 to 100 times more concentrated than in the pond. Silica was about an order of magnitude greater but ortho-phosphate was roughly equal to the in-pond concentration. Groundwater is clearly a source of nitrogen and silica to the system.

The Upper Lagoon Pond discharges through a herring ladder at the Madeiras run. This freshwater pond experienced a severe algae bloom with chlorophyll a concentrations rising from 8.2 micrograms per liter in mid-May to over 50 by mid-June. A second bloom began in mid-August, peaked at 193.5 µg/L on August 19 and continued through the last sample round on September 12.

The data collected hint at a complex cycle of phytoplankton populations, grazers including jellyfish, water quality, and shellfish survival at the MVSG Hatchery. The jellyfish were numerous this year and, it is suspected, their feeding on copepods and other grazing organisms freed up primary phytoplankton growth, which is influenced by both the availability of nitrogen and silica. Low levels of nitrogen in the system probably subdued the phytoplankton bloom that, as a result, was not as excessive as perhaps previous years.

AN UPDATE ON BLUE MUSSEL CULTURE IN LONG IS-LAND SOUND. Lawrence Williams, Jessie D., Inc., 68 Anchorage Drive, Milford, CT 06460; **Tessa S. Getchis,** Connecticut Sea Grant, University of Connecticut, 1080 Shennecossett Road, Groton, CT 06340-6048; **Inke Sunila,** Connecticut Department of Agriculture, Bureau of Aquaculture, P.O. Box 96, Milford, CT 06460.

As fisherman and commercial shellfish harvesters continue to struggle with fisheries disasters in Long Island Sound including disease, drought, storms, invasive species, etc., they are able to depend less and less on traditional fisheries. Maritime industry members have partnered with state and federal agencies to investigate the possibility of new/alternative species for culture in Long Island Sound.

In 2001, a pilot project was initiated to investigate long line culture of blue mussels (*Mytilus edulis*) in Long Island Sound. A natural set of seed mussels was collected on the long lines in the area of Charles Island off the coast of Milford, Connecticut, USA. Newly set mussels were observed on the long lines in the spring, summer, and fall of 2001 and 2002. The mussels reached market size of approximately 2.5 inches in less than 10 months.

An investigation into the feasibility of a commercial-scale operation involving blue mussel culture in Long Island Sound has been proposed.

ABSTRACTS OF TECHNICAL PAPERS

Presented at The 95th Annual Meeting

NATIONAL SHELLFISHERIES ASSOCIATION

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ENVIRONMENTAL EFFECTS ON *PERKINSUS MARINUS* INFECTION RATES, GROWTH AND SURVIVAL AMONG DERMO-DISEASE-FREE JUVENILE OYSTERS IN THE PATUXENT RIVER, MARYLAND DURING DROUGHT CONDITIONS. George R. Abbe,* Candace A. Morrell, Academy of Natural Sciences Estuarine Research Center 10545 Mackall Rd. St. Leonard, MD 20685; **Carol B. McCollough** and **Christopher F. Dungan,** Sarbanes Cooperative Oxford Lab, Oxford, MD21654.

In September 2000 specific pathogen-free (SPF) oysters were transplanted to 3 sites in the Patuxent River, Maryland to investigate environmental effects of *Perkinsus marinus* on infection rates, growth and survival. During the first year, salinity at Holland Point (upper river), Gatton (mid) and Town Creek (lower) averaged 11, 13 and 14, respectively, but during the second year averaged 13, 16, and 17. Thirty oysters were collected from each site for assay of *P. marinus* infections by the whole body burden technique allowing an estimate of time to initial infection and subsequent progression of disease. An additional 30 oysters from the natural bar at each site were checked by rectal tissue assay. Oysters at HP, GAT and TC grew 23, 34 and 27 mm, respectively, and survival was 95, 98 and 94% during the first year. During the second year, growth was slightly better at HP (21 mm) than at GAT (16mm) or TC (19mm). By August 2002, mortalities at HP, GAT and TC were 60, 98 and 97%, respectively, and HP reached 97% 2 months later.

ECONOMIC IMPACT OF THE CULTURED HARD CLAM INDUSTRY IN FLORIDA. Charles Adams, Elfie Philippakos, Alan Hodges, David Mulkey, Dorothy Comer and Leslie Sturmer*, P.O. Box 89 Cedar Key, FL 32625 USA.

The hard clam aquaculture industry in Florida went from producing 10 million clams in 1991 to 140 million clams, with farm gate sales totaling $15 million, in 2001. This paper determines the economic impact of this rapidly growing industry. Not only does the industry contribute in terms of product sales and employment, it produces a greater economic benefit because of the activity it generates among the firms that provide inputs to the clam culture firms. Further, employees within the industry generate economic activity when they spend their income. Thus, the economic benefits resulting from clam culture extend beyond the local area to the general economy. For the cultured clam industry to thrive, it is essential that water quality standards remain high, permitting and regulatory measures continue to be favorable, and effective marketing efforts are employed. Given the need for this support, the state and local decision-makers must understand the value of clam sales to their economy.

PRODUCTION OF TETRAPLOID SUMINOE OYSTERS *C. ARIAKENSIS*. Standish K. Allen Jr.*, A.J. Erskine, Elizabeth Walker, and Ronald Zebal, Aquaculture Genetics and Breeding Technology Center, Virginia Institute of Marine Science, Gloucester Point, VA 23062; **Gregory A. DeBrosse,** Haskin Shellfish Research Lab, Rutgers University, Port Norris, NJ 08349.

Tetraploids have now been produced in three species *Crassostrea*, with the addition of the Suminoe oyster (*C. ariakensis*) in 2002. Tetraploids are produced by a unique genetic manipulation of eggs from triploids. This presupposes that there will be triploid females with exceptional fecundity. We found 1-10% of 2- and 3-year old triploid C. ariakensis attained a level of fecundity sufficient for accomplishing 21 tetraploid spawns in summer 2002 resulting in 12 that yielded spat. Number of spat obtained ranged from about 30 to 4800; the proportion of tetraploids ranged from 0% (one case) to 90%, averaging about 65%. Fecundity of triploids ranged from 1.25M to 75.2M eggs. Average time in culture to first eyed larvae was 15 days, ranging from 14 to 18 days. Eyed larvae attained exceptional size before setting, averaging 424 μm, compared with 340 μm in diploid *C. ariakensis* and 390 μm in triploids. We also experimented with decreased dosage of cytochalasin B. Half the working dose (0.25 μg/ml) worked as well as our typical working dose (0.50 μg/ml) as indicated by increased initial survival (6.5% and3.2%, respectively) and the same percentage tetraploidy (74% and 76%, respectively).

GROUND TRUTHING HYDROACOUSTIC DATA WITH COMMERCIAL OYSTER DREDGING. Yvonne C. Allen, Charles A. Wilson, Harry Roberts, John Supan, Buddy Pausina, Coastal Fisheries Institute Louisiana State University Baton Rouge, LA 70803.

Traditional methods used to assess oyster reef distribution and condition are only able to provide subjective point information, which is often poorly georeferenced. Maps of oyster habitat in shallow waters are therefore typically extremely generalized, giving few details about the true distribution, character, extent and dynamics of reefs. Sidescan sonar offers a significant advantage for quick and accurate assessment of oyster reefs in the turbid waters of coastal Louisiana.

We conducted four years of side scan surveys over the same area of oyster reef in south Louisiana. We compared the resulting imagery to the volume of shell from quadrant sampling and were able to establish a strong quantitative relationship. In the summer of 2002, we integrated dredge sampling into our ground truthing efforts in the hope of making our results more relevant for the oyster industry. We found a similar relationship between shell present in dredge samples and pixel intensity. There is a very promising future in using sidescan sonar and GIS to monitor a productive oyster lease. This combination of tools will be very powerful in helping the oyster grower to plant, harvest, monitor and track changes to the lease - focusing efforts on productive areas.

OYSTER IRRADIATION: PATHOGENIC *VIBRIO* RESPONSE AND CONSUMER DIFFERENCE TESTING. L.S. Andrews*, B. Posadas, D. Burrage, Michael Jahncke. Coastal Research and Extension Center Mississippi State University 2710 Beach Blvd. Suite 1E, Biloxi, MS 39531.

Pathogenic strains of Vibrio (*Vibrio vulnificus* and *V. parahaemolyticus*), although natural inhabitants of estuarine and ocean environments, can cause serious illness and death in susceptible persons when consumed along with raw half-shell oysters.

Objectives of this study were 1) establish the irradiation dose needed to reduce pathogenic *Vibrios* to nondetectable levels and 2) determine consumer's ability to differentiate between irradiated and control oysters.

Live oysters, with naturally incurred and artificially inoculated pathogenic *Vibrios*, were exposed to 0–3 kGy dose Cobalt-60 gamma radiation for microbial response. Consumer volunteers were asked to determine differences between treated (1 kGy) and untreated oysters by triangle difference testing.

Vibrio vulnificus (MO-624) was reduced from 106 cfu/g oyster meat to nondetectable levels (<3 mpn/g oyster meat) at a dose of 0.75 kGy. *Vibrio parahaemolyticus*, 03:K6 (TX-2103), required 1.0 kGy for reduction to nondetectable levels. Sensory triangle difference tests by 146 volunteers resulted in confirmation that consumers, many of whom work in the seafood industry, could not distinguish between control and irradiated oysters (p <0.01).

RESPONSE OF *VIBRIO VULNIFICUS* AND *V. PARAHAEMOLYTICUS* 03:K6. Linda S. Andrews* and Susan DeBlanc. Mississippi State University, Coastal Research and Extension Center, 2710 Beach Blvd., Suite 1E, Biloxi, MS 39531.

Vibrio vulnificus and *V. parahaemolyticus* are natural inhabitants of estuarine environments world wide. Pathogenic strains of these bacteria can cause serious illness and death in susceptible persons when consumed along with raw half-shell oysters.

Objectives of this study were to determine the time/temperature parameters needed to reduce *Vibrios* in shell stock oysters to nondetectable levels (<3 mpn/g oyster meat) using hot water pasteurization followed by cold shock. Secondly, sensory evaluation studies were conducted to determine sensory changes associated with the process.

Oysters containing naturally incurred *Vibrio* and artificially contaminated pathogenic strains of *V.vulnificus* (MO624) and *V. parahaemolyticus* 03:K6 (TX2071) (106 cfu/g oyster meat) were pasteurized at 52o C for up to 22 minutes. Oysters, for sensory testing were harvested during the winter months and also processed in 52o C circulating water bath for up to 22 minutes.

Pathogenic strains of V. v. and V.p.O3:K6 proved to be more process resistant than nonpathogenic environmental strains found in Gulf of Mexico waters. High levels of *Vibrio* (106 V.v. and V.p 03:K6 cfu/g oyster meat) were successfully reduced to nondetectable levels (<3 mpn) when internal oyster temperature achieved

>500C for 10 min. Processing at this T/T did not adversely affect the sensory qualities consumers expect in raw half-shell oysters.

POPULATION COLLAPSE, DEPENSATION EFFECTS, AND THE TIME-SCALE OF RECOVERY OF HARD CLAM (*MERCENARIA* SPP.) FISHERIES. William S. Arnold*, Florida Fish & Wildlife Conservation Commission Marine Research Institute 100 8th Avenue SE St. Petersburg, FL 33701.

The commercial fishery for naturally occurring hard clams has a brief but eventful history in Florida waters. The first known fishery, initiated in the early 1900's on the southwest coast of the state, constituted one of the largest hard clam fisheries on record. The population that supported that fishery collapsed in the 1940s ` and has never recovered. Smaller fisheries developed in the early 1980's and the early 1990's in the Indian River on the Florida east coast. Those fisheries also collapsed, apparently in response to freshwater inputs, and similarly have not recovered. The observed lack of recovery of any of those populations may result from depensation effects at low population density. Recovery may be protracted even with intervention. An alternative management approach, taking into account the vagaries of hard clam recruitment and population survival, is proposed for consideration.

INFLUENCE OF CONGENERIC AQUACULTURE ON HARD CLAM (*MERCENARIA* SPP.) POPULATION GENETIC STRUCTURE. William S. Arnold*, Sarah L. Walters, Sarah C. Peters, Theresa M. Bert, Jon S. Fajans. Florida Fish & Wildlife Conservation Commission Marine Research Institute 100 8th Avenue SE St. Petersburg FL 33701.

An aquaculture-based hard clam industry is developing on the west coast of Florida. There, the species *Mercenaria campechiensis* predominates in the natural clam population whereas *M. mercenaria* is the predominant species utilized by the industry. The species hybridize extensively, and this study was conducted to measure the genetic impact of *M. mercenaria* aquaculture on the natural population of *M. campechiensis* near Cedar Key, Florida. Clams (N = 257) were analyzed for genetic composition, age, and the presence and stage of gonadal neoplasia. Results indicate that the genetic composition of the clam population has changed since the 1993 advent of aquaculture. *Mercenaria mercenaria* were nonexistent prior to the initiation of aquaculture but increased in abundance post-aquaculture, as did hybrid clams. There was no difference in abundance of *M. campechiensis* pre- vs. post-aquaculture. All taxa exhibited a high incidence (> 80%) of gonadal neoplasia, but it is not clear if this high incidence results from the introduction of aquaculture or if neoplasia predates that introduction. These results indicate that *Mercenaria* culture can influence naturally occurring congeneric populations in the vicinity of the culture operation, although the long-term implications of that influence remain to be seen.

IN SITU DETERMINATION OF *PERKINSUS MARINUS* TRANSMISSION DYNAMICS.

Corinne Audemard*, Lisa M. Ragone Calvo, Kimberly S. Reece, Eugene M. Burreson, Kennedy T. Paynter, Virginia Institute of Marine Science, The College of William and Mary, Gloucester Point, Virginia 23062.

Dermo disease, caused by the protozoan parasite *Perkinsus marinus*, is currently the most widespread and lethal infectious disease of the oyster, *Crassostrea virginica*. During the last decade, it has spread into low salinity areas raising questions about parasite transmission dynamic. Our objective is to determine the transmission dynamics of *P. marinus* in low to moderate salinity areas. The functional relationship between disease-related mortality, ambient parasite abundance, and infection acquisition by naive oysters was examined in three Chesapeake Bay tributaries-the Magothy, Patuxent, and James Rivers. From June through October 2002, water samples were collected weekly and parasite cell numbers were quantified using real-time PCR. Concurrently, on a monthly basis, naive sentinel oysters were deployed and monitored for *P. marinus* acquisition and local oysters were monitored for mortality and infection levels. The three studied rivers showed very different *P. marinus* abundance: the high salinity site in the James river showed up to 3000 cells/l, whereas the Patuxent site showed less than 20 cells/l during the whole study and, the Magothy showed no parasite detection. These abundances and *P. marinus* incidence in sentinel oysters were significantly correlated with mortality of local oyster population and with salinity.

FUNCTIONAL GENOMICS: A POWERFUL APPROACH TO STUDY THE IMMUNE RESPONSE OF THE PACIFIC OYSTER *CRASSOSTREA GIGAS*.

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Most of knowledge on oyster innate immunity is based on biological activities, and molecular features of immune effectors remain largely unknown. To progress in oyster immune gene characterization we generated expressed sequence tags (ESTs) from a hemocyte cDNA library built from *Crassostrea gigas* subjected to bacterial challenge. A total of 1142 randomly selected clones were single-pass sequenced. According to sequence similarities, a putative function could be assigned to 54% of the clones (for more details, visit the database web site http://www.ifremer.fr/GigasBase). Among them, 20 genes potentially involved in immunity were identified. To investigate the expression pattern of these genes, cDNA arrays were developed. Oysters were experimentally injected with several *Vibrio* strains isolated from moribund animals during mortality outbreaks, and gene expression was compared with unchallenged animals. First results showed that some of these genes were over-expressed after bacterial challenge suggesting their involvement in defense mechanisms. Likewise, cDNA arrays were designed with cDNAs encoding proteins involved in physiological functions such as immunity, wound healing, cell proliferation or cell motility, in order to assess the effect of environmental stresses on oyster health.

BIOLOGICAL ASSESSMENT OF STORM EFFECTS ON THE LOUISIANA PUBLIC OYSTER RESOURCE: TROPICAL STORM ISIDORE AND HURRICANE LILI.

Patrick D. Banks, P.O. Box 98000 Baton Rouge, LA 70898.

Effects of Tropical Storm Isidore and Hurricane Lili on Louisiana's public oyster resource were determined using a combination of square-meter and dredge sampling. Pre and post storm samples were statistically analyzed for differences in percent mortality and density of oysters (*Crassostrea virginica*). Results were discussed in relation to environmental parameters such as salinity, precipitation, and storm surge. Although percent mortality of oysters in square meter samples significantly increased on some public oyster grounds following the storms, it was generally < 40%. Oyster density data from square meter samples yielded mixed results and dredge samples indicated a slight increase in percent mortality of oysters in the Lake Pontchartrain basin following the storms. Negative effects of Tropical Storm Isidore and Hurricane Lili on the public oyster resource exhibited large spatial variation (likely due, in part, to extensive spatial variation of Louisiana's oyster habitat) with significant effects only occurring on some of the public grounds sampled.

CLEARANCE RATES AND FEEDING SELECTIVITY OF *CRASSOSTREA VIRGINICA* AND *MERCENARIA MERCENARIA*; IMPLICATIONS OF INCREASED EUTROPHICATION IN THE SUWANNEE RIVER ESTUARY.

Carla D. Beals* and Shirley Baker, Department of Fisheries and Aquatic Sciences, University of Florida, 7922 NW 71st Street, Gainesville, FL 32653.

The objective of this study is to examine the potential effects of increased eutrophication of the Suwannee River Estuary on the feeding biology of clams and oysters. My hypotheses are that 1) the presence or absence of particular phytoplankton species will affect bivalve clearance rates; and 2) bloom concentrations of some phytoplankton species will reduce the particle selection and clearance rates of bivalves. Oysters and clams collected from the Estuary will be subjected to two concentrations of plankton (average and Suwannee bloom densities) and four types of plankton assemblages: 1) natural phytoplankton, 2) monospecific cultures of phytoplankton (not included in selectivity experiments), 3) laboratory-manipulated phytoplankton assemblages, and 4) phytoplankton and micro-zooplankton combinations. Changes in clearance rate or particle selection ability will have implications for the future productivity of clams and oysters in the Suwannee River Estuary.

ENGINEERING AND ECONOMICS AS RELATED TO OYSTERS GROWN IN THE GULF OF MEXICO. Donald L. Bishop, Bishop Aquatic Technologies Inc., P.O. Box 669, 110-B Bonnechere St. Eganville, Ontario, Canada. K0J 1X0 / Engineering and Economics as Related to Oysters Grown in the Gulf of Mexico.

Globally the demand for a quality, safe, consistently available shellfish continues to outpace production. To deliver to the current consumers as well as to yet to be developed markets will take the focused involvement of biological, engineering and business planning aspects to further develop the industry. Currently there is little correlation between the individuals that specialize in these areas, yet solutions have been implemented and proven by a small minority of shellfish producers that understand how to match together these dynamics. Husbandry technologies have been developed that allow for the control of shell shape, appearance, size, meat yield and even shelf life pre harvest. Unfortunately many within the scientific community are unfamiliar with this area. These technologies also input control to bio fouling, and production management further enhancing yield and profitability. Economics related to new technologies with return on invested capital per acre per year are significant. In the past economic models have been created for the oyster industry based on past input, output and cost of operation numbers. New husbandry technology and processes change this processes significantly, a discussion relating these together with physical and biological aspects will be covered.

PERSISTENCE OF ATRAZINE IMPACT ON ANEUPLOIDY IN THE PACIFIC OYSTER, *CRASSOSTREA GIGAS*. Karine Bouilly*, Helen McCombie, Alexandra Leitão, and Sylvie Lapègue, IFREMER, Laboratoire de Génétique et Pathologie, Avenue de Mus de Loup, 17390 La Tremblade, France.

Aneuploidy is the alteration of the normal diploid chromosome number. In the Pacific oyster, *Crassostrea gigas*, *hypodiploid aneuploid* cells have regularly been reported as has a negative correlation between this phenomenon and growth and evidence for a genetic basis. We previously demonstrated a positive relationship between a pollutant, atrazine, and aneuploidy in *Crassostrea gigas* adults and juveniles. To evaluate the persistence of this impact, the present study focused on a sample of the same juveniles exposed to different atrazine treatments (0.01 mg/l which represents a peak value found in a polluted environment and 0.1 mg/l) for three and a half months and evaluated them for aneuploidy after another two and a half months in non polluted conditions. Their aneuploidy level remained significantly different between the treatments applied. In addition, our study examined the offspring of the same adult population previously treated and found that these offspring exhibited significantly higher aneuploidy levels when the parents had been exposed to atrazine. These results demonstrate the persistence of the atrazine impact on Pacific oyster aneuploidy in time

within and between generations and also support the genetic basis previously found for this phenomenon.

IMPACT OF AN INVASIVE TUNICATE IN ATLANTIC CANADA: RECRUITMENT AND COMPETITION. Daniel Bourque*, Thomas Landry DFO, P.O. Box 5030, Moncton, New Brunswick, E1C 9B6. Jeff Davidson, University of Prince Edward Island, 550 University Avenue, Charlottetown, Prince Edward Island, C1A 4P3; Neil McNair, PEI Department of FAE, P.O. Box 2000, Charlottetown, Prince Edward Island C1A 7N8.

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. *S. clava* has had a negative impact on mussel culture, attaching in high densities to mussel socks and equipment, competing for food resources and fouling equipment. This tunicate is spreading rapidly in the waters of PEI and seems to be mainly from anthropological mode as opposed to natural mode. Recruitment, abundance and growth of *S. clava* were studied on a temporal scale. The impact of this new fouling organism was investigated by evaluating its competition for food in relation to the mussels. 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 SIMULATION MODEL OF THE POPULATION GROWTH OF HARD CLAMS (*MERCENARIA MERCENARIA*). III. EFFECTS OF BROWN TIDE. V. M. Bricelj*, J. N. Kraeuter, E. N. Powell, J. M. Klinck, E. E. Hofmann, R. Grizzle, and S. Buckner, Institute for Marine Biosciences National Research Council Canada 1411 Oxford Street Halifax, NS B3H 3Z1.

Brown tides of *Aureococcus anophagefferens* have occurred in Great South Bay, NY, since the mid-1980's. Peak concentrations usually occurs in June or July and have been attributed a role in the decline of hard clam populations. Using a physiologically-based model, simulations were run to examine the effect on clam population growth by a) timing of blooms, b) *Aureococcus* concentration (105 to 2x106 cells ml-1), c) bloom duration, and d) food supply. Brown tide effects were incorporated into the model by assuming dose-dependent feeding inhibition of juveniles/adults. A brown tide-induced, larval mortality function was generated based on laboratory results obtained with bay scallop larvae. Sensitivity of the model output to variation in larval mortality was assessed. Effects of a time-dependent, juvenile mortality function, based on published data for 2 mm clams which experienced high moralities after prolonged (4-wk) exposure to high brown tide levels (4x105 cells ml-1) were also tested. Preliminary results of a modeled brown tide effects on individual scope for growth and egg produc-

tion over a clam's lifespan for individuals varying in initial body size and genotype will be presented.

DETERRENTS TO BLACK DRUM PREDATION ON OYSTER LEASES. Kenneth M. Brown*, Gary Peterson, Mike McDonough, Patrick Banks, and Brian Lezina, Department of Biological Sciences, Louisiana State University, Baton Rouge, Louisiana 70803.

Preliminary experiments indicated large black drum were effective predators, and small oysters were preferred, but that salinity did not affect feeding. Further laboratory experiments indicated scent of dead black drum (as hypothesized by lease holders) did not lower fish feeding. Field experiments in Barataria Bay in 2 seasons indicated scent reduced feeding by 10–20 %, but only at one site in one season. We conclude scent is not effective under most conditions. Mortalities to all predators ranged from 60 % to 90 % within the four weeks on leases. The importance of black drum and southern oyster drills varied among sites, as did temporal patterns of mortality. Fish caused 74 % of mortality, and oyster drills 23 %, and fish and oyster drill predation were inversely related. Laboratory experiments with sound deterrents indicate "drumming" by male black drum in the frequency range of 40–60 Hz does not deter predation. Sounds in the range of 10–30 Hz may deter fish, but are impractical because they require considerable power to broadcast over leases. We hope in future work to determine whether limited gill netting or trot line fishing can decrease oyster mortality to black drum without impacting other fish populations through by-catch.

STRESS RESPONSES IN SCALLOPS AND HARD CLAMS TO HEAT AND COLD SHOCK. Nicole T. Brun*[1,2], V. Monica Bricelj[2], Emmanuel E. Egbosimba[2], Thomas H. MacRae[1], and Neil W. Ross[2], [1]Biology Department, Dalhousie University, Halifax NS B3J 1Z1, [2]National Research Council, Institute for Marine Biosciences, Halifax NS B3H 3Z1.

In response to various stressors, such as temperature, organisms increase production of stress proteins (SPs). Heat shock SP responses have been studied in mussels, but limited information is available for other bivalves. Cold shock Stress Protein Response (SPR) has not been previously investigated. Sea scallops *Placopecten magellanicus*, a deeper water species, and the estuarine bay scallop *Argopecten irradians irradians* differ in temperature tolerance: the former is susceptible to high temperatures, whereas the latter may be more vulnerable to low temperatures. Juvenile hard clams *Mercenaria mercenaria* suffer heavy mortalities during over wintering in Atlantic Canada and the mid-Atlantic US. The SPR to acute heat shock, determined by SP-70, was compared in the two scallop species (10°C increase for 3h). No differences in SP-70 expression were observed in sea scallops, except at 21d,

when levels were significantly lower than initial, control levels. In contrast, SP-70 in bay scallops increased significantly during and following heat shock, attaining a maximum by 12h, and exceeded control levels after 8d-recovery. The SPR of bay scallops and hard clams to acute cold shock (17°C decrease for 3h) was examined to determine if this stressor also modulates SP-70. The latter increased significantly in both bivalves, with levels still increasing after 8d and 24h respectively. The same samples are being analyzed using SP-40. Characterization of the SPR to acute temperature shock may have application in acquired thermo tolerance of bivalves transferred from hatchery to field growout sites.

PERKINSUS CHESAPEAKI AND *PERKINSUS ANDREWSI* ARE THE SAME SPECIES. Eugene M. Burreson*, Kimberly S. Reece, Karen L. Hudson, Christopher F. Dungan. Virginia Institute of Marine Science Gloucester Point, VA 23062.

Perkinsus chesapeaki was described from the soft-shell clam, *Mya arenaria*, and *Perkinsus andrewsi* was described from the Baltic macoma, *Macoma balthica*, both from Chesapeake Bay. Sequence analysis of the internal transcribed spacer region (ITS), the large subunit ribosomal RNA gene and actin genes from clonal *Perkinsus* cultures derived from both hosts revealed that the two species are synonymous. Multiple DNA clones of each region were sequenced from each clonal isolate. Phylogenetic analyses based on all three sequences placed isolates derived from the two different hosts into a monophyletic group. Polymorphisms were detected at each locus and sequence variation was observed within clonal isolates at the multi-copy genes. ITS sequences from each isolate were found in each of two monophyletic sister clades. One clade included the GenBank deposited ITS sequence for *Perkinsus chesapeaki*, and the sister clade included *P. andrewsi* ITS sequences. These results suggest variation observed among ITS sequences of these isolates is representative of polymorphisms within a single parasite species from two different hosts. GenBank deposited *P. chesapeaki* and *P. andrewsi* ITS sequences represent sequence variants from a single *Perkinsus* species. The name *P. chesapeaki* has priority under Article 23 of the International Code of Zoological Nomenclature.

EVALUATING SHELL QUARANTINE DURATION TO LIMIT THE TRANSFER OF *PERKINSUS MARINUS* WHEN PLANTING OYSTER CULTCH. David Bushek*, Donnia Richardson, Yvonne Bobo, Loren Coen and Jennifer Cardinal. Baruch Marine Field Lab, University of South Carolina, PO Box 1630, Georgetown, SC 29442.

Freshly shucked oyster shell can carry harmful organisms such as predators, non-natives or pathogens in remaining tissues. Thus, planting fresh oyster cultch may spread harmful organisms. Decomposing *Perkinsus marinus* infected oyster tissue is a major source of infective stages of *P. marinus*. Therefore, we examined

changes in *P. marinus* abundance in tissues of shucked and whole Gulf Coast oysters deployed in replicate shell piles between March and July 2002 in Charleston, SC. Parasite abundance was determined by RFTM body burden assay, and parasite enlargement in RFTM used to indicate viability. After 31 days, only 13% of shucked oysters contained any tissue and total parasite abundance had declined to 0.05%. No tissues remained in subsequent samples. Tissues decayed much slower in whole oysters, but parasite abundance still declined rapidly with just 1% remaining after only 31 days. After 115 days, only two whole oysters contained any observable tissue and total parasite abundance was a mere 0.005% of the original number. The impact of climate and shell pile configuration should be more closely evaluated, but simply quarantining oyster shell for one month or more on land can dramatically reduce the abundance of *P. marinus,* minimizing the potential for transmission.

HIGH HYDROSTATIC PRESSURE INACTIVATION OF VIRUSES. Kevin R. Calci*, US FDA PO Box 158 Dauphin Island, AL 36528.

Viruses have been epidemically linked to the majority of the illnesses associated with consumption of raw shellfish. The majority of the implicated shellfish were traced back to growing areas in approved status which were thought to have become contaminated by illegal overboard discharges or failures of proximal wastewater treatment facilities. High hydrostatic pressure (HHP) processing is in use commercially to reduce *Vibrio sp.* in shellstock oysters. Investigations are underway, to determine if HHP might serve as a post-harvest treatment process to improve the safety of oysters as related to viruses. Viruses under study include hepatitis A virus and SM-17, a surrogate for Norwalk-Like virus. Oysters (*Crassostrea virginica*) accumulated virus in a flow through seawater system. Shucked meats were packaged in plastic pouches before subjecting to HHP processing. Results show HAV to be the more pressure resistant requiring pressures $\geq$ 400 MPa to achieve a 3 log10 reduction in 1 min. A similar reduction could be achieved with SM-17 in 1 min at 275 MPa. The wide range of pressures required to inactivate different viruses may make it difficult to select a pressure that will be effective in destroying all viral contaminates in oysters without damaging the quality of the oysters.

HOST GENETIC ORIGIN AN IMPORTANT DETERMINANT OF QPX DISEASE. Lisa M. Ragone Calvo*, Gene M. Burreson, Susan E. Ford, John N. Kraeuter, Dale F. Leavitt, Roxanna Smolowitz, Virginia Institute of Marine Science, College of William and Mary, Gloucester Point, VA 23062.

Epizootics of QPX (Quahog Parasite Unknown) a protistan pathogen of hard clams, *Mercenaria mercenaria* have occurred in maritime Canada and Massachusetts, New York, New Jersey, and Virginia, USA. Although it has been found in wild hard clam populations, the parasite has most seriously affected cultured hard clams, suggesting that aquaculture practices may promote or predispose clams to the disease. In this investigation we examined the influence of host genetic origin and geographic location on QPX susceptibility. Five clam strains, originating from Massachusetts, New Jersey, Virginia, South Carolina, and Florida were produced at a single hatchery and evaluated for growth, survival, and QPX susceptibility at three QPX endemic sites (Massachusetts, New Jersey and Virginia). Severe winter-associated clam losses occurred at the Massachusetts site precluding completion of the study at that location. At both the New Jersey and Virginia sites the South Carolina and Florida clam stocks exhibited significantly higher QPX prevalences and lower survival than the New Jersey and Massachusetts clam stocks; while clams from Virginia had QPX prevalences and survival rates that were intermediate to the more " northern" and " southern" clam stocks. These results suggest that genotype-environment interactions are important determinants of QPX disease.

EXPERIMENTAL EVALUATION OF CROSSES WITHIN AND AMONG FIVE COMMERCIAL STRAINS OF HARD CLAMS, *MERCENARIA MERCENARIA,* ACROSS A SALINITY GRADIENT IN VIRGINIA WATERS. Mark D. Camara*, Standish K. Allen Jr, Aquaculture Genetics and Breeding Technology Center Virginia Institute of Marine Science PO Box 1346 Gloucester Point, VA 23062.

Cultured *Mercenaria mercenaria* are a multi-million dollar industry in Virginia. Grow-out sites vary from ocean salinity outside to mid-salinity estuarine sites inside the Chesapeake Bay. Presently, the industry uses essentially undomesticated genetic stocks, and we know very little about the suitability of stocks to varying environmental conditions. We evaluated the genetic influence of hard clam strain selection on growth along a salinity gradient in Virginia as well as the potential for enhancing production by outcrossing available strains. We first created all fifteen possible combinations within and among five brood stock strains in the hatchery. We subsequently raised the juveniles in common conditions until they reached approximately 10 mm, at which point we split the groups for planting at five sites encompassing the range of salinities at which clams are grown. We measured them and compared the growth of these groups in the hatchery, nursery, and field, estimated the correlations among the performance measures between life stages, compared the performance of within- and among-strain crosses, and assessed site-specifity. We discuss the results and their implications for strain selection, hatchery spawning procedures, and future efforts in selective breeding for superior hard clam strains.

GROWTH OF QUAHOGS (*MERCENARIA MERCENARIA*) AND SOFTSHELL CLAMS (*MYA ARENARIA*) IN RESPONSE TO EUTROPHIC-DRIVEN CHANGES IN FOOD SUPPLY AND HABITAT. **Ruth H. Carmichael*, Andrea C. Shriver, Erica T. Weiss**, and **Ivan Valiela**, Boston University Marine Program, Marine Biological Laboratory, 7 MBL Street, Woods Hole, MA 02543.

In recent years increased urbanization has increased nitrogen loads to coastal estuaries, prompting eutrophication and changing estuarine features. Increased N loads increase phytoplankton and microphytobenthos concentrations, result in accumulation of organic matter from detritus of algae, reduce sediment and water column oxygen content, and may change sediment composition. These changes likely affect growth and survival of commercially important bivalves like quahogs and soft-shell clams. To determine how eutrophication-related changes affect these bivalves, we transplanted juveniles into estuaries of different land-derived N loads, measured changes in sediment and water column properties, and recorded growth and survival of bivalves. We used N stable isotopes to link responses of bivalves to their food supply and land-derived sources of N for management. We found growth rates of quahogs and soft-shell clams increased as land-derived N loads increased their food supply. Water column food sources had a greater effect on growth than sediment sources, and low salinity and high particulate organic matter may have limited growth in some areas despite increased food supply. N stable isotope analysis linked these growth responses to land-derived N primarily from wastewater sources.

DEVELOPMENT OF A SINGLE NUCLEOTIDE POLYMORPHISM (SNP) MARKER SET FOR THE HARD CLAM, *MERCENARIA MERCENARIA*. **Ryan B. Carnegie*, Mark D. Camara, Lisa M. Ragone Calvo, Kimberly S. Reece**, and **Patrick M. Gaffney**, Virginia Institute of Marine Science P.O. Box 1346 Gloucester Point, VA 23062.

In aquaculture, molecular genetic markers can be used to evaluate the diversity of wild shellfish stocks to be introduced into hatchery breeding programs, to control pedigrees in hatchery lines, and to track the performance of outplanted seed. While progress has been made in developing molecular markers for *Crassostrea spp.*, the hard clam *Mercenaria mercenaria*, an extremely valuable commercial species in eastern North America, has received relatively little attention. Our objective was to develop a set of single nucleotide polymorphism markers (SNPs) for *M. mercenaria*. Hemocyte and mantle complementary DNA (cDNA) libraries were created in plasmid vectors and then sequenced. Screening for SNPs is being done using a panel of clams encompassing the genetic diversity of VIMS hatchery stocks and reflecting the wide geographic distribution of *M. mercenaria*. SNPs demonstrating Mendelian inheritance will be immediately useful for evaluating the relative performance of clams produced from Massachusetts,

New Jersey, Virginia, and South Carolina broodstock that are now deployed at two QPX-enzootic and three QPX-free sites in Virginia. The markers will also be useful for characterizing wild *M. mercenaria* germplasm diversity, and may begin to reveal allelic variation underpinning the variable susceptibility of East Coast clams to QPX.

TROPHIC INTERACTION BETWEEN HARD CLAMS AND NATURAL ASSEMBLAGES OF PLANKTON. **Robert M. Cerrato*, Amy E. Streck**, and **Darcy J. Lonsdale**, Marine Sciences Research Center Stony Brook University Stony Brook, NY 11794-5000.

To examine whether intensive grazing by hard clams or copepods shifts the composition of the plankton community toward species of different nutritional quality, we conducted experiments in 400-liter tanks at three locations in Great South Bay, NY. Treatments were created by varying adult clam and copepod abundances. After a 2-week acclimation period, several juvenile (2 mm) clams were added to each tank and allowed to grow for 4 weeks. In one location, where growth under ambient conditions was high, juvenile growth declined by 57% in the treatment with high adult clam grazing, suggesting that juveniles and adults were competing. In the other two locations, where growth under ambient conditions was moderate to poor, juvenile growth improved by 60 to 200% in treatments with high adult clam grazing. Plankton composition was altered in the high adult copepod treatments, but no effect on juvenile hard clam growth was observed. Examination of clearance and assimilation rates of naïve clams exposed to treatment water indicated that observed increases in juvenile clam growth were related to food quality rather than quantity. Our results suggest that intense grazing by hard clams can have a positive effect on the nutritional value of the plankton.

PRESENCE OF PATHOGENIC BACTERIA IN THE LAGOON SYSTEMS LA MANCHA AND ALVARADO VERACRUZ, MEXICO IN WATER AND OYSTER (*CRASSOSTREA VIRGINICA*). **Maria del Refugio Castañeda Chávez*, Erasmo Orrantia B., Violeta Pardio Sedas, Fabiola Lango Reynoso**, Carr. Veracruz-Cordoba Km 12 C.P. 94290, Boca del, Ver. México.

Mexico maintains the 6th place in world-wide oyster production, contributing the Gulf of Mexico with 76% of the total volume. In this coastal area, 30 and 36 sampling stations were established in the coastal lagoons of Alvarado, and La Mancha. Water and oyster samples were taken during one annual cycle, and microbiological analysis were performed to determine according to the Mexican Official Norm NOM-031-SSA1-1993. Three stocks of pathogenic *vibrios* were isolated from water samples of Lagoon of Alvarado, *Vibrio alginolyticus, V. cholerae* (INABA) and *V. cholerae* No-01, besides Salmonellas and total coliforms. The *V.*

cholerae serotype INABA was reported in Alvarado during the months of July, August and September. The *V. alginolyticus* was reported in January. *V. cholerae* No-01 was reported in the La Mancha during the rainy season exclusively. Analysis for *V. cholerae* no-01 from oyster samples of the Alvarado is not significantly different to those reported from the oyster banks of La Mancha. It was concluded that fecal discharges is the main cause of pollution representing a health problem that must be considered due to the possibility of survival of microorganisms when oysters are raw consumed and not subjected to depuration.

MUSSEL GROWTH AND FOOD UTILIZATION IN RELATION TO WATER COLUMN CONDITIONS ON RAFT SYSTEMS IN PUGET SOUND, WASHINGTON.

Daniel P. Cheney*, Andrew D. Suhrbier, Aimee E. Christy, Hector S. Beltran, Jonathan P. Davis, Kenneth M. Brooks, and Frank J. Smith. 120 State Ave. NE #142 Olympia, WA 98501.

Suspended mussel and oyster culture on the U.S. west coast is predicted to increase significantly in coming years. Description of the changes associated with the culture of these crops is essential for the siting and evaluation of new culture facilities and in improving yield and production of existing facilities. This research had three general objectives: 1) to assess at large-scale farm sites, mussel growth and yield against a suite of measured physical, chemical and biological variables; 2) to compare the same suite of variables with measurements of mussel feeding and biodeposit production; and 3) to utilize available nutrient and yield models to estimate potential mussel carrying capacity in the farming area. During a two year period (2001-03), multiple observations were made of water currents, water chemistry, phytoplankton, mussel growth, seston removal and absorption, fouling, and fish utilization at commercial mussel raft culture sites in Totten Inlet and Penn Cove, Washington. Although parameters, such as water currents and phytoplankton abundance varied markedly inside and outside the raft units and under different tidal regimes, these effects were localized and did not correlate with mussel growth. This research is supported by a Sea Grant National Marine Aquaculture Initiative grant.

ASSESSING THE EFFECT OF HABITAT ALTERATION ON SHELLFISH POPULATIONS.

Marnita M. Chintala*, U.S. EPA, NHEERL, Atlantic Ecology Division, Narragansett, RI 02882; and Karin A. Tammi, NOAA/R.I. Dept. of Environmental Management, Narragansett, RI 02882.

Habitat provides a variety of life support functions for many species, such as providing shelter, substrate, food, and nursery areas. Habitat alteration is one of the most important causes of declines in ecological resources in North America, and habitats essential to the well being of shellfish species are rapidly being affected by many land-use activities. As a result, many restoration efforts have been conducted to restore many of the altered areas back to their original habitat value. What is not always clear is how to define the value of a habitat to a particular species of interest. This information is important to assess the impacts of habitat alteration on species that utilize those areas. The impacts of these types of alterations to the critical life support functions of shellfish populations will be reviewed. A characterization of the habitat conditions that support the survival and continued viability of shellfish populations is needed to properly assess habitat alteration and evaluate the success of restoration efforts.

DESIGN AND IMPLEMENTATION OF A SURVEY OF COMMERCIAL BLUE CRAB EFFORT IN THE MARYLAND PORTION OF THE CHESAPEAKE BAY.

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The Maryland Department of Natural Resources (MD DNR, requires estimates of the fishing effort expended by commercial crab fisheries in the Chesapeake Bay. We designed a three-prong approach to obtaining instantaneous estimates of effort in the bay. We collected field data on the commercial pot and trotline crab fisheries, and telephone surveys for supplementary information. Sampling included ~160 stratified random transects for pots each month to obtain estimates of spatially explicit pot densities. Survey stations were modified transects; planar boards were used to delineate the width of each transect. The trotline surveys were performed using both aerial flyovers and roving intercept surveys to quantify the mean number of lines per boat and mean trotline length. We describe methods for merging this information in ways that can be used to estimate effort for similar fisheries.

AN INTEGRATED APPROACH TO BIVALVE DOMESTICATION: INTRODUCTORY REMARKS.

Fu-Lin E. Chu*, Virginia Institute of Marine Science, College of William and Mary, Gloucester Point, VA 23062; Jean-Francois Samain*, Ifremer Centre de Brest, BP 70, 29820, Plouzané, France.

Environmental and disease stresses are worldwide problems and have caused severe mortality in many cultivated and feral bivalve populations. For years, scientists in France and US have devoted time and effort in an attempt to improve the yields via multi-disciplinary research. To coordinate activities of researchers from various scientific disciplines in US and France, a US-France Workshop on "Domestication of bivalve molluscan shellfish" was held in La Tremblade, France, 2002. Via the meeting several short-term US-France collaborative projects have been developed. To accelerate information and technology exchange, ideas for future technical workshops have been established. Currently a five-year

multi-disciplinary research project on *Crassostrea gigas* summer mortality is being conducted in France. Six disciplines are contributing together to test the hypothesis of a complex interaction between oyster, environment and opportunistic pathogens. The study focuses on mortality dynamics in the field and determines the relative role of different putative factors in contributing to the mortality.

EVALUATING THE IMPACTS OF HARVESTING PRACTICES, BOAT WAKES AND ASSOCIATED SHORELINE EROSION ON INTERTIDAL CREEK HABITATS IN THE SOUTHEASTERN U.S.: MANAGERS AND RESTORATION PROGRAMS TAKE NOTE. Loren D. Coen* and Majbritt Bolton-Warberg, Marine Resources Research Institute, SCDNR, 217 Fort Johnson Rd., Charleston, SC 29412 and Graduate Marine Biology Program, Grice Marine Lab, College of Charleston, 205 Fort Johnson Rd., Charleston, SC, 29412.

In areas where oysters are intertidal and fringe marsh-lined creeks, they can act as shoreline 'stabilizers'. Recent work (FL, SC, and NC) suggests that harvesting and boating, in addition to natural phenomena, can significantly impact natural intertidal habitats and restoration/enhancement efforts. We assessed oyster populations prior to applied treatments, evaluating the direct impacts of four common harvesting practices on oyster population recovery at 12 sites, paired with controls. Concurrently, recruitment, survival, and growth were also examined annually and populations reassessed ~3 years later to evaluate 'recovery'. Simulated boat wake experiments used shell treatments (with and without mesh) to evaluate impacts of wakes on restoration efforts. Results are discussed and current larger-scale study designs applying our findings are summarized. Four study sites were established in 1999 to measure shoreline erosion. Over 25-38mo, rates ranged from ~0-23cm/month; overall bank losses were from 69-154 cm. In 2001, we expanded sampling at nine additional sites using our SCORE program. Erosion rates (4-16mo.) ranged from ~2-8cm/month, with overall losses from 13-104cm. These and other results suggest that anthropogenic impacts may be having much greater impacts on critical intertidal habitats than previously perceived.

HISTORY OF POST-HARVEST TREATMENT TO REDUCE *VIBRIO* SP. IN SHELLFISH. David W. Cook, Food & Drug Administration Gulf Coast Seafood Lab P.O. Box 158 Dauphin Island, AL 36528.

Vibrio vulnificus was first recognized as the cause of primary septicemia in humans and its relationships to shellfish consumption established in the early 1970's. *V. vulnificus* is a naturally occurring pathogen and it densities in shellfish at harvest are related to growing water temperature. To control illnesses caused by this bacterial species, several approaches including time-temperature controls, harvest restrictions and consumer education have been initiated. Research into post-harvest processing mitigation strategies to reduce *Vibrio* numbers without destroying the raw characteristic of the shellfish was undertaken. In 1996, the first commercial post-harvest treatment process, a mild heat treatment, was recognized as capable of reducing *V. vulnificus* in shellfish to a non-detectable level. Two other processes, freezing and high-hydrostatic pressure processing, were validated in 2002 by commercial processors. Other processes under study are depuration, relaying of shellfish to waters free of *V. vulnificus* and irradiation. Post-harvest processes for reducing *V. parahaemolyticus* in shellfish to non-detectable levels are also being validated.

FRESHWATER PEARL CULTURE AND PRODUCTION IN CHINA. Hua Dan*, Freshwater Fisheries Research Center (FFRC), Chinese Academy of Fisheries Sciences, Wuxi City 214081, Jiangsu Province, CHINA

Lustrous pearls have been called the queen of jewels, but the occurrence of quality pearls in wild mussels is rare. The technologies of freshwater pearl culture were developed in China some 2,000 years ago. However, commercial pearl culture dates back only to the late 1960s. Gradual changes in technology and in the type of mussel used (*Hyriopsis cumingii*), resulted in the production of greater quantities of larger and more lustrous round, near-round, and baroque cultured pearls of various colors. Today, there is a great demand for cultured freshwater pearls, and China produces 95% of those pearls sold in the world market. China produces an estimated 800 to 1000 metric tons of freshwater cultured pearls annually, of which roughly 400 to 500 metric tons are exported to different continents and countries worldwide. Pearls 8 mm and larger represent a large percentage of those exported. This presentation will review the techniques of freshwater pearl culture in China, to include principles of pearl formation, mussel operation procedures, and mussel culture post-implantation.

POPULATION GENETICS OF THE BLUE CRAB (*CALLINECTES SAPIDUS*) IN THE GULF OF MEXICO. Richard L. Darden* and Brian R. Kreiser, Department of Biological Sciences, University of Southern Mississippi, Hattiesburg, MS 39406.

Gene flow among populations of the blue crab (*Callinectes sapidus*) is determined by larval dispersal and adult crab movements. Assessment of population genetic structure allows inferences about historic and contemporary patterns of gene flow. A total of 1,920 crabs were collected from 26 locations around the Gulf of Mexico coast between Naples, Florida and Brownsville, Texas during 2001-02. A 650-base pair portion of the mitochondrial cytochrome oxidase 1 (COI) gene was amplified and sequenced for individuals from each location. Preliminary results seem to indicate that Gulf of Mexico blue crab populations are not

genetically homogeneous. We will place these results into the context of blue crab life history as well as prevailing theories concerning blue crab dispersal and migration.

between cumulative mortality and burden of pathological conditions was significant.

GROWTH AND MORTALITY OF DIFFERENT *OSTREA EDULIS* STOCKS CULTURED IN THE RÍA DE AROUSA (GALICIA, NW SPAIN). Patricia M. da Silva*, Antonio Villalba, and José Fuentes, Centro de Investigacións Mariñas, Aptdo. 13, 36620 Vilanova de Arousa, Spain.

Nowadays, Bonamiosis is the most important constraint for the Galician oyster industry. The development of a disease-resistant stock by a selective breeding program seems a promising measure. Oysters harvested from four genetically different populations were used as broodstock to obtain 5 families from each stock in a hatchery. Two of these stocks were obtained from two *B. ostreae*-free areas in Ireland and Greece, and the other two from Coroso and Ortigueira, two Galician areas where the parasite is present. Spat of every family is being cultured in the Ría de Arousa since Sept 2001. Growth and mortality data for one-year culture period are analyzed in this presentation. Results show significant differences in growth and mortality, both among stocks and families. On average, Galician and Greek stocks perform better (faster growth and lower mortality) than the Irish one. However, the importance of the differences detected among families in both variables diminishes the relevance of those among stocks.

DIFFERENCES IN DISEASE SUSCEPTIBILITY AMONG *OSTREA EDULIS* STOCKS CULTURED IN GALICIA (NW SPAIN). Patricia M. da Silva*, Antonio Villalba, María J. Carballal, and José Fuentes, Centro de Investigacións Mariñas, Aptdo. 13, 36620 Vilanova de Arousa, Spain.

Bonamiosis is the bottleneck for Galician oyster industry. A program to develop a *Bonamia ostreae* resistant strain is being performed. Oysters from different populations were selected as broodstock: Ireland and Greece bonamiosis-free areas, and two Galician areas, Ortigueira (bonamiosis is epizootic), and Coroso (low bonamiosis pressure). Five families per stock were transferred to a raft in the Ría de Arousa on September 2001. Mortality is estimated monthly and samples of each family are taken and historically processed. The most prevalent pathological conditions detected until October 2002 were intranuclear inclusions, suggesting viral infection, and disseminated neoplasia. RLO in digestive epithelia and Haplosporidium-like plasmodia were rare. Haemocytic infiltration, granulocytomas and necrosis were also observed. *B. ostreae* was detected in September and October 2002 with very low prevalence, although increment is expected in the second year. Significant differences in the burden of pathologic conditions were detected among stocks and families. Correlation

CROSSBREEDING IN PACIFIC OYSTERS. Joth Davis*, Taylor Shellfish Farms, Quilcene, WA 98376; Dennis Hedgecock, Bodega Marine Laboratory, Bodega Bay, CA.

Intraspecific hybrid lines of Pacific oysters (*Crassostrea gigas*) were made in 2001 by crossing inbred oysters in a full factorial mating design at the Taylor Shellfish Farms bivalve breeding facility in Quilcene, WA. Two cohorts of hybrid oysters were reared from inbred lines produced by the Molluscan Broodstock Program. Families generated from individual pair-matings were reared and set using standard techniques. Seed from individual families was reared in the field in a 2 month replicated experiment to test for differences in yield among hybrid families. Oysters were subsequently redeployed in replicate cages for a 12-month yield trial. Final yield measurements (total count and biomass) made in August 2002 demonstrated a positive correlation between yield at the seed stage and yield in harvest-ready oysters. Inbred lines and hybrid combinations that generated superior yield at both the seed and harvest stages were identified. Stock improvement via crossbreeding emphasizes yield testing at the seed stage to help predict final yield in oyster production, and offers some advantages over the cost and effort associated with traditional selection and breeding programs.

THE EFFECT OF ALGAL TOXINS ON THE ISOLATED VENTRICLE OF THE HARD CLAM, *MERCENARIA MERCENARIA*. Lewis E. Deaton, Biology Department, University of Louisiana at Lafayette, Lafayette, LA 70504.

While many species of algae have been associated with mass mortalities of shellfish, relatively little is known about the specific effects of algal toxins on the organ systems of mollusks. Isolated ventricles in aerated seawater were exposed to varying concentrations of saxitoxin, brevetoxin 2 and brevetoxin 9. Saxitoxin had no effect, even at a concentration of $1 \times 10\text{-}6$ M. Brevetoxin 2 caused a prolonged negative inotropy in the ventricles; the threshold is about $1 \times 10\text{-}9$M and the effect is dose-dependent. Brevetoxin 9 ($1 \times 10\text{-}9$ M) caused a decrease in the amplitude and increase in the diastolic tone; these effects were transitory. Higher doses ($10\text{-}8$, $10\text{-}7$ M) of brevetoxin 9 did not increase the inhibitory effect. The hearts of bivalves are myogenic, and are not affected by the neural Na+ channel blocker, tetrodotoxin. The lack of any effect of saxitoxin is therefore unexceptional. Brevetoxins open Na+ channels; whether this is the mechanism of their inhibition of the *Mercenaria ventricle* will require further study.

GENETIC BASIS OF SUMMER MORTALITY IN JUVENILE CUPPED OYSTERS.

Lionel Dégremont*, Pierre Boudry and Patrick Soletchnick, LGP-LCPC, F-17390 La Tremblade; Edouard Bédier, LCB, F-56470 La Trinité; Michel Ropert, LCN, F-14520 Port-en-Bessin; Arnaud Huvet, Jeanne Moal and Jean François Samain, LPI, F-29280 Plouzané.

The French project "Morest", coordinated by IFREMER, aims to understand the causes of the summer mortalities in *Crassostrea gigas*. In 2001, three sets of families were bred following a nested half-sib mating design. 17 half-sib families (HSF) were obtained in this first generation (G1) and reared in 3 sites. Significant differences in survival were observed among HSF, and some HSF showed high levels of mortality in all sites, clearly indicating a genetic basis for survival. In 2002, a second generation (G2), including divergent selection and inbred lines, was constituted. Monitoring of survival and growth of G2s were the same as in 2001. Significant differences in survival were found between the offspring of the "high" and "low" selected groups and between inbred lines. The high realized heritability for survival indicates that selective breeding programs could efficiently improve survival of juvenile oysters.

MUCIN SECRETIONS AND NACRE DEPOSITION IN THE FORMATION OF PEARLS.

Leonard DiMichele* and Stephan Towers, Department of Wildlife and Fisheries Sciences, Texas A&M University, College Station, TX 77843; Donald Shepherd, Professional Pathology Laboratories, Ltd, P.O. Box 326, Tow, TX 78672

Cultured pearls originate within a pearl-sac formed by the insertion of a nucleus and graft tissue into a surgically created pouch. Within the pouch, the host animal initiates a classical wound healing response and then nacre-secreting cells from the graft proliferate, lining the lumen of the pouch. Maturing pearl-sac epithelia from a freshwater mussel (Unionidae: *Cyrtonaias tampicoensis*) were examined. Mucopolysaccharide secretions gradually increased after 30 days of development. By day 45, all mucins were actively secreted by host epithelia. Although the pearl-sacs were morphologically mature, there was no evidence of calcium secretion. However, natural pearl-sacs in the same mussels exhibited calcium secretions. The various proteins and calcium secretions formed an aragonite - protein laminate (nacre). Using atomic force microscopy and acid extractions, we characterized natural pearls and shell nacre of *Cyrtonaias tampicoensis*. Our results were similar to those reported from several salt water species and were consistent with evidence from Asian freshwater mussels.

IMPACT OF ENVIRONMENTAL AND NUTRITIVE CONDITIONS ON DEFENCE MECHANISMS OF OYSTERS DURING AN ANNUAL CYCLE.

Maryse Delaporte*, Philippe Soudant, Jeanne Moal and Christophe Lambert, Maryse Delaporte Laboratoire de Physiologie des Invertébrés Centre Ifremer de Brest, BP 70 29280 Plouzané (France).

In the frame of MOREST project, a common biological material, resulting of a mixture of different families produced in experimental hatchery, was reared in two different environmental fields: Normandy and Charente. Concomitantly, a pool was conditioned at the Ifremer Argenton hatchery with three different algae levels: 4%, 8% and 12% of algal dry weight per oyster dry weight. During the experiments, five immune parameters were studied in parallel with survival rate and reproductive status (stages and intensity).

Site location, seasonal variations and experimental diet level clearly influenced oyster immune parameters. Hemocyte counts were higher for oysters reared in Normandy than those reared in Charente and in hatchery. Granulocyte percentage was drastically reduced in hatchery conditions compared to in situ conditions. Moreover, hemocyte activities were also affected by the in situ conditions and dietary treatments in relation to reproductive cycle and mortality events. In example, in vitro haemocyte adhesion capacities were more affected by pathogenic *Vibrio* when oysters suffered mortality.

REPRODUCTION IN FLAME SCALLOPS, *LIMA SCABRA SCABRA* (BORN 1778), FROM THE LOWER FLORIDA KEYS.

Angela K. Dukeman*, 100 8th Avenue SE St. Petersburg, FL 33701, Norman J. Blake and William S. Arnold.

Sex ratio, gonadal characteristics, and the reproductive cycle of the flame scallop, *Lima scabra scabra* (Born 1778), collected from Boca Chica Key, FL were investigated over a 21-month period from January 1998 through September 1999. Gametogenic cycles were examined using qualitative and quantitative methods and the results were analyzed within the context of environmental variation. Gamete development was initiated in winter and coincided with cooler water temperature and moderate food concentration. Maximum gamete ripeness and size occurred in late summer, when water temperatures were at maximum values (33 C), and food quantities were increasing (>0.2 ug/l). Both quantitative and qualitative results indicated a clearly defined spawning event that occurred in autumn in association with decreased female gonad size, increased presence of partially spawned, spent, and early gametogenic gonads, rapidly decreased water temperature (~7 degrees), and maximum measured chlorophyll-a concentrations (1 ug/l). Less defined periods of spawning activity occurred in February and June but could not be related to specific changes in environmental conditions. The presence of ripe and partially spawned flame scallops and adequate chlorophyll-a concentrations throughout the year suggests a continuous spawning reproductive strategy, common in tropical marine invertebrates.

IN VITRO PROPAGATION OF *PERKINSUS* SP. PARASITES FROM JAPANESE MANILA CLAMS, *RUDITAPES PHILIPPINARUM.* Christopher F. Dungan*, Maryland DNR, Cooperative Oxford Laboratory, Oxford, MD 21654; Kimberly S. Reece, and Karen L. Hudson, Virginia Institute of Marine Science, Gloucester Point, VA 23062

Perkinsus sp. is destructive parasites of Manila clams, *Ruditapes philippinarum* from Korea, Japan, and Spain, but parasite isolates are not reported from this host. Gills of Japanese Manila clams collected in Gokasho Bay, Mie prefecture were infected by *Perkinsus sp.* parasites at 97% prevalence and moderate infection intensities. Parasite cells in gill and gonad tissue samples were enlarged for 48h at 28C in Ray's fluid thioglycollate medium; then inoculated into DME: Ham's F-12 *Perkinsus sp.* culture medium. Enlarged parasite cells zoosporulated to produce hundreds of motile zoospores, which subsequently gave rise to schizogonic in vitro cell lines that zoosporulated intermittently at low frequency. Four *Perkinsus sp.* isolates were propagated, cryopreserved, and cloned. In vitro cell morphologies and cell cycles of these isolates differed from those reported for other *Perkinsus sp.*, and DNA sequences suggest that at least one of our isolates is genetically distinct from described *Perkinsus* species.

THE ROLE OF HEAT SHOCK PROTEINS IN TOLERANCE TO PARASITIC STRESS IN THE EASTERN OYSTER, *CRASSOSTREA VIRGINICA*. Vincent G. Encomio*; Fu-Lin E. Chu. Virginia Institute of Marine Science, College of William and Mary, Gloucester Point, VA 23062.

Thermal stress could affect disease resistance mechanisms by depressing immune defense and physiological fitness . We are investigating the relationship between heat tolerance and *P. marinus* resistance among Dermo "resistant" and "susceptible" oyster stocks and the role of heat shock proteins (hsps) in protection of oysters from thermal and disease stress. Results revealed that Chesapeake stocks had higher thermal tolerance than Louisiana stocks. Levels of hsp 70 did not vary between these two stocks and only increased slightly as water temperatures increased. No consistent differences in thermal tolerance were found among Chesapeake resistant and susceptible stocks, and a resistant hatchery strain. Exposure of oysters to a sublethal heat shock improved their survivorship when subsequently exposed to a lethal temperature. We are presently examining how induced thermo tolerance and hsps mediate interactions between parasitic and thermal stress in uninfected and *P. marinus* challenged oysters This study is supported by ODRP, Sea Grant, NOAA (Award#: 114926-GL10014, Project# VA-OD-01-05).

REPRODUCTIVE STRATEGY: VARIABILITY OF REPRODUCTIVE PATTERN IN TWO POPULATIONS GENETICALLY DETERMINED OF *CRASSOSTREA GIGAS*. Martha Enríquez-Díaz*, Stéphane Pouvreau, Caroline Fabioux, Yvette Le Coguic, Jean Claude Cochard, Marcel Le Pennec; UMR PE2M, IFREMER; BP70, 29280 Plouzané, France.

In the literature, the reproductive cycle *of C. gigas* has been well described and is generally characterized in three steps: (1) energy storage; (2) gamete development and (3) spawning. But the genetic intra-variability of this cycle has been scarcely investigated in *C. gigas.* During the French MOREST program, a genetic selection based on the survival criteria allowed to obtain a resistant stock (named " R") and a susceptible stock (named " S"). The gametogenic activity of these two stocks was characterized in field (South Brittany, France) on the basis of quantitative histology of the gonad (gonad volume, number and egg size) and by the expression of the vasa gene, specific marker of the germinal cell. Results showed that the reproductive strategy, especially the reproduction effort and the spawning intensity, was strongly different between the two groups and these results suggest that a genetic triggering mechanism might exist for the onset and flexibility of gametogenesis.

HISTOLOGICAL EXAMINATION OF GAMETOGENESIS IN GENETIC TRIPLOID *CRASSOSTREA ARIAKENSIS* IN CHESAPEAKE BAY. A.J. Erskine* and Standish K. Allen, Jr., College of William and Mary, Virginia Institute of Marine Science, P.O. Box 1346, Gloucester Point, VA 23062.

Combating the loss of the oyster resource in Chesapeake Bay has been ongoing for decades. Recently, focus has turned to the non-native Suminoe oyster, *Crassostrea ariakensis* and the possibility of its introduction as reproducing diploid or a triploid for aquaculture only. In field tests, triploid *C. ariakensis* has exhibited high survival, growth, and disease tolerance in Chesapeake Bay. As reported for several other shellfish species, triploidy often results in abnormal or arrested gametogenesis. Documenting the extent of gamete development in triploid *C. ariakensis* is an important biological variable addressing the risk associated with non-native introduction. Nine diploid females and one tetraploid male were used as parents for this triploid spawn. These genetic triploids were deployed at six sites along Chesapeake Bay ranging from low salinity (~13‰) to high salinity (~35‰). Diploid native controls were sampled at each site to track the "normal" cycle of gametogenesis. Paraffin histology of triploids revealed abnormal gamete production typical of triploid. However, a few sites produced unusually mature ova and spermatozoa for triploids. Samples late in the season indicated spawning had occurred in both diploid and triploid males and females.

EFFECTS OF INBREEDING ON PERFORMANCE TRAITS IN PACIFIC OYSTERS (*CRASSOSTREA GIGAS*). Ford Evans*, Sean Matson, John Brake, and Chris Langdon, Hatfield Marine Science Center, Oregon State University, Newport, OR, 97365.

Understanding the effects of inbreeding is critical to the long-term viability of shellfish breeding programs. Inbreeding depression in shellfish is well documented among the offspring of self fed individuals and full-sib crosses. This study was conducted to determine if crossing more distantly related parents would result in measurable inbreeding depression of performance traits in adult raised in a commercial inter-tidal growing environment. Families were created with average estimated inbreeding coefficients (F) of 0, 1/16, and 1/5. Average family yield, individual growth rate, and survival were recorded after the first and second growing seasons. After two growing seasons, significant inbreeding depression of yield and individual growth rate was observed in families with F = 1/16 and F = 1/5. Significant depression of survival at harvest was observed only in families with F = 1/5. These results emphasize the importance of maintaining pedigree records in breeding programs to help avoid the deleterious effects of inbreeding depression, even among crosses of distantly related parents.

OYSTER VASA-LIKE GENE: A SPECIFIC MARKER OF THE GERM CELL LINEAGE IN *CRASSOSTREA GIGAS*. Caroline Fabioux*, Arnaud Huvet, Frederic LeRoux, Marcel LePennec, Jean-Claude Cochard, UMR PE2M, Ifremer, BP70 29280 Plouzane, France.

Identification of physiological mechanisms implied in reproduction of *Crassostrea gigas* is essential to improve control reproduction in hatchery. Origin and developmental pattern of first germ cells in oyster are steel unclear underlying the need of markers for gametogenesis initiation. The vasa gene, isolated from several organisms such as Drosophila, Caenorhabditis, Xenopus or Zebrafish are specifically expressed in germ cells and are essential for gonad differentiation. We isolated and characterized an homologue of the vasa gene in *C. gigas* by RT-PCR. The spatio-temporal expression pattern of vasa gene was established by In Situ Hybridization or real-time PCR. Results showed that vasa is only expressed in germ cells and not in somatic cells. Moreover, vasa appeared differentially expressed during gametogenesis: from high expression in oogonia and spermatogonia to zero in gametes. Oyster Vasa-like gene appeared to be a relevant marker of germ cells for further studies such as the analysis of environmental effect on the kinetic of gametogenesis and reproductive effort of *C. gigas*.

TRACKING THE SPREAD OF AN INVASIVE MUSSEL (MYTILIDAE: *PERNA VIRIDIS*) IN FLORIDA. Jonathan S. Fajans*, Patrick Baker, Department of Fisheries and Aquatic Sciences, University of Florida, 7922 NW 71st ST, Gainesville, FL 32653

The green mussel *Perna viridis* was introduced to Tampa Bay, Florida in 1998. Since April 2002, we have been conducting surveys to chart the population growth of the mussel and monitor its spread. Three sites within the Bay were chosen as representatives of estuarine, introduction epicenter, and oceanic environments. Monthly collections were made for population density estimates. Densities within the Bay have reached 4033, 3675, and 4117 per square meter, respectively. Coastal sites throughout Florida were visited annually to determine presence or absence of *P. viridis*. As of January 2003 the range of populations has been extended to Fort Myers Beach to the south of Tampa Bay and Indian Rocks Beach to the north. Additionally, a new population has been found south of St. Augustine extending to Ponce Inlet on Florida's east coast, and several specimens have been reported from Pensacola in the panhandle.

MANIPULATION OF ENVIRONMENTAL PARAMETERS FOR OUT-OF-SEASON EGG AND LARVAL PRODUCTION IN BLUE CRAB BROODSTOCK (*CALLINECTES SAPIDUS*). Andrea Findiesen*, Oded Zmora, Moti Harel, and Yonathan Zohar, Center of Marine Biotechnology, University of Maryland Biotechnology Institute, Baltimore, MD; Alicia Young-Williams and Anson H. Hines, Smithsonian Environmental Research Center, Edgewater, MD.

Blue crab production techniques are being developed at the Center of Marine Biotechnology (COMB) to evaluate the possibility of restocking the diminished Chesapeake Bay blue crab population. Mature mated females were introduced into 2 m3 tanks with phase-shifted environmental conditions. By manipulating photoperiod, temperature and salinity, we have successfully induced females to ovulate, produce egg masses (sponges) and provide viable larvae all year-round. We also have been able to produce up to four successive sponges per female. Sponge production seems to be affected by a combination of photoperiod and temperature: long photoperiod (14 hours light: 10 hours dark) and high temperature (23oC) generated the most sponges. Our data indicates that high temperatures, though optimal for sponge production, increase susceptibility to disease when exposed over long durations of time. Sand is necessary for sponges to adhere properly to a female's abdomen. There doesn't seem to be any difference between maintaining the females at 25 or 30 ppt. Future work may include hormonal manipulation of broodstock to provide more predictable ovulation and larval production.

MANAGING AND MONITORING INTERTIDAL OYSTER REEFS WITH REMOTE SENSING IN COASTAL SOUTH CAROLINA.

Mark Finkbeiner*, Bill Stevenson, Bill Anderson, Mike Yianopolous, Loren Coen, Ginger Martin, and Karen Cullen. NOAA Coastal Services Center 2234 South Hobson Ave. Charleston, SC 29405.

Intertidal oyster reefs are a keystone species in South Carolina's estuaries and a major commercial and recreational resource. The South Carolina Dept. of Natural Resources (SCDNR) is responsible for conserving oyster reefs and regulating their harvest. The current oyster reef database for South Carolina was developed by field assessment in the 1980s and an update is needed to assess resource status and trends across the coastal zone. Coastal development and associated waterway usage are suspected of altering the extent and density of the state's oyster resources. The NOAA Coastal Services Center is working with SCDNR to develop methods for using high-resolution remote sensing data to assess intertidal oyster reefs along the South Carolina coast.

The objective of the project is to provide SCDNR with a new methodology for assessing intertidal oyster resources. The project examined digital and analog aerial photography in two pilot areas located in Charleston and Beaufort Counties. A variety of image processing and photogrammetric methods were evaluated including manual delineation, spectral clustering, and digital texture analysis. These methods focused on determining the perimeter and spatial characteristics of oyster reefs. Results of this study will support future efforts to update the entire state database.

IS COPPER REQUIRED FOR EASTERN OYSTER SETTING AND METAMORPHOSIS?

William S. Fisher. U.S. Environmental Protection Agency National Health and Environmental effects Research Laboratory Gulf Ecology Division Gulf Breeze, FL 32561.

Recent field research with eastern oysters demonstrated higher defense activities, including hemocyte numbers, locomotion and bactericidal ability, associated with locations exhibiting relatively high chemical contamination. Copper and zinc, found in high concentrations in tissues of oysters collected from these sites, are known to accumulate almost exclusively in amebocytes. These data have led to a re-evaluation of potential roles for copper and zinc in oyster physiology. A role for copper in setting and metamorphosis of oysters was previously proposed by Herbert F. Prytherch (1934), who found that larval oysters would not set or metamorphose without 0.05 to 0.6 mg L-1 copper for at least short durations in the surrounding water. High concentrations were not toxic for these short durations, and setting was stimulated within minutes of copper addition. Salinity altered the amount of time required for larvae to fix to the substrate but was not ultimately critical to setting. Consequently, oyster setting near river mouths may be due to incoming copper rather than the variable salinity to which it is sometimes attributed. If true, our understanding of oyster distributions and larval setting success would be greatly altered. Yet, by all appearances, these observations have never been validated.

COMPARISON OF PACIFIC OYSTER (*CRASSOSTREA GIGAS*) REARING RESULTS (SURVIVAL, GROWTH, QUALITY) IN FRENCH FARMING AREAS, AFTER A 10-YEARS MONITORING (1993-2002) BY THE IFREMER/REMORA NETWORK.

Pierre-Gildas Fleury*, Erwan Le Ber, Serge Claude, Florence Cornette, Florence d'Amico, Patrice Guilpain, Hubert Palvadeau, Stéphane Robert, Patrick Le Gall, Michel Ropert, Charlotte Simonne, Catherine Vercelli, IFREMER, F-56470 La Trinité-sur-mer, France.

Since 1993 the network IFREMER /REMORA has carried out annual standard monitorings of survival, growth and quality criteria of the Pacific oyster (*Crassostrea gigas*) among the main French farming areas. The network provides data series for each site, mean values (references) and allows multifactorial analysis of oyster rearing results. It must be pointed out that no general correlation was found between growth and mortality. A large range of results was exhibited both between years and between sites. However, unusual mortalities, annual variations of growth, or increasing infestation by the worm Polydora could be focused and quantified. Moreover, local trends may be of interest for collective oyster management. At last, REMORA data may support various types of studies, such on oyster quality, biological indicators for coastal waters or explanatory models of the oyster-farming ecosystems.

EVALUATION OF RARITAN AND SANDY HOOK BAY HARD CLAM, *MERCENARIA MERCENARIA*, STOCKS FOR FISHERY MANAGEMENT.

George E. Flimlin, Jr.*, Michael Celestino, John N. Kraeuter, Robert J. Macaluso, Michael Kennish, Rutgers Cooperative extension 1623 Whitesville Rd. Toms River, NJ 08755.

The hard clam fishery in the Raritan and Sandy Hook Bays took a twenty year hiatus started by a hepatitis outbreak in the early 1960's. The use of a clam relay and depuration allowed clammers to re-enter the fishery in 1983. Since then the fishery has grown steadily to about 200 full and part-time participants supplying clams to two depuration plants with others relaying their catch to approved beds in another county for purging.

A stock assessment was done the State in 1983 with no further evaluation until 2000 when the Bureau of Shellfisheries covered the same area again. Simultaneously, studies were done examining the age and growth of the shellfish as well as a natural mortality study. Armed with this information, the industry and the state can better work together to manage the harvest pressure and the participation in the area. Analysis of the data indicates that the stocks are at higher levels than when harvest restarted in 1983, possibly allowing for further exploitation.

POTENTIAL PATHOGENS ASSOCIATED WITH ABNOR-MAL MORTALITIES. Céline Garcia,* Isabelle Arzul, Franck Berthe, Bruno Chollet, Jean-Pierre Joly, Nolwenn Kerdudou, Laurence Miossec, Maeva Robert and **Jean-Louis Nicolas**, Genetic-Pathology and Aquaculture Shellfish Research Laboratory, IFREMER 17300 La Tremblade, France.

In France abnormal mortalities of mollusks affect many species of bivalves. They occur mainly in summer and concern all the French coastline. For *Crassostrea gigas*, they affect all life's stages but more particularly spat. A pathology monitoring network, REPAMO, was created at the beginning of the nineties in France in order to answer European requirements as regards mollusk pathology. REPAMO observes whether there is abnormal mortality and keeps track of the health situation of mollusk stocks including the presence of pathogens notifiable to the European Union and OIE.

When mortalities occur, the network REPAMO, samples the populations and performs different types of analysis (histology, bacteriology, viral detection) in order to detect potential pathogens. In France, different agents have been sometimes associated with abnormal mortalities of bivalves such as herpes-virus in *Crassostrea gigas*. Bacterial agents can be also involved. Indeed hemolymph of moribund oysters from open sea and from hatchery are often invaded by one *Vibrio* species belonging to *V. splendidus* group or *V. aestuarianus*. These observations suggest that *Vibrio* could induce or aggravate mortality in oysters weakened by environmental or physiological (maturation) factors.

SEASONAL VARIATION IN THE PHYSIOLOGICAL STATUS OF THREE SPECIES OF MUSSELS IN THE ALLEGHENY RIVER, PA. Catherine M. Gatenby*, The Academy of Natural Sciences 1900 Benjamin Franklin Parkway Philadelphia, PA 19103; **Danielle A. Kreeger, Deborah Raksany, and Richard J. Neves.**

A necessary precursor to identifying suitable feeding regimes for maintaining endangered freshwater mussels in captivity is defining their nutritional requirements. Similarly, a better understanding of their physiological status and use of food resources is needed to assess their role in natural systems and develop management plans that protect existing populations from further decline. We quantified the seasonal and interspecific variation in condition index and tissue biochemistry of representative unionid families from a large bed in the Allegheny River. Condition peaked in July and was similar between November and May for all species. Protein content peaked in November and May for *Elliptio dilatata* and *Lasmigona costata* (>40%), but did not differ seasonally in *Actinonaias ligamentina* (>30%). Lipid content was high in November and May for *A. ligamentina* and *E. dilatata* (>29%), but peaked in July in *L. costata.*(23%). Carbohydrate content was similar among species and times. The overall physiological status and specific demands for protein and lipid varied considerably

among seasons and species. Hence, the formulation of diets for maintaining captive mussels should target these changing demands. As well, efforts to assess the ecological importance of mussels should anticipate variation in physiological rate functions.

CHARACTERIZATION OF *VIBRIO* ISOLATED FROM PACIFIC OYSTERS' SPAT SUFFERING FROM SUMMER MORTALITY OUTBREAKS. Mélanie Gay*, Laboratoire de Génétique et Pathologie IFREMER 17390 La Tremblade France, **Guenaelle Lancelot, Bruno Chollet, Tristan Renault, Nathalie Cochennec, Franck Berthe, Christophe Lambert, Gwenaelle Choquet, Christine Paillard, Manolo Gouy, Frédérique Le Roux, Philippe Goulletquer.**

The pathogens related to summer mortality outbreaks are a herpes virus and two bacterial strains one belonging to *Vibrio splendidus* biovar II and the other to *Vibrio splendidus spp.* However, the feature pathogen/opportunist of these strains is still unknown. Several strains belonging to the genus *Vibrio* have been identified as pathogen for different mollusk species.

In the context of the French program Morest, experiments of cohabitation have been used to demonstrate the potential presence of a transmissible infectious agent in batches of oysters suffering from summer mortality outbreaks. More than one hundred *Vibrio* strains have been isolated from these experiments. These strains have been phenotypically and genotypically characterized. Their virulence has been evaluated by infection trials.

Two *Vibrio lentus* strains have been selected. The mortality rate induced by them injected together is always higher than the mortality rate induced by each strain injected individually. A histological examination of injected animals showed damaged hemocytes and muscle. However, bacteria have only been observed in the tissue surrounding the muscle and in the kidney. We have shown that physiological and genetic factors had an effect on the sensitivity of Crassostrea gigas to the experimental model of bacterial infections.

RESTORATION OF BAY SCALLOPS IN HIGHLY MODIFIED AND RELATIVELY PRISTINE HABITATS ON THE WEST COAST OF FLORIDA, USA. Stephen P. Geiger* and **William S. Arnold**, Florida Fish and Wildlife Conservation Commission Marine Research Institute 100 8th Avenue S.E. St. Petersburg, FL 33701 USA.

The density of scallops in many populations within Florida has declined greatly while other populations have remained healthy enough to allow recreational harvest. We have been attempting to restore four of the populations that experienced declines. Two of these populations exist in coastal areas with expansive seagrass meadows and low impact from development. Two populations exist in embayments which have been modified by anthropogenic impacts such as hardened shorelines, filled wetlands, channelization, and construction of causeways. In one coastal population,

adult density has returned to harvestable levels. Good management practices, natural variability, and restoration efforts may have all played a role. The density in the second coastal community has also increased but not to harvestable levels. Neither population in embayments has recovered despite restoration efforts. Evidence from surveys of adult scallops and recruitment of spat in these four populations as well as three additional populations where no restoration efforts occurred suggest that habitat alteration may amplify negative variations in the population. One example is the rate of recovery from declines related to harmful algal blooms. Continued development in northwest Florida may exacerbate the population declines, especially if those regions serve as a source for recruits in other areas.

FLOW CYTOMETRY AS A TOOL TO QUANTIFY OYSTER PHAGOCYTOSIS, RESPIRATORY BURST AND APOPTOSIS. Michael Goedken*, and Sylvain De Guise, Department of Pathobiology and Veterinary Science, University of Connecticut, 61 N Eagleville Road, U-89, Storrs, CT 06269.

The parasites *Perkinsus marinus* and *Haplosporidium nelsoni* have generated losses in the hundreds of millions of dollars. 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 sub-populations of hemocytes (granulocytes, hyalinocytes and intermediate cells) were identified with unique functional characteristics. Granulocytes were most active at phagocytosis and peroxide production while hyalinocytes were relatively inactive. TUNEL assay application allowed quantification of hemocyte apoptosis, which was more frequent in dividing cells. 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 and susceptibility to disease.

INTEGRATION OF MODELING AND GIS IN STUDIES OF CARRYING CAPACITY FOR BIVALVE AQUACULTURE. Jon Grant, Marie Archambault*, Cedric Bacher, and Peter Cranford, Dpt Oceanography Dalhousie University Halifax, NS B3H 4J1 Canada.

Estimation of carrying capacity for bivalve culture is important in predicting the effect of the environment on culture yield, as well as the effect of culture on the environment. Areas of Prince Edward Island (Canadian Maritimes) appear saturated with respect to mussel farms, and there is a requirement for estimation of culture density relative to sustainability for growth rates and ecosystem health. We have combined field studies, biophysical modeling, and

GIS to examine the effects of culture density and location on seston depletion in Tracadie Bay, an important site in the PEI mussel industry. Models have been constructed at several levels including box models of the estuary, and fully coupled physical-biological models set up on a detailed hydrodynamic grid. In the latter case, maps of seston depletion and biodeposition are generated as a function of culture density and distribution. Model results are integrated as data layers in the GIS, and calculations are made within grid cells using spatially explicit conditions to predict mussel growth and bioenergetics. A comprehensive field program including moorings with current meters, particle sensors, sediment traps, and surveys with a towed vehicle was used to provide boundary conditions as well as groundtruthing of model results.

MAPPING AND CHARACTERIZING EASTERN OYSTER (*CRASSOSTREA VIRGINICA*) REEFS USING UNDERWATER VIDEOGRAPHY AND QUADRAT SAMPLING. Jennifer Greene*, Ray Grizzle and Jamie Adams, Jackson Estuarine Laboratory University of New Hampshire 85 Adams Point Rd. Durham, NH 03824.

This project attempts to develop an economical technique to map oyster (*Crassostrea virginica*) reef boundaries as well as characterize the general health of oyster populations using videography and quadrant sampling. In New Hampshire, oyster monitoring by resource managers has been impeded by lack of an effective methodology for determining distribution and abundance. Videography was conducted in Great Bay, NH by systematically imaging multiple sampling cells in a grid covering two study reefs. In each cell, a 5-10 s digital video image was recorded (0.25 m2 area) with location determined by DGPS. A representative still image was selected for each cell and combined into a photomontage overlaid onto a geo-referenced base map using ArcView GIS. Quadrat samples (0.25 m2) were collected from 8-10 of the imaged areas on each reef and all live oysters were counted, measured and returned to the reef. Initial results suggest that systematic videography can accurately delimit reef boundaries, yield quantitative data on shell densities, and provide information on reef characteristics and structure. Additional reefs will be sampled in 2003 using a combination of continuous video transects with periodic camera drops in an attempt to provide finer scale determination of reef boundaries.

THE EFFECTS OF BACKGROUND CONCENTRATIONS OF THE BROWN TIDE ALGA *AUREOCOCCUS ANOPHAGEFFERENS* ON GROWTH AND FEEDING IN THE BIVALVE *MERCENARIA MERCENARIA*. Dianne I. Greenfield*, Darcy J. Lonsdale, Robert M. Cerrato, and Glenn R. Lopez, Marine Sciences Research Center, Stony Brook University, Stony Brook NY 11794-5000.

This study examined the extent to which background levels, defined as concentrations too low for toxicity to inhibit feeding, of *Aureococcus anophagefferens* (brown tide) influenced the growth

and feeding physiology of hard clams, *Mercenaria mercenaria*, in the laboratory compared to other phytoplankton common to Long Island, NY, waters. Juvenile clams were fed either unialgal cultures or diets mixed with background levels of brown tide. Absorption efficiency (AE) was determined using the 14C:51Cr dual-tracer method and growth was determined by biomass change. Results showed that unialgal diets resulting in the highest AE, specifically *Isochrysis galbana* and *Thalassiosira pseudonana*, resulted in rapid *M. mercenaria* growth. A unialgal diet of *Nitzschia closterium* resulted in a comparatively low AE and loss in clam biomass. Diets mixed with brown tide resulted in a significantly lower AE than the corresponding unialgal diet for all phytoplankton species *except N. closterium*. Additionally, mixed diets resulted in slightly less clam growth than unialgal diets. This suggests that when brown tide occurs in the field at background levels, clams may suffer subtle, chronic effects. Moreover, the responses of *M. mercenaria* to each diet have implications for understanding how phytoplankton community composition influences bivalve growth in the field.

A SIMULATION MODEL OF THE GROWTH OF HARD CLAMS (*MERCENARIA MERCENARIA*), IV. EFFECTS OF CLIMATE CHANGE. Raymond E. Grizzle*, Eileen E. Hofmann, John M. Klinck, Eric N. Powell, John N. Kraeuter, V. Monica Bricelj, and Stuart C. Buckner, Jackson Estuarine Laboratory University of New Hampshire 85 Adams Point Rd. Durham, NH 03824.

A physiologically-based model that simulates individual growth of the hard clam, *Mercenaria mercenaria*, in response to changes in environmental conditions has been developed. We are applying this base model to evaluate the effects of possible climate change scenarios. Thus far, our climate change modeling has focused on water temperature and the timing of spring and fall phytoplankton blooms because these are major factors that control growth of hard clams. Actual water temperature data sets from Great South Bay, NY as well as sites in Chesapeake Bay and North Inlet, SC were used to simulate the long-term warming trend predicted by all major climate change models. Each data set was used in combination with different spring and fall phytoplankton bloom scenarios. When bloom times were held constant, long-term warming resulted in increased growth and the predicted rates matched published values for clams from each area from which water temperature data were used. The timing of blooms had a dramatic effect on growth, suggesting that year-to-year variations may be more important than overall temperature trends as climate change ensues. Details on the modeling results will be presented and discussed.

STATUS OF BLUE CRAB POPULATIONS IN LOUSIANA BASED ON FISHERY INDEPENDENT DATA COLLECTIONS (1967-2002) WITH OBSERVATIONS ON RELATIVE ABUNDANCE IN OTHER GULF STATES. Vincent Guillory*, Louisiana Department of Wildlife and Fisheries, P.O. Box 189, Bourg, Louisiana 70343; Harriet Perry, Center for Fisheries Research and Development, Gulf Coast Research Laboratory, College of Marine Sciences, the University of Southern Mississippi, P.O. Box 7000, Ocean Springs, Mississippi 39566-7000; and the Blue Crab Technical Taskforce, Gulf States Marine Fisheries Commission.

The 35-year (1967-2000) database from the Louisiana Department of Wildlife and Fisheries bottomfish/shrimp monitoring program is the most extensive and continuous fishery independent blue crab data set in the Gulf of Mexico. Long term and recent trends in recruit (<40 mm CW), juvenile (40-99 mm CW), sub-legal (100-124 mm CW), and legal (>125 mm CW) blue crabs were examined, as well as overall catch per unit of effort sub-legal crabs did not significantly change over the long term, although overall CPUE for these size groups showed a significant increase from 1967-1989 with a significant decrease in recent years (1990-2002). Catch per unit of effort of recruits significantly increased over the long term with a downward trend noted in current data. Trends in relative abundance are discussed in relation to habitat changes in northern Gulf of Mexico estuaries and to biological factors such as predation.

BREEDING AND EVALUATION OF EASTERN OYSTER STRAINS SELECTED FOR MSX, DERMO AND JOD RESISTANCE. Ximing Guo*, Susan Ford, and Gregory De-Brosse, Haskin Shellfish Research Laboratory, Rutgers University, 6959 Miller Avenue, Port Norris, NJ 08349; Roxanna Smolowitz, Marine Biological Laboratory, 7 MBL Street, Woods Hole, MA 02543; Inka Sunila, Bureau of Aquaculture and Laboratory, Milford, CT 06460.

Rutgers University has been breeding oysters for disease-resistance since the early 1960s and produced strains showing strong resistance to MSX and some resistance to Dermo. Breeding at the F.M. Flower Oyster Company has produced a strain (FMF) showing superior growth and JOD-resistance. We undertook a project to evaluate the Rutgers NEH strain, the FMF strain and their hybrids (HYB) along with a global susceptible control (ME) and local controls that were normally cultured at each of the four deployment sites. Oysters were produced in June 2000, deployed in July 2000 and evaluated for 27 months. Dermo exposure was heavy at most sites, while MSX and JOD infections were low or absent. At Cape Shore (NJ) where infection was the heaviest, NEH and HYB had the lowest cumulative mortality, 43.5% and 43.6% respectively, compared with 82.3% for FMF, 99.4% for ME and 81.1% for the local control (Delaware Bay wild). In growth, HYB was the same as FMF and faster than NEH, while ME and the local

controls grew the slowest. The hybrid offered the highest yield by surviving as well as the NEH strain and growing as fast as the FMF strain.

MARKETING IMPLICATIONS OF CONSUMER ATTITUDES TOWARD OYSTERS. Terrill R. Hanson*, Lisa O. House, Benedict C. Posadas, Dept. of Agricultural Economics P.O. Box 5187 Mississippi State, MS 39762.

As US consumption of oysters declined during the 1990's an understanding of why consumers purchase and consume oysters is important to marketing oysters effectively and reversing this trend. In 2000-2001, a survey was administered to U.S. residents on the topic of seafood consumption. Results and findings of this survey are useful for sellers to use in targeting consumers likely to increase their oyster consumption and for processors to use in designing programs likely to improve food safety considerations.

Reasons for eating oysters included enjoyment of the flavor (80% of consumers) and addition of variety to their diet (37%). Oyster consumer's identified price (38%), product safety (29%), and lack of availability of fresh product (20%) as the main reasons for not consuming oysters more often. Forty-three percent of oyster consumers and 54% of those concerned about product safety indicated their consumption of oysters would increase if depuration methods were used to 'guarantee' oyster safety. Sixty-one percent stated a willingness to pay of $0.34/oyster over the raw oyster price for a 'guaranteed' safe product. This option may be profitable if depuration costs do not exceed the increases in consumer's willing to pay.

HOW MANY LARVAE STAY AT HOME? MEASURING PATTERNS OF LOCAL OYSTER RECRUITMENT USING MOLECULAR MARKERS. Matthew Hare*, D. Merritt and K. Paynter, Biology Department University of Maryland College Park, MD 20742; S.K. Allen, Jr., E.M. Burreson, M.D. Camara, Ryan Carnegie, M. Luckenbach and K.S. Reece,

Recruitment enhancement is one of the primary objectives of oyster restoration in the Chesapeake Bay. Given the tremendous spatial and temporal variability in recruitment patterns that have been documented in Chesapeake Bay oysters, correlations between broodstock plantings and spat set provide only a partial and potentially misleading index of recruitment strength from enhancement efforts. We are using the unique genetic signature of disease-tolerant selected strains to measure the geographic scale of recruitment provided by restored reefs. With the highly variable DNA polymorphisms that we have developed, every newly settled oyster is "tagged" with a genotype that links it to its parents and its source population. In cooperation with several agencies and organizations we planted CROSBreed selected strains in the Great Wicomico River in Virginia and the Little Choptank River in Maryland during the summer of 2002. Genetic markers provide better than 95%

accuracy for assigning individual oysters to their source, selected strain or natural broodstock. CROSBreed oysters planted in Virginia averaged 6 cm, large enough to potentially breed during 2002. We will present analyses of 2002 Virginia spat indicating the proportion of recruitment derived from planted oysters versus natural broodstock.

SUITABILITY OF OYSTER CLUSTERS AS HABITAT FOR REEF-RESIDENT FISHES AND DECAPOD CRUSTACEANS IN THE CALOOSAHATCHEE ESTUARY. Leslie H. Haynes,* Arielle Poulos, Lacey K. Smith, Aswani K. Volety and S. Gregory Tolley, 1227 S.W. 25th St. Cape Coral, FL 33914.

The habitat suitability of oyster clusters for reef-resident fishes and decapod crustaceans was examined in the Caloosahatchee Estuary. Lift nets representing three habitat treatments were deployed during three seasonally wet and three seasonally dry months on an oyster reef located in the lower estuary. Nets contained either no oyster shell (control), dead, articulated clusters, or live oyster clusters. Based on the results of Hester-Dendy sampling conducted at the same site, nets were deployed for a period of 1 month to ensure full recruitment. Analysis of variance indicated that articulated oyster shell (dead or living clusters) exhibited greater species richness, biomass, and dominance than did the controls. Furthermore, organism abundance was higher in living oyster clusters compared to dead, articulated clusters; both treatments with oyster shell exhibited significantly greater abundances than the control. In addition, biomass of all treatments was significantly greater during the dry season than during the wet season. The results of this study suggest that the habitat value of oyster clusters to reef-resident fishes and decapod crustaceans lies primarily in the three-dimensional structure created: dead, articulated oyster shell exhibited levels of associated biomass and species richness similar to that of clusters containing living oysters.

ALGAL FOOD QUANTITY AND QUALITY AFFECT IMMUNE FUNCTION IN OYSTERS STRESSED BY HIGH TEMPERATURE. Hélène Hégaret*, Gary Wikfors, NOAA Fisheries, Milford, CT, USA, Philippe Soudant, LEMAR, IUEM-UBO, Plouzané, France, and Jean-François Samain, Laboratoire de Physiologie des Invertébrés, IFREMER, Brest, France.

Oyster seed from a hatchery must resist environmental stresses when planted in the sea. We conducted an experiment to analyze the influence of nutrition on oyster, *Crassostrea viriginica's*, immune capability. Cultured microalgal diets were varied factorially in quantity (10 and 50% dw/dw microalgae/oyster soft tissue per day) and quality (Skeletonema, Tetraselmis, and a 50/50 mix), with unfed controls. Oysters were fed five weeks at 20°C and then temperature-stressed for one week at 28°C. Before and after heat stress, we used flow-cytometry and multivariate statistics to analyze the following hemocyte functions: viability, aggregation,

phagocytosis, and respiratory burst. Discriminant Analysis showed significant effects of food quantity and quality on hemocyte function. Principal Components Analysis revealed the main effects of heat stress to be increased respiratory burst and decreased phagocytosis; this decoupling of the two steps in pathogen defense was more severe in starved or poorly-fed oysters.

ASSESSING FEASIBILITY OF STOCK ENHANCEMENT FOR CHESAPEAKE BLUE CRABS (*CALLINECTES SAPIDUS*).

Anson H. Hines*, Jana L.D. Davis, Alicia Young-Williams, Smithsonian Environmental Research Center, Edgewater, Maryland 21037,USA; Yonathan Zohar, Oded Zmora, University of Maryland Biotechnology Institute, Baltimore, Maryland 21202, USA.

In overexploited, recruitment-limited fisheries, enhancement with hatchery-produced juveniles, coupled with traditional management techniques and habitat restoration, may be required for effective stock management. Enhancement, used most frequently for finfish stocks, has rarely been attempted with crustaceans. The Chesapeake blue crab stock exhibits key characteristics as an appropriate candidate for enhancement: 81% decline in biomass over the past decade, recruitment limitation, and extensive habitat with reduced juvenile mortality and densities below carrying capacity. The goals of this work were (1) to determine the enhancement potential of blue crab subpopulations by releasing hatchery-reared crabs (25,000 juveniles <25mmCW) on spatial scales of 10-15 ha, and (2) to identify factors influencing survivorship of hatchery crabs in the wild. In four separate cohorts (3,700-9,500 juveniles) that were sampled over 8-16 weeks, released tagged hatchery crabs successfully enhanced local subpopulations, growing rapidly and surviving to contribute to the spawning stock. Hatchery and wild crabs were similar in most respects, but differed initially in burial rate, carapace morphology, and susceptibility to predation. However, differences disappeared within days in the field, suggesting ways to improve success of future released crabs. These initial results contribute to determining whether enhancement on a larger scale is possible.

A SIMULATION MODEL OF THE POPULATION GROWTH OF HARD CLAMS (*MERCENARIA MERCENARIA*). I. MODEL DEVELOPMENT AND IMPLEMENTATION.

Eileen E. Hofmann*, John M. Klinck, Eric N. Powell, John Kraeuter, Monica Bricelj, Ray Grizzle, Stuart Buckner, CCPO, Crittenton Hall Old Dominion University Norfolk, VA 23529.

A physiologically-based model that simulates the population growth of hard clams, *Mercenaria mercenaria*, in response to temperature, salinity and food supply has been developed and applied in Great South Bay. The model structure model allows independent simulation of shell and tissue growth, which permits calculation of animal condition as a diagnostic. Also, length and age are independently tracked, thereby allowing specification of size-frequency and age-frequency distributions to describe population structure, and more importantly to define age dependent, as well as size-dependent, processes. The model structure includes a genetic component that permits simulation of a range of genotypes, which are combined into cohorts to construct a population. The simulated hard clam growth obtained using environmental conditions characteristic of Great South Bay match weight and length values that are observed for populations in this region. The extension of the simulations of individual clams to cohort and populations scales shows the importance of assimilation rate and the apportionment between reproductive and somatic growth in determining inter-cohort variability and overall long-term population characteristics. The results of these simulations as well as those that examine model sensitivity to assumptions made for key model processes and parameterizations will be presented.

COMPARISON ALONG THE NEW ENGLAND COAST OF EPIDEMIC SHELL DISEASE IN THE AMERICAN LOBSTER, *HOMARUS AMERICANUS.*

Andrea C. Hsu*, Boston University Marine Program Marine Biological Laboratory Woods Hole, MA 02543; Roxanna M. Smolowitz, Marine Resources Center Marine Biological Laboratory Woods Hole, MA 02543; Andrei Y. Chistoserdov, Department of Biology University of Louisiana at Lafayette Lafayette, LA 70504; and Hemant M. Chikarmane, Marine Resources Center Marine Biological Laboratory Woods Hole, MA 02543.

During the last six years, shell disease has been found at high levels in wild lobsters along the New England coast. This study utilizes a combination of scanning electron microscopy (SEM), denaturing gradient gel electrophoresis (DGGE), and histological analysis to describe and define bacterial cells on the infected carapace of wild-caught lobsters. Diseased lobsters used in this study were collected starting from Eastern Long Island Sound, New York, up toward Cape Cod Bay, Massachusetts, with control animals from Maine.

SEM analysis revealed and statistical tests verified five separate morphological types of bacteria present in shell lesions. Halo-like holes surrounding all bacterial types suggest boring as their causative mechanism for degrading the lobster carapace. Preliminary DGGE data indicated up to fourteen independent phylotypes of bacteria were present in lobster lesions. At least two of them were found in all diseased lobsters used in this study. Histopathologically, the carapace matrix was usually absent or lattice-like cuticular remnants were found attached to underlying less degraded cuticle. Bacteria were the predominate organisms found at the leading edge of erosions. Combined results from SEM, DGGE, and histological analyses present evidence that an assemblage of bacteria may be the cause of New England epidemic shell disease.

TENNESSEE'S PEARL CULTURE INDUSTRY. Don Hubbs, Mussel Program Coordinator, Tennessee Wildlife Resources Agency, P.O.B. 70, Camden, TN 38320.

The Tennessee Wildlife Resources Agency (TWRA) regulates freshwater pearl culture in Tennessee. Administrative rules, proclamations and contracts are employed to regulate the industry, and protect and manage its utilization of the native mussel resource. Although experiments in pearl culturing began in the 1960's, governing regulations were not developed until 1988. A panel composed of TWRA fisheries personnel and industry representatives drafted the first regulations. The washboard, *Megalonaias nervosa* (Rafinesque, 1820), is the primary freshwater mussel species used by the pearl culture industry. Because washboards command the highest price in the commercial shell market, and legal-sized individuals can be scarce, industry experts convinced the TWRA to permit the use of sub-legal sized washboards for economic reasons. Contracts, seasons, and quotas were established to control the harvest of wild washboard mussels for the pearl culture industry. Permission for use of sub-legal sized washboards for pearl culture proved unpopular with many commercial shell harvesters and wholesale shell dealers.

EVIDENCE OF A COLD SHOCK RESPONSE IN *VIBRIO VULNIFICUS*, A HUMAN PATHOGEN TRANSMITTED VIA RAW EASTERN OYSTERS, *CRASSOSTREA VIRGINICA*, FROM THE GULF OF MEXICO. Kristi L. Huels*, 203 Swingle Hall Auburn University, Auburn, AL 36049-5419, **Yolanda J. Brady, Mary A. Delaney, Joel A. Bader**.

This study examined the response of *Vibrio vulnificus* to incubation at 13 and 4o C. It focused on changes in protein expression using one and two dimensional gel electrophoresis. Although different proteins were expressed following cooler temperature exposure no major cold shock protein was identified. As hypothesized, longer incubation times at 13o C resulted in increased variations. Proteins expressed at the cooler temperature were only transiently expressed, classical of stress responses. These preliminary results suggest there is a cold shock response active in *V. vulnificus* that requires further investigation in order to properly evaluate and alter the general management practices for collection and processing of the Eastern Oyster, *Crassostrea virginica*, from the Gulf of Mexico.

PREVALENCE AND ABUNDANCE OF *PERKINSUS MARINUS* AND *PERKINSUS CHESAPEAKI/ANDREWSI* IN CHESAPEAKE BAY OYSTER BEDS. Karen L. Hudson*, Kimberly S. Reece, Christopher F. Dungan, and Rosalee M. Hamilton, Virginia Institute of Marine Science, Gloucester Point, VA 23062.

Three described species of *Perkinsus* have been reported in the Chesapeake Bay region of the United States. *Perkinsus marinus* is a well known pathogen of the eastern oyster, *Crassostrea virginica*. *Perkinsus chesapeaki* and *Perkinsus andrewsi* are more recently described species from the soft-shell clam, *Mya arenaria* and the Baltic clam, *Macoma balthica*, respectively. Recent molecular studies, however, suggest that these two species are synonymous (Dungan et al. 2002). In 2001, Coss et al. reported *P. andrewsi* infections in oysters. The routine test used to diagnose *P. marinus* infections from oysters, Ray's fluid thioglycollate medium (RFTM) however, is not species-specific. The objective of this study was to survey oyster beds in the Chesapeake Bay area located adjacent to a variety of clam species in order to assess prevalence and abundance of *Perkinsus* species in oyster and clam hosts. Prevalence was assessed by standard PCR using two species-specific PCR primers: one *P. marinus*-specific and the other *P. chesapeaki / andrewsi*- specific. Abundance was accomplished using quantitative PCR using the same species-specific primers. Two species-specific in situ hybridization probes were developed and tested. Results of the assay development and environmental screening will be presented.

A FISHERY-ORIENTED MODEL OF MARYLAND OYSTER POPULATIONS. Stephen J. Jordan* and Jessica Vanisko, USEPA, Gulf Ecology Division 1 Sabine Island Drive Gulf Breeze, FL 32561.

We used time-series data to calibrate a model of oyster population dynamics for Maryland's Chesapeake Bay. Model parameters were fishing mortality, natural mortality, recruitment, and carrying capacity. We calibrated for the Maryland bay as a whole and separately for 3 salinity zones. Simulations indicated that a long-term declining trend in the Maryland bay-wide stock of harvestable oysters could be reversed by controlling fishing mortality and enhancing recruitment. For example, an exponential increase in stock size was predicted by simulating a 40% reduction in fishing mortality; initial losses to the fishery were more than compensated by large gains after a few years. In the low salinity zone, where the harvestable stock has been maintained largely by relaying seed oysters, recruitment rates are too low to support a significant population increase, but stocks in the medium and high salinity zones appear to have potential for recovery within 10-20 years. The model is sensitive to mortality and recruitment rates, but not to carrying capacity, which is much larger than current stock sizes. Measures of uncertainty for model predictions include (1) confidence limits for mean predicted trends, and (2) percentages of iterative simulations that satisfy specified criteria.

DEVELOPMENT OF BIOMARKERS FOR PERKINSUS MARINUS RESISTANCE IN THE EASTERN OYSTER (*CRASSOSTREA VIRGINICA*). Stephen L. Kaattari* and Christopher Earnhart, Department of Environmental and Aquatic Animal Health, Virginia Institute of Marine Science, College of William and Mary, Gloucester Point, VA 23062.

The development of biomarkers for the determination of *Perkinsus marinus* resistance in the eastern oyster would be of great utility to the oyster industry and would also serve as an important tool in the study of disease pathogenesis. To achieve such a goal we have capitalized on the observation of the ability of *P. marinus* cells to respond in a specific manner to extracts of susceptible oyster tissue. Generally co-incubation of *P. marinus* with host tissue extracts can elicit a variety of effects including altered cellular differentiation, protease expression, growth rates, infectivity, and parasite lethality. The application of this analysis to stock assessment and deployment decisions, as well as their use in the selection of future oyster broodstock could provide a needed competitive edge to the American oyster industry. Further, investigation in this arena should yield useful models for the analysis of the developmental process of oyster protozoan parasites.

EVALUATION OF COMMERCIAL POST HARVEST TREATMENTS FOR CONTROL OF *VIBRIO VULNIFICUS* IN OYSTERS. Marilyn B. Kilgen*. Department of Biological Sciences, Nicholls State University, Thibodaux, LA 70310.

Post harvest treatments of freezing, low dose ionizing irradiation, and hydrostatic high pressure (HHP) were commercially tested in collaboration with oyster industry members from the gulf and east coasts. Six vinegar-based oyster marinades were also developed in collaboration with the NSU Chef John Folse Culinary Institute. All reduced *Vibrio vulnificus* levels from 240,000 MPN/g to non-detectable levels (<3MPN/g) after 24 hours of marinating at 35F, and one received the highest sensory score from 1,116 tasters (80%) at the Louisiana Boat Show. Commercial cryogenic (liquid CO2) freezing of half-shell oysters reduced *V. vulnificus* levels from 460,000 MPN/g to 0.74 MPN/g by 6 weeks post freezing. Commercial ionizing irradiation with Co60 reduced levels of *Vibrio vulnificus* in live shellstock oysters from 460,000 MPN/g to <0.3 MPN/g at 1.0 kilogray (KGy). In recent studies, live oysters were treated with hydrostatic high pressure for the first time. In commercial applications, 35,000 psi for 3 minutes at 70F was determined to be most economically feasible and was validated to reduce *V. vulnificus* from >100,000 to <3 MPN/g. It was also initially discovered in these studies that oyster adductor muscle proteins were denatured at the shell attachment resulting in mechanical shucking of the oyster.

THE BLUE CRAB FISHERY OF THE HUDSON RIVER ESTUARY. Gregg Kenney*, Andrew Kahnle, Kathy Hattala, and Steven H. Jury, 21 South Putt Corners Road New Paltz, NY 12561.

Despite its economic and recreational importance, there has been relatively little systematic inquiry into blue crab (*Callinectes sapidus*) abundance, distribution, and habitat utilization in the Hudson River Estuary. Blue crab abundance is generally considered to have increased in this system as indicated by expanding recreational and commercial fisheries. The New York State Department of Environmental Conservation has implemented a program to investigate the extent of the commercial fishery and seasonal movements of blue crabs in the estuary. The commercial fishery catch was monitored during the 2000, 2001 and 2002 crabbing seasons and fishery independent sampling was conducted weekly throughout the 2002 season. Catch per unit effort was found to fluctuate temporally and spatially in a manner similar to that found in other temperate estuaries. The relationship between blue crab abundance and changes in temperature and salinity is presently being analyzed. This project will provide data that can be used to monitor changes in relative abundance and distribution of blue crabs in the Hudson River to fulfill the goal of maintaining a sustainable fishery.

POLINICES PULCHELLUS: THE JAMES DEAN OF GASTROPODS: LIVING FAST, DYING YOUNG. Peter Kingsley-Smith*, VIMS P.O. Box 1346 Gloucester Point, VA 23062.

The gastropod, *Polinices pulchellus*, is patchily distributed on subtidal muddy sand in Red Wharf Bay, Wales, UK. Competent pediveligers metamorphosed in response to sediment collected from the adult habitat, such that the adult distribution may be explained by preferential larval settlement. *Polinices pulchellus* densities were significantly higher in summer than in winter, which it is proposed arose from mating aggregations. Small individuals (4-5 mm) were present throughout the year indicating an extended period of low-level recruitment, which was reflected in the year-round production of egg collars in the laboratory. Larger females had higher fecundities than smaller females, however, smaller females continued to lay egg collars later in the year. Small females (4-5.9 mm) grew rapidly during the warm, summer months (April to August), attained sexual maturity at 8-9.9 mm and began laying egg collars in mid-September. The relationship between shell length and statolith diameter was determined for newly hatched larvae through to fully-grown adults (16 mm). Estimates of mean shell length at the formation of the first and second prominent rings supported the conclusion that prominent statolith rings are formed annually. *Polinices pulchellus* reaches its maximum size in 2-3 years.

OBSERVATIONS ON THE UNUSUAL ABUNDANCE OF TROPICAL *CALLINECTES* SPECIES IN THE SOUTH ATLANTIC BIGHT IN FALL 2002, AND REMARKS ON THE NON-INDIGENOUS *CHARYBDIS HELLERII*. David M. Knott*, Elizabeth L. Wenner, and Susan L. Thornton, Southeastern Regional Taxonomic Center at the Marine Resources Research Institute, South Carolina Department of Natural Resources, Charleston, SC 29412.

Tropical species of *Callinectes* typically appear in the South Atlantic Bight only occasionally, and then usually only in isolated occurrences. In fall 2002, however, commercial fishermen near Charleston reported the capture of mature *C. exasperatus* and *C. bocourti* in abundances great enough to warrant inquiry about their identity and the legality of selling them. Although quantitative estimates are not available (landings reports do not include species composition), we believe that these species were fairly common and widespread in the vicinity, beginning in mid-October. A single specimen of *C. larvatus* was also collected by SCDNR staff in mid-November, at about the same time that the last *C. bocourti* was seen. The more recent collection of moribund *C. exasperatus* on December 10, after water temperatures had dropped below 11°C, indicates that these species may be unable to survive typical winter condition in the area. Possible explanations for this unusual event will be discussed. Additional remarks will address the invasive portunid *Charybdis hellerii* in the SAB, and the original report of its occurrence in the western Atlantic will be updated.

A SIMULATION MODEL OF POPULATION GROWTH OF HARD CLAMS (*MERCENARIA MERCENARIA*). II. EFFECTS OF FISHING. Kraeuter*, Haskin Shellfish Research Laboratory Rutgers University 6959 Miller Avenue Port Norris, NJ 08349; Powell, Hofmann, Klinck, Grizzle, Bricelj and Buckner.

A physiologically-based model that simulates hard clam, *Mercenaria mercenaria*, growth in response to environmental conditions of temperature, salinity and food supply has been developed. We are applying this base model to evaluate the effects of various fishing scenarios on Great South Bay, New York clam populations. Comparison of fishery-independent samples with landings suggests the population was heavily over fished in the late 1970 until at least the mid 1980's. Base simulations illustrate the effect of changing salinity and food environments. The spawner/recruit relationship is based on limited data so the effects of variation in this parameter have been evaluated. Fishing simulations evaluate the effects of proportional fishing (all marketable sizes of clams are removed in proportion to their abundance) at various percentages of removal. In addition, exclusive removal of various percentages of commercial size categories; littleneck, topneck, cherrystone or chowder is explored. Finally, population recovery rates are evaluated under scenarios of a total fishing ban or limited percentage removal. In general, simulations indicate recovery time

is on the order of a decade or more, and fishing proportionally or on littlenecks at greater than 25 to 35% of adult standing stock will decrease fishing yields and clam populations.

GENOMIC SIGNATURE TAGS: A NOVEL METHOD FOR GENOMIC PROFILING WITH APPLICABILITY TO SHELLFISHERIES RESEARCH. Maureen K. Krause*, Dept. of Biology, Hofstra University, Hempstead, NY 11549; John J. Dunn, Daniel van der Lelie, Sean McCorkle, Biology Dept., Brookhaven National Laboratory, Upton, NY 11973.

Genomic Signature Tags (GSTs) are the products of a new high-throughput, direct sequence-based approach for characterizing genomes that does not rely on *a priori* knowledge of the genome. Our technique produces large numbers of positionally defined tag sequences that can, in principle, provide limited representation of all the DNA molecules in a sample. A GST analysis of the 4.7 Mb *Yersinia pestis* EV766 genome validates that the GST technique provides a route to obtaining numerous sequence tags that can be used to identify the DNA source. Additionally, our data show that the presence or absence of particular tags can be used to characterize intraspecific genetic variability. One exciting extension of GST analysis, ribosomal GSTs, shows tremendous potential for analyzing microbial communities, including those that may be associated with shellfish disease. Overall knowledge of microbial communities associated with diseased versus non-diseased shellfish remains poor due to constraints of culturability and microscopy. Ribosomal GST profiles have the potential to not only determine what microbial species are present, but their relative abundance, as well. The power of our approach is that it is both qualitative and quantitative, and can directly provide sequence information without electrophoretic isolation of amplicons.

SPATIAL AND TEMPORAL VARIATION IN OYSTER FITNESS IN SAN ANTONIO BAY, TEXAS, 1998-2002. D. Kreeger*, R. Thomas, H. Hertler, and D. Raksany, Patrick Center for Environmental Research, Academy of Natural Sciences, 1900 Ben Franklin Parkway, Philadelphia, PA 19103.

Adult oysters (*Crassostrea virginica*) were sampled eleven times between October 1998 and May 2002 from four locations in San Antonio Bay to quantify spatial and temporal variability in body size, condition index, and tissue biochemical composition. All measures of oyster fitness varied spatially, seasonally and among years. Seasonal differences were consistent with expected norms for healthy adults that undergo an annual reproductive cycle (fall/winter conditioning; winter/spring gametogenesis and spawning). This pattern was not observed every year at every location, however, and spatial and inter-annual variability interacted strongly. Spatial variation was greatest along an axis extending from the upper to lower bay, and inter-annual differences were greatest at upper bay locations. Seaward oysters were consistently

fit. but oyster fitness in the upper bay varied widely between dry and wet years. Following major floods. upper bay oysters had a smaller size (or were morbid) and demonstrated a subdued seasonal conditioning cycle compared to seaward oysters: whereas. in drier years upper bay oysters were largest and attained the highest condition of all locations. The stable oyster beds in the lower bay appear to serve as critical broodstock that provide larvae to replenish upper bay stocks following major disturbance events.

RECONSTRUCTING THE GROWTH OF HARD CLAMS, *MERCENARIA MERCENARIA*, UNDER BROWN TIDE CONDITIONS. Cathy A. Laetz* and Robert C. Cerrato. 4501 Greenwood Ave N Apt # 102 Seattle WA 98103.

Hard clams have been an important resource in Great South Bay. New York for decades despite severe population declines. One suspected cause of declines in recent years is brown tides, or blooms of the phytoplankton *Aureococcus anophagefferens* which have been found to cause a slowing or cessation of feeding activity in various shellfish. Growth in hard clams planted in Great South Bay for one year during a brown tide bloom was similar to growth in clams measured in years prior to brown tides. Similarly. archived shells from the Town of Islip's annual shellfish surveys showed comparable growth rates between brown tide and pre-brown tide years. Rapid shell growth was observed in the spring and fall, whereas no growth occurred when water temperatures fell below 6°C. Although there was no relationship between brown tide concentration and clam growth, a strong relationship was observed with temperature, which accounted for 65% of the variation in shell growth rate. In contrast to other shellfish, brown tide does not appear to have as great a negative effect on the growth of hard clams in Great South Bay, possibly due to acclimation, growth compensation, or population selection over time.

IMMUNOLOGICAL STATUS OF SELECTED *CRASSOSTREA GIGAS* FAMILIES AND DESCENDANTS, REARED IN DIFFERENT ENVIRONMENTAL CONDITIONS. Christophe Lambert*, Laboratoire des sciences de l'environnement marin (LEMAR) Institut Universitaire Européen de la Mer (IUEM) Université de Bretagne Occidentale (UBO) Place Copernic. technopole Brest Iroise 29280 Plouzané. FRANCE: Philippe Soudant, Gwénaelle Choquet, Christine Paillard, Stéphane Frouel, Lionel Degremont, Maryse Delaporte, Jeanne Moal, Pierre Boudry, Patrick Soletchnick, Michel Ropert, Edouard Bédier, Tristan Renault, Béatrice Gagnières Arnaud Huvet and Jean-François Samain.

Defense mechanisms variability in *Crassostrea gigas* is suspected to result from genetic factors. In the French program MOREST, bi-parental families, obtained from a nested half-sib crossing design. were reared four months in three culture sites. Six families were selected on their survival performance ("good" and

"bad"). Their immunological status was compared after four months in the three sites. Another comparison was performed after 13, 15, and 17 months but only in one site. Concomitantly. immunological parameters of triploïdes and diploïde oysters were followed during summer in Charentes. Significant differences were measured between good and bad families but were less marked during the year 2. Triploïdes and diploïdes presented clear differences. To discuss the possible genetic transmission of immune parameters, status of 8 divergent families from crossed good or bad families was studied.

OPTIMIZATION OF SPERM CRYOPRESERVATION FOR THE PACIFIC OYSTER *CRASSOSTREA GIGAS:* EVALUATION OF COOLING RATE. Paul Lang* and Chris Langdon. Coastal Oregon Marine Experiment Station, Hatfield Marine Science Center, Oregon State University. Newport, Oregon, 97365.

Sperm suspension with a concentration of 10% dimethyl sulfoxide was prepared from calcium-free Hanks' balanced salt solution (~800 mOsmol/kg) and sperm of five Pacific oysters (*Crassostrea gigas*). Plastic straws (2.5-mL) were filled with 2 mL suspension. placed in a chamber previously cooled to either $-30°C$ or $-70°C$, and plunged into liquid nitrogen ($-196°C$) when internal straw temperature fell within ~2°C of the chamber temperature (7 min at $-30°C$, 4 min at $-70°C$). Straws were thawed in a water bath ($70°C$) for 30 sec. Eggs of females ($n = 5$) were fertilized using thawed or fresh (control) sperm at equal sperm-to-egg ratios (20:1), and incubated in 10-mL tubes. Fertilization (the percentage of eggs to have reached the 4-cell stage) was $22\% \pm 9\%$ for eggs fertilized with sperm cooled to $-30°C$, $51\% \pm 8\%$ for $-70°C$, and $57 \pm 4\%$ for fresh sperm. Larval development (the percentage of initially fertilized eggs to have reached D-stage) was $29\% \pm 10\%$ for eggs fertilized with sperm cooled to $-30°C$, $62\% \pm 13\%$ for $-70°C$. and $72\% \pm 5\%$ for fresh sperm.

FAMILY-BASED SELECTION IMPROVES YIELDS OF PACIFIC OYSTERS, *CRASSOSTREA GIGAS*. Chris Langdon*, Sean Matson, John Brake, Ford Evans. Coastal Oregon Marine Experiment Station and Dept. Fisheries and Wildlife, Oregon State University, Newport, Oregon 97365.

The Molluscan Broodstock Program (MBP) was established in 1995 to improve yields of Pacific oysters on the West coast, U.S., through family-based selection. Parental families (P1) in three cohorts of about 60 families each were selected based on superior live weight and meat yields at harvest. Live weight yields of progeny (F1) from crossing P1 selected families were significantly greater than those of non-selected control families in four out of seven trials (ANOVA, $p < 0.001$), resulting in an average gain of 9.5% after one generation of selection. ANOVA indicated a significant ($P < 0.01$) genotype by environment interaction effect on yields for families planted at both inter-tidal and sub-tidal sites.

Nonetheless, it was possible to identify four to six "generalist" families that were among the top ten families at both sites. Further evaluation is needed to determine if the best strategy to improve oyster yields will be to select "generalist" families or whether it will be more effective to develop site-specific lines instead.

AN EVALUATION OF SEA SCALLOP CLOSED AREA BOUNDARIES IN THE MID-ATLANTIC. J. David Lange, Jr., William D. DuPaul*, and David B. Rudders, VIMS P.O. Box 1346 Gloucester Point, VA 23062-1346.

A formal area management strategy for the U.S. sea scallop fishery is being developed under Amendment 10: Sea Scallop Fishery Management Plan. Area closures impacting the sea scallop fishery occurred in 1994 on Georges Bank to protect groundfish resources. Also, in 1998, area closures in the mid-Atlantic (Hudson Canyon and Virginia Beach) were enacted to protect concentrations of pre-recruit scallops. This study determined if scallop abundance was reflective of closed area boundaries designated by co-ordinates on navigational charts.

Data was collected among two post-closure stock abundance surveys. A total of 329 standard survey tows were conducted both inside and outside the closed areas. Survey data were evaluated and results indicate that the use of electronic vessel monitoring systems to track fishing activity can be an effective tool in the enforcement of area management strategies. The effective boundary is described as the location at which the scallop population differed as a result of an absence of fishing mortality due to the protection provided by the area closures.

THE ROLE SUBSTRATE CHARACTERISTICS HAVE IN ALTERING THE BEHAVIOR, GROWTH AND SURVIVAL OF JUVENILE (POST-SETTLEMENT) *MERCENARIA MERCENARIA*. Amy A. Larson*, and Robert M. Cerrato, Department of Biology San Diego State University 5500 Campanile Drive San Diego, California 92182-4614.

Indirect effects can be the primary structuring mechanism in soft-sediment communities, but can be overlooked in experiments that do not test for effects at appropriate levels of habitat complexity. Interactions between physical factors (azoic sediments) and biotic factors (faunal communities from the different substrates) on growth and predation of small *Mercenaria mercenaria* were tested in different habitat types: sand, shell hash, large pieces of shell and a control with no substrate. In the sand, competition between *small M. mercenaria* and infauna reduced growth of *M. mercenaria*. Shell hash and the associated fauna had no effect on growth. On large pieces of shell, both competition and effects of the substrate were important, and the combined effect of the two was additive, resulting in the slowest growth rate overall. Predation rates were approximately equivalent in the different habitat types, but the relative importance of physical and biotic factors on

M. mercenaria abundance differed and was nonadditive. Sediment and faunal effects in shell hash were not different, although there was some indication the sediment effect may be greater. In sand and large shell pieces, alternative prey availability may be more important for small *M. mercenaria* survival than physical refuge from predation.

ONE MAN'S DREAM: AMERICAN CULTURED PEARLS. Gina Latendresse*, American Pearl Company, 807 Watts Lane #B, Nashville, TN 37209.

The late John Latendresse was the visionary behind pearl culture in the United States. His forty-five year journey from local fisherman, entrepreneur, shell exporter, pearl importer and finally to the originator of the American cultured pearl walks us through the many facts of his life and details of his business success and failures. His venture into pearl culture started with a challenge from a Japanese colleague. Later he would be known as an evangelist for pearl culture in the United States. To accompany this presentation will be a display of exceptional American natural pearls and jewelry designs with American cultured pearls, to include coin, bar, triangle, loaf, cabochon, teardrop and round.

ZOOPLANKTON INGESTION BY BIVALVES—MORE FOOD FOR THOUGHT. Clare Lehane* and John Davenport, Dept. of Zoology & Animal Ecology, University College Cork, Lee Maltings, Prospect Row, Cork, Ireland.

Bivalves have generally been thought of as herbivorous, gaining nutrition from phytoplankton. However, since the 19th century researchers have reported finding zooplankton species in the stomachs and excreta of bivalves. A study was carried out to determine if four species of bivalves, namely blue mussels, common cockles, queen scallops and horse mussels could ingest zooplankton. Though a series of suspended cage experiments and sampling bivalves from their natural habitats, it was determined that all four species ingested zooplankton representative of that found in the water column on the days of experiment. A second experiment dealt with determining if a fabricated bed of blue mussels could deplete zooplankton in overflowing water. It was found that zooplankton numbers were lowest in the middle of the bed, signifying that mussels may have the ability to affect zooplankton populations to some degree.

SPECIES-SPECIFIC VARIATION IN THERMAL TOLERANCE DURING LARVAL DEVELOPMENT IN BLUE MUSSELS, *MYTILUS SPP.* Susan J. Limbeck*, and Paul D. Rawson, School of Marine Sciences, University of Maine, Orono, ME 04469.

Two species of blue mussel, *Mytilus edulis* and *Mytilus trossulus*, are sympatric throughout much of the Canadian Maritime Provinces and into the Gulf of Maine. While the distribution of *M.*

edulis extends south to the Mid-Atlantic, that of *M. trossulus* ends abruptly in the Gulf of Maine. We hypothesized that species-specific variation in larval thermal tolerance influences differences in distribution. Previously, we found that *M. trossulus* experienced higher mortality than *M. edulis* when larvae were held at temperatures above 15°C throughout development. Our current experiment examines whether species-specific sensitivity to elevated temperatures is dependent upon larval age. Growth and mortality were monitored for larvae exposed to three experimental temperatures at three time points during development. Preliminary analysis suggests that *M. trossulus* larvae experience higher mortality at 18°C and 22°C but the effect is dependent on larval age. The importance of these findings with respect to patterns of larval dispersal and coastal water temperatures in the Gulf of Maine will be discussed.

VARIATIONS IN THE SIZE STRUCTURE OF LOBSTER (*HOMARUS AMERICANUS*) POPULATIONS WITHIN THE OFFSHORE FISHERY.

Susan A. Little*, Winsor H. Watson, III, and Rudman Hall, Dept of Zoology University of New Hampshire Durham, NH 03824.

The offshore lobster fishery is currently managed as one unit (Area 3), although it extends from New Jersey to Maine. To determine if there were differences in the size structure of lobster populations within Area 3, we examined 36,815 lobsters from three regions: North: Georges Bank and offshore Gulf of Maine; Middle: offshore Massachusetts to south of Cape Cod, and; South: offshore Rhode Island to New Jersey. Each region included mid-shelf areas (30-40 fathoms) out to continental shelf canyons (120-150 fathoms). In the North 2% of the catch was sub-legal, compared to 40% in the Middle and 29% in the South. This pattern was reflected in the average size in each region: North = 110mm CL (carapace length); Middle = 89mm and; South = 91mm. There were also more lobsters >100mm CL in the North (37%) than in the Middle (4%) and South (3%), and, conversely, more lobsters <65mm CL in the Middle (3%) and South (5%), than in the North (<1%). Despite similarities in depth and bottom temperature, the populations differ between the three regions examined, which may have important management implications. These patterns may result from regional differences in growth rates, migratory patterns or fishing pressure.

OYSTER REEF HABITAT RESTORATION: A REVIEW OF RESTORATION APPROACHES AND AN AGENDA FOR THE FUTURE.

Mark W. Luckenbach* and Loren D. Coen, Virginia Institute of Marine Science P.O. Box 350 Wachapreague, VA 23480.

Reefs produced by the Eastern oyster have been degraded by many factors, including overfishing, disease, sedimentation, pollution, hydrographic alterations and boat wakes. Numerous oyster reef restoration projects are currently underway or have been initiated within the past few years. New urgency is given to evaluating the success of these efforts by the serious consideration currently being given to the introduction of an exotic oyster species. We will summarize these projects and assess what is known about their success. Patterns emerging from this review indicate some unifying themes, but also point to the importance of site specificity. Several studies reveal the importance of reef architecture (size, shape and spatial configuration) and substrate composition, but questions remain about how to optimize the placement of material. Hatchery-produced and wild oysters have been transplanted onto reefs in a variety of locations, but the efficacy of this has only occasionally been tested. Altered hydrographic regimes and changing water quality conditions in many estuaries increase the complexity of restoring these habitats. The importance of improving our understanding of the genetic implications of restoration strategies, larval dispersal patterns, factors affecting early post-settlement survival, disease dynamics and landscape-level patterns in restoring oyster reefs is emphasized.

PROGRESS IN THE DEVELOPMENT OF A CHEMO-THERAPEUTIC PROTOCOL FOR ELIMINATING/REDUCING DERMO DISEASE IN INFECTED OYSTERS.

Eric D. Lund*, Fu-Lin E. Chu and Ellen Harvey, Virginia Institute of Marine Science, College of William and Mary, Gloucester Point, VA 23062.

There is a need for protocols to eliminate/reduce *Perkinsus marinus* infection of oysters for hatchery, aquaculture operations and basic research on the disease, yet no practical method for treating infected oysters currently exists. Studies on the antimicrobial drug triclosan have shown that the drug is a specific inhibitor of Fab1, an enzyme in the type II class of fatty acid synthetases which are found in bacteria, plants and some protozoans, but not animals. The effects of triclosan on 7, 15 and 48-day-old *P. marinus* cultures were tested. Results revealed that triclosan (5-10 micromolar) added to the medium inhibits fatty acid synthesis and stops the proliferation of *P. marinus* meronts in vitro. The inhibitory effects of triclosan were highest in the 7-day cultures and somewhat attenuated in older cultures. These results suggest that triclosan may be a useful chemotherapeutic agent for treating oysters infected with *P. marinus*. This study has been supported by NSF award #0131108.

STRIKING SUCCESSION OF MUSSELS AT NEWLY FORMED DEEP-SEA HYDROTHERMAL VENTS.

Richard A. Lutz*, Timothy M. Shank, and Daniel J. Fornari, Institute of Marine and Coastal Sciences, Rutgers University, New Brunswick, NJ 08901.

In April, 1991 a volcanic eruption obliterated existing biological communities within extensive regions along the crest of the East Pacific Rise between 9°45'N and 9°52'N (depth 2520 m). The

initiation of hydrothermal venting during the eruptive process afforded the unique opportunity to follow, over a 10+ year period, temporal changes in biological community structure from the "birth" of numerous hydrothermal vents. Vestimentiferan tubeworms, amphipods, copepods, octopods, and galatheid and brachyuran crabs gradually colonized the vents during the first 2 years. Noticeably absent during this time was any evidence of the mussel *Bathymodiolus thermophilus*. Small mussels (<1 cm shell length) were first observed on basaltic substrates 3 1/2 years after the eruption, but were noticeably absent on the tubes of the dominating vestimentiferan *Riftia pachyptila* during this period. By 4 1/2 years after the eruption, mussels with shell lengths >5 cm were common in the region and had begun to colonize the tubes of *Riftia pachyptila*, concomitant with declining concentrations of H2S in the venting diffuse flow fluids. Over the next 5-6 years, the abundance of mussels increased dramatically until most of the existing communities previously dominated (in biomass) by tubeworms were now dominated by extensive populations of mussels.

SUSPENSION-FEEDING BIVALVES, MARINE AGGREGATES AND THE ACCESSIBILITY OF SMALL PARTICLES. M. Maille Lyons*, J. Evan Ward, Department of Marine Sciences, University of Connecticut, Groton CT 06340.

Marine aggregates (i.e., marine snow, organic aggregates, flocs, and detritus) are common in coastal waters where large populations of bivalves dominate benthic communities. Suspension-feeding bivalves actively pump seawater through their pallial cavities and extract particles for food. Retention efficiencies of small, freely suspended, particles (~1μm) are generally low. Small particles are often concentrated within aggregates. The retention efficiency of the larger, amorphous aggregates (>10 μm) should be 100%. However, the percentage of aggregates ingested, compared to the percent rejected as pseudofeces, is not known. The focus of this study is to determine the fate of the particles embedded within aggregates. Determining the ingestion rate of aggregates is an important step in assessing the role aggregates play in trophic interactions between water column microbiota and benthic bivalves. To test the hypothesis that the presence of marine aggregates increases the accessibility of small, otherwise poorly retained, particles, experiments were designed using artificial aggregates generated on a rolling table. Fluorescent beads (1 μm and 10 μm) were incorporated into aggregates in order to track the fate of embedded particles. The percentage of beads in the bivalves gut, evaluated by direct counts, was compared to controls (fluorescent beads not incorporated into aggregates). Preliminary results indicate species specific differences, increasing the accessibility of small particles for the sea scallop (*Placopecten magellanicus*), but showing no significant increase in accessibility for the marsh mussel (*Geukensia demissa*).

SHELLFISH RESTORATION: IT'S NOT JUST BIOLOGY THAT MATTERS. Sandra L. Macfarlane, Coastal Resource Specialists P O Box 1164 Orleans, MA 02653.

Shellfish restoration projects have been practiced in most coastal states for years. But as stocks decline, water quality degrades and population pressures increase throughout the coastal zone, restoration projects have become more urgent in many sectors. While biological factors such as predator/prey relationships and physical factors such as current and sedimentation are important for the success of a restoration program, other less tangible factors can be equally important. Increasingly, the success of a project may depend on a holistic approach of embayment management that often includes land use issues, topics such as stormwater runoff, nutrient loading, proliferation of docks or erosion control structures and other human use impacts, issues that may not considered when planning a restoration effort. As coastal area population increases, land use and human marine use issues may have greater influence on the success of the restoration effort than traditional biological and physical factors. And yet, as land uses continue to degrade water quality, shellfish restoration projects are being considered as a counter measure, using the natural filtering capacity of shellfish to boost water quality. This paper discusses these issues as challenges to shellfish restoration efforts.

EVIDENCE FOR NATURAL SELECTION FOR RESISTANCE TO PSP TOXINS IN EARLY LIFE HISTORY STAGES OF THE SOFTSHELL CLAM, *MYA ARENARIA*. S. MacQuarrie*, V. Monica Bricelj, 1411 Oxford St. Halifax, Nova Scotia, Canada, B3H 3Z1.

Our prior research has demonstrated that sensitivity to paralytic shellfish poisoning (PSP) toxins, measured by behavioral and physiological indices, varies significantly among *Mya arenaria* populations with differing toxin exposure histories. Populations from PSP-affected areas are predominantly resistant whereas naïve populations are dominated by sensitive individuals. An extensive survey of *M. arenaria* populations supports this correlation over a wide geographical range. This study identifies the life history stages susceptible to selective pressures and demonstrates the potential for strong selection. Effects of toxin exposure were determined for larvae and spat from a population previously characterized as sensitive. Veliger larvae exposed to bloom levels of a highly toxic *Alexandrium tamarense* strain (PR18b) in a mixture with non-toxic algae showed no increased mortality relative to controls. However, spat (3.5mm) exposed to a monospecific suspension of PR18b suffered 95% mortality after 1-week exposure, resulting in a population dominated by resistant clams. Video observations suggest that anoxia of the pallial cavity may be responsible for mortalities. Ingestion of toxic cells is necessary to induce mortality and a single bloom of sufficient toxicity is capable of selecting for resistance at these stages. Results will be discussed in context of ecological relevance and fitness consequences.

OPTIMIZING OYSTER PRODUCTIVITY IN CARAQUET BAY: COORDINATING RESTORATION AND AQUACULTURE. **J. F. Mallet***, and **T. Landry**, DFO, P.O. Box 5030, Moncton NB, E1C 9B6.

Caraquet Bay represents the most northern location with a sustainable oyster (*Crassostrea virginica*) population. In recent years, a decrease in the productivity of oysters from the natural beds has generated interest in restoration projects. To determine the benefits of restoration activities, information on the distribution, abundance and population structure of oysters was collected in 1999. Over 60% of the oysters found were pre-recruits (35-75mm). These oysters were mainly found in the northern portion of the bed, which is locally renowned for its "stunted oysters". In 2001, "stunted" oysters along with control oysters were transferred to four stations and monitored for growth. Results to date show that growth oysters are associated with site and bottom conditions. In 2002, samples of "stunted" oysters were placed at three stations in various holding situations to evaluate the effects of vertical positioning in the water column and tidal position. The results from this experiment revealed that oyster productivity varies in relation to their geographical location. They will provide key information for the oyster fishers and aquaculturists to develop management strategies.

ROSEIMARINA CRASSOSTREAE (GEN. NOV., SP. NOV.) ASSOCIATED WITH JOD-SIGNS IN THE ABSENCE OF SIGNIFICANT MORTALITIES, AND FIRST ISOLATION FROM A NEW YORK EPIZOOTIC. **Aaron P. Maloy***, 5735 Hitchner Hall University of Maine Orono ME, 04469; and **Katherine J. Boettcher**.

The alpha-proteobacterium *Roseimarina crassostreae* (gen. nov., sp. nov.) has, to date, been isolated exclusively from episodes of juvenile oyster disease (JOD) in Maine. With few exceptions, isolates have been recovered from animals that probably would have died from the disease. Mortalities have been reproduced by experimental exposure to the bacterium, although without typical JOD-signs (e.g. conchiolin). Herein we describe induction of conchiolin in oysters challenged with *R. crassostreae*. Further, we report a 90% correlation between conchiolin and colonization by *Roseimarina* in a natural (but unusual) occurrence of JOD. The affected animals were from Maine's Damariscotta River where cumulative mortalities were < 5% in 2002 (down from 50% in 2001). Thus, these bacteria were isolated in the absence of significant mortalities. In fact, most of the 9% of animals with conchiolin appeared otherwise healthy. Finally, we document the first isolation of *R. crassostreae* from JOD outside of Maine. Analyses of the 16S-23S rDNA internal transcribed spacer region revealed that isolates from a 2002 New York epizootic were the same genotype (GT1) as those from Maine epizootics in 1997 and 1998. For unknown reasons, a slightly different genotype (GT2) appeared in

Maine in 2000, and thereafter replaced GT1 as the etiological agent here.

FINDING THE WHEAT IN THE CHAFF—OYSTER LARVAL FEEDING IN TURBID, LOW SALINITY CONDITIONS. **Roger Mann*** and **Peter Kingsley-Smith**, VIMS P.O. Box 1346 Gloucester Point, VA 23062.

Oyster restoration efforts in the Middle Atlantic States focus on a combination of benthic habitat refurbishment and brood stock supplementation, predominantly in low salinity sanctuaries from endemic disease. Central to this approach is the assumption that efforts increase local recruitment, yet we are ignorant of the influence of low salinity, elevated turbidity, and limited food availability on the survival and growth of oyster larvae. We suggest that in high turbidity regions available food is essentially diluted by indigestible inorganic material, and larvae may be food limited despite an apparently adequate absolute concentration of food because the relative food concentration is low. If this is the case then watershed management practices emphasizing nutrient reduction policies in excess of concomitant sediment load reduction may serve to reduce larval survival in receiving water bodies, and compromise restoration efforts. We offer numerical estimates of the impact of elevated turbidities on oyster restoration through decreased larval survival. We then investigate larval feeding behavior, as a proxy for overall larval viability, under both controlled salinity-turbidity conditions in the laboratory, and along a salinity-turbidity cline in the York-Mattaponi river systems of the Chesapeake Bay.

CHARACTERIZATION OF SUMMER MORTALITIES OF *CRASSOSTREA GIGAS* OYSTER IN RELATION TO PHYSIOLOGICAL PARAMETERS. **M. Mathieu***, **B. Dubois**, **K. Costil, C. Heude, A. Huvet, K. Kellner, S. Pouvreau**. Physiologie Ecophysiologie des Mollusques Marins IFREMER Université de Caen, 14032 Caen Cedex, France.

Pacific oysters are characterized by high fecundity, and follow a seasonal breeding pattern beginning in autumn with gamete maturation in spring and early summer. Summer mortalities occur during spawning period, but according to the environmental conditions gametes are spawned or reabsorbed. In Normandy, which is the northern more oyster cultivation area in France, spawning is often partial or absent. Mortalities were observed in both situations but more often when gamete release is delayed. The implication of hemocytes in gamete resorption as in storage tissue restoration was observed. The level of fecundity varies with trophic conditions. Storage material is accumulated in specific cells mainly in autumn and winter, and then mobilized to support gametogenesis. Resorption of gametes contributes to storage tissue development. Incorporation of metabolites in storage cells is performed by diffusion through cell membrane and by two transport systems sensitive to

internal regulation. The storage tissue follows a seasonal cycle regulated by internal factors, with reversion of its metabolism in summer.

PERKINSUS MARINUS INFECTION RATES IN SPECIFIC-PATHOGEN-FREE JUVENILE OYSTERS PLANTED IN THE PATUXENT RIVER, MARYLAND.

Carol B. McCollough* and Christopher F. Dungan, Sarbanes Cooperative Oxford Laboratory, Oxford, MD 21654; George R. Abbe and Candace A. Morrell, Academy of Natural Sciences Estuarine Research Center, St. Leonard, MD 20685.

Specific-pathogen-free (SPF) oysters were set and reared in artificial seawater and transferred to four sites in the Patuxent River along a salinity gradient. Three sites were adjacent to natural oyster bars and one, Sandgates, was remote from existing oyster populations. Samples of 30 oysters were assayed at 2 and 4 weeks post-deployment for infection by *P. marinus* using an enhanced RFTM whole body burden technique. Assays continued at 4-week intervals. Deployments were made in May and September 2002. From the May deployment, SPF oysters placed at Sandgates, remote from existing populations, acquired infections by day 27 (13% prevalence), as did juveniles deployed at TC (7%) and HP (3%). At all sites oysters acquired infections within 62 days, with prevalences of 10%, 63%, 43%, and 37% (TC - HP). By 91 days post-deployment all sites, with the exception of TC, had infection prevalences greater than 90%, and these elevated prevalences continued through 127 days. At 91 days TC prevalence remained low at 10%, but by 127 days it also increased, to 53%. In October prevalences declined at TC, GA, and SG (154 days), and all remained above zero into mid-November. SPF juveniles deployed in late September acquired *P. marinus* infections by 27 days at all sites, however prevalences were low and declined at 55 days, with infections detected then only at HP.

IS *MERCENARIA MERCENARIA* A HOST FOR *PERKINSUS* SPECIES?

Ayana McCoy*, Shirley Baker, Ruth Francis-Floyd, and Anita Wright, University of Florida Department of Fisheries and Aquatic Science 7922 NW 71st St Gainesville, Fl 32653.

Perkinsus marinus is an endoparasitic protistan that infects the Eastern oyster, *Crassostrea virginica*. This parasite has caused mass mortalities of oysters along the Atlantic and Gulf coasts. The commercially important *Mercenaria mercenaria* is cultured in areas naturally populated by *C. virginica*. Whether the hard clam, *M. mercenaria*, is susceptible to *Perkinsus* infection or serves as an intermediate host has not been well studied. Therefore, the objectives for this study were (1) to examine the diversity of *Perkinsus* species associated with *M. mercenaria* and *C. virginica* in the environment, and (2) to experimentally test the susceptibility of hard clams to *P. marinus* and *P. andrewsi* infections. *M. mercenaria* and *C. virginica* were collected from the Cedar Key area on

the Gulf Coast of Florida. Both species-specific PCR assays and standard Ray's Fluid Thioglycollate Media assays were used to identify associated parasites and determine levels of infection. Laboratory studies are in progress to determine virulence of *Perkinsus* species in *M. mercenaria*. This project should help to provide an indication of virulence potential of *Perkinsus* species for the hard clams on Florida's Gulf Coast and the possible threat of these parasites to the rapidly growing aquaculture industry in the region.

RECOMMENDATIONS TO OYSTER HARVESTERS ON REMOVING HOOKED MUSSELS, *ISCHADIUM RECURVUM*.

Earl J. Melancon, Jr.*, Biology Department, Nicholls State University, Thibodaux, La 70310; Dale Diaz, Mississippi Department of Marine Resources, Biloxi, Miss. 39530; Badiollah Asrabadi, Math Department, Nicholls State University.

Our results indicate that high salinity as a physiological factor to kill mussels is of minimal value to removal success. Predation, often by the blue crab, is the driving force on the removal of mussels from oysters. In addition, the physical process of harvesting with a dredge, using water cannons to move oysters on deck and then again to plant overboard can resulted in as much as a 33-38% direct mussel mortality; in turn, the dead and dying mussels attract predators. Planting to down-bay (high salinity) habitats will remove mussels within a short period of time if predators are present; perhaps in as little as week in summer water temperatures (25-30°C). Use of water cannon to move oysters on deck suppresses temperatures within the pile and allows relaying during summer months without harming oysters. The spray from the hose, the dripping from the stacked oysters and evaporative cooling all work together to keep air temperatures well below the heat tolerance of mussels and oysters. Observations also suggest that cultivation by breaking up oyster clusters may reduce mussel fouling in mid-bay and down-bay sites, but not necessarily at up-bay (low-salinity) sites.

A COMPARISON OF CRYOGENIC FREEZING TECHNIQUES AND THEIR USEFULNESS IN REDUCTION OF *VIBRIO VULNIFICUS* IN RETAIL OYSTERS.

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Freezing the oysters and storing them at freezing temperatures suppress the number of recoverable *V. vulnificus* from the oyster meat. There are various methods that can be used to achieve a frozen product. In this study the effectiveness of carbon dioxide and nitrogen was analyzed. A comparison of freezing whole oysters versus half shell oysters with this two freezing methods was also studied.

The oysters were processed using the commercial practices at each of the three seafood-processing plants. The samples were analyzed using the guidelines provided by the FDA Bacterial Ana-

lytical Manual. An initial sample of fresh unfrozen oysters was analyzed to determine the initial *Vibrio vulnificus* load followed by analysis of frozen samples at 1, 7, 14 and 21 days after storage at -10°C.

The study demonstrates that there is lower number of recoverable *V. vulnificus* when CO_2 is used for freezing than when nitrogen is used, but the overall decrease in *V. vulnificus* load in the fresh to frozen product is by 200,000 organisms per gram of oyster meat. There are few organisms recovered by 21 days regardless of the freezing method.

USING MOLECULAR GENETIC TECHNIQUES TO ASSESS OYSTER RESTORATION PROGRAMS AND PROJECTS. Coren A. Milbury* and Patrick M. Gaffney, College of Marine Studies, 700 Pilottown Road, Lewes, De 19958.

Restoration efforts are becoming essential in managing many of our ecological resources. Equally important are the assessment and monitoring of restoration programs. Recent advances in genetic techniques allow for the use of high-throughput and cost effective methods in restoration assessment and monitoring. We have used molecular methods to assess a restoration project by the Maryland Oyster Recovery Partnership and the University of Maryland. Four million Louisiana oysters were planted in the Choptank River, Maryland. *Crassostrea virginica* exhibits regionally diagnostic mitochondrial haplotypes, which provide a means to genetically differentiate Gulf Coast oysters from native oysters. Detection of newly recruited spat possessing the Gulf Coast haplotype in the Choptank River confirms the survival and propagation of the outplanted oysters and the contribution of new progeny. A high-throughput, synthesis-by-sequencing technique (Pyrosequencing) was used to determine the mitochondrial haplotypes of spat collected in the Choptank River. Of 4,566 spat analyzed, 94.2% possessed the North Atlantic haplotype, 5.3% had the South Atlantic haplotype, and 0.1% possessed the Gulf Coast haplotype. The results demonstrate the contribution of the outplanted Louisiana oysters to the resident Choptank River population, and show that effective monitoring of stock enhancement projects can be achieved with high-throughput molecular genotyping techniques.

CREATING SALT MARSHES TO ENHANCE PRODUCTION OF FISHERY SPECIES. Thomas J. Minello* and Lawrence P. Rozas, National Marine Fisheries Service, Southeast Fisheries Science Center, Galveston Laboratory, 4700 Avenue U, Galveston, TX 77551, U.S.A.

Salt marshes in the northern Gulf of Mexico are valuable nursery habitats for fishery species such as penaeid shrimps and blue crabs. Extensive marsh loss has led to numerous restoration projects in the region, but little design information has been available for optimizing fishery productivity from these created wetlands.

We have sampled the small-scale spatial distributions of shrimps and blue crabs in natural and created marsh systems and developed models to 1) estimate populations of these fishery species in marshes of different land-water configurations and 2) simulate population changes in created marshes with different land-water patterns. The amount of vegetation-water interface or edge in salt marshes is an important characteristic that can determine the fishery value of these habitats. Marsh creation projects that maximize edge are likely to be most productive for commercially important decapod crustaceans. Terracing and the formation of small marsh islands are two restoration techniques that produce a great amount of marsh edge and should provide productive habitats for penaeid shrimps and blue crabs.

GENETIC VARIABILITY IN REPRODUCTION AND SUMMER MORTALITY IN *CRASSOSTREA GIGAS*. Jeanne Moal*, Edouard Bédier, Pierre Gildas Fleury, Aimé Langlade, Yvette LeCoguie, Lionel Dégremont, Pierre Boudry, Jean René Le Coz, Stéphane Pouvreau, Martha Enriquez-Diaz, Christophe Lambert, Philippe Soudant, Jean François Samain. Ifremer, centre de Brest BP 70 29280 Plouzane, France.

Bi-parental families were produced in hatchery and tested in the field in 2001. Two sets of 5 families were constituted, selected on their high (R) and low (S) survival. These two sets were reared in Brittany from March to November 2002. Samplings were performed twice a month to obtain data on biometry, survival, reproductive cycle, biochemical composition, adenylate energy charge, hemolymph parameters (ions and defense system) and muscle strength.

"R" and "S" oysters exhibited different reproductive effort and spawning strategy. "R" oysters allocated less energy in gonad than "S" ones and presented a complete spawning at the end of August contrary to the "S" which spawns partially. Mortality started in July when the seawater temperature reached 19°C and affected mainly "S" oysters. Concentrations of Na and Cl ions in hemolymph were different for "S" and "R" from May to June. A bacterial increase in hemolymph (R and S) was observed during the same period. The adenylate energy charge was more lowered for "R" than for "S" oysters, just before the spawning event However, other physiological and immunological parameters were similar between the two sets during the phases of maturation and mortality but discriminated groups after the spawning peak.

PRELIMINARY PATHOLOGICAL INVESTIGATION OF THE WHITE ABALONE, *HALIOTIS SORENSENI*. James Moore*, Thea Robbins, Carolyn Friedman, Neal Hooker, Thomas McCormick, Melissa Neuman, Bodega Marine Laboratory P. O. Box 247 Bodega Bay CA 94923 USA.

Populations of white abalone *Haliotis sorenseni*, deep water inhabitants, were severely exploited in the 1970s following serial depletion of several other species found in shallower water. This

species appears to be nearing extinction and in 2000 became the first marine invertebrate to be listed under the federal Endangered Species Act. Acquiring health information is critical for planning recovery of this species. White abalone broodstock were collected in 1999-2000 prior to federal listing. Deaths of eleven of these animals appeared to be related to collection injuries or water quality problems rather than infectious disease. The etiological agent of withering syndrome (WS-RLP, withering syndrome associated Rickettsiales-like prokaryote), was not detected in any of the dead animals by histology or PCR. Juveniles produced from broodstock were held at 12, 15 and 18C and were exposed to the WS-RLP. Marked losses of and pedal atrophy in animals with severe WS-RLP infections demonstrated that white abalone are susceptible to withering syndrome. As in other abalone species, cool water provided some refuge from WS-RLP pathogenicity. No other significant pathogens were observed. The susceptibility of white abalone to WS must be considered in the formulation of recovery plans.

UTILIZATION OF POST-HARVEST TREATMENT AS A STRATEGY FOR REDUCING *VIBRIO VULNIFICUS* ILLNESSES. Ken B. Moore, ISSC 209-2 Dawson Road Columbia SC 29223.

Illnesses and deaths associated with the consumption of raw molluscan shellfish continue to be a significant public health concern for the Interstate Shellfish Sanitation Conference (ISSC). In 1995, the ISSC highlighted three main approaches for reducing *V. vulnificus*-related illnesses and deaths involving high-risk consumers. These included education of "high-risk" groups to avoid raw shellfish, more rapid post-harvest refrigeration of shellfish to prevent increases in numbers of the pathogen, and encouraging and promoting shellfish post-harvest treatments to reduce *Vibrio vulnificus* to non-detectable levels. The role of post-harvest treatment as a strategy to control *Vibrio vulnificus* has become more significant since the passage of the mandatory *Vibrio vulnificus* Illness Reduction Plan by the ISSC Voting Delegates in 2000. The establishment of collective illness reduction goals for core states has created a proactive approach for addressing *Vibrio vulnificus*-related illnesses and deaths. The ISSC remains committed to *Vibrio vulnificus* illness reduction and is continuing efforts to identify additional effective strategies.

CHARACTERIZATION OF NATURAL KILLER CELL-LIKE ACTIVITY IN THE EASTERN OYSTER, *CRASSOSTREA VIRGINICA*. Brenda M. Morsey* and Sylvain De Guise, Department of Pathobiology and Veterinary Science, University of Connecticut, 61 N Eagleville Road, U-89, Storrs, CT 06269, USA.

Natural killer (NK) cells are an important part of the innate immune system of mammals. However, little is known about NK-like cell activity in the Eastern Oyster, *Crassostrea virginica*. NK-like cell activity of oyster hemolymph cells was measured by a flow cytometric assay in which oyster hemocytes were incubated with DiO-labeled K-562 target cells, and propidium iodide to label dead target cells. For every individual oyster tested, higher effector-to-target cell ratios resulted in higher levels of target cell death. Moreover, NK-like activity of individual oysters was further enhanced by recombinant human interleukin-2. Enhancement of NK-like cell activity by interleukin-2 was more pronounced in pooled oyster hemolymph compared to individual oyster hemolymph samples. Our data demonstrate for the first time the presence of NK-like cell activity in a marine invertebrate. This activity can be enhanced by physiologically relevant concentrations of mammalian interleukin-2 which further suggest that some structural and functional homologues of the mammalian innate immune functions are conserved in invertebrates such as the oyster. The importance of oyster NK-like activity in protection against disease and pathogen control will be assessed.

FOOD AVAILABILITY IN A MUSSEL RAFT. Jessica Munro* and Carter Newell, Great Eastern Mussel Farms, P.O. Box 141, Tenants Harbor, Maine, 04860.

Current speed, phytoplankton concentration, detritus concentration, mussel biomass and mussel density are important determining factors in the growth rate of raft cultivated mussels. Periodic measurements of flow and food with depth inside and outside mussel rafts are used to determine seasonal and site specific food availability and mussel raft consumption. Field data is collected with Seabird CTD and current meter casts, water sampling, and weighing mussel lines with a crane scale. Seasonal stratification causes vertical variation of food availability to mussel rafts in Maine waters. The depletion of available food is a function of the biomass of a mussel raft and mussel raft hydrodynamics.

COMPARING TWO *MYA ARENARIA* POPULATIONS AS POTENTIAL CANDIDATES FOR SEEDING OPERATIONS. Bruno Myrand*, Station Technologique Maricole des Iles-de-la-Madeleine, Cap-aux-Meules, Canada, G0B 1B0, Réjean Tremblay, Société de Développement de l'Industrie Maricole, Gaspé, Canada, G5X 1T5; Lise Chevarie, Société de Développement de l'Industrie Maricole, Cap-aux-Meules, Canada, G0B 1B0; Fabrice Pernet, Université du Québec à Rimouski-Centre Aquacole Marin de Grande-Rivière, Grande-Rivière, Québec, G0C 1V0; and Diego Mantovani, Institut des biomatériaux du Québec, Université Laval, G1K 7P4.

It is important to identify a source of clams for seeding in Iles-de-la-Madeleine. Two populations were examined: Havre-aux-Basques (HB) and Dune-du-Nord (DN). No neoplasia were found. Both populations belong to the same stock and have a low multilocus heterozygosity. Growth was better at DN site for both populations and better for the DN clams at both sites. The HB clams had a very limited growth. These results will be interpreted according to scope for growth measurements. The fragility of the

shell was higher for HB clams. Therefore, the HB clams appear unsuitable for seeding.

PROPAGATION OF FRESHWATER MUSSELS FOR FRESHWATER PEARL PRODUCTION. Richard J. Neves*, Jess W. Jones, William F. Henley, and Rachel A. Mair, Freshwater Mollusk Conservation Center, Virginia Cooperative Fish and Wildlife Research Unit, Virginia Tech, Blacksburg, VA 24061.

The commercial harvest of mussel species suitable for pearl production could provide an incentive to replace wild-caught adults with laboratory-reared juveniles to sustain populations. The Freshwater Mollusk Conservation Center at Virginia Tech was the first facility in the United States to begin an annual propagation and release program focused on endangered mussel species. Initial research to identify host fishes, develop production and culture methods, and test culture technology required nearly 10 years of experimentation. Endangered juvenile mussels were released first in 1997, and subsequent annual releases total nearly 370,000 juveniles of 10 species. A new facility dedicated to propagation was completed in 2002, with capacity to address commercially harvested species, as well as those under federal protection. Should the harvest of particular species such as those with colored nacres increase, then culture techniques are now available to replace harvested specimens with progeny produced from the parental population.

AN EXPERT SYSTEM FOR THE OPTIMIZATION OF SHELLFISH RAFT CULTURE. Carter Newell* and John Richardson, Great Eastern Mussel Farms P.O. Box 141 Tenants Harbor, Maine.

An expert system combining computer-based methodologies for determining tidally driven flows, wave heights, flow through shellfish raft systems, and consumption of food by the shellfish with specially designed data collection techniques is being used to improve shellfish production on mussel rafts in Maine. Elements of the expert system are being incorporated into a single computer that operates in a "point and click" manner. A large scale flow model develops tidal flow boundary conditions for the three dimensional computational fluid dynamics (CFD) raft model, and predicts wave conditions relative to mooring specifications and site risk assessment. The detailed CFD raft model predicts current speed and chl a consumption relative to ambient flow speed and direction, shellfish biomass, and density distribution. Field data collection involves flow profiles, wave gauges, CTD casts, sediment traps and feeding chambers. Mussel biomass on culture ropes is monitored using a crane scale. Optimization of production cycles on shellfish rafts involves careful consideration of raft hydrodynamics, seasonal changes in food availability, and stocking densities.

LINKING HARD CLAM (*MERCENARIA MERCENARIA*) REPRODUCTION TO PHYTOPLANKTON COMMUNITY STRUCTURE: II. PHYTOPLANKTON COMMUNITY STRUCTURE AND FOOD COMPOSITION. Roger I.E. Newell*, Horn Point Laboratory UMCES Cambridge, MD 21613. Christopher Gobler, and Stephen T. Tettelbach.

Hard clam, *Mercenaria mercenaria*, recruitment has declined in some southern bays of Long Island, NY and we hypothesized that this was associated with changes in the phytoplankton community structure and overall patterns of primary production. We collected hard clams over an annual cycle for analysis of reproductive condition from five south shore bays of Long Island. Concurrently, ambient water was filtered for analyses of organic carbon and nitrogen, total and size-fractionated chlorophyll, and microscopic counts for the harmful brown tide picoplankter, *Aureococcus anophagefferens*. We found appreciable differences in seston composition that related to the observed differences in hard clam reproductive and tissue condition. Bay Shore and Patchogue had the highest total Chl a levels and organic carbon nitrogen and carbon of any bay. Paradoxically, clams from this location had the lowest condition index and reproductive effort. The size fractionated Chl a data, however, showed that the high levels of organic material at these two locations was mainly contributed by cells < 2 μm which are too small to be efficiently retained by adult hard clams and hence have no nutritional value. In addition, both Bay Shore and Patchogue had brown tide blooms at cell concentrations that inhibit adult hard clam feeding. We conclude that changes in the floristic composition of the phytoplankton community in at least some of the Long Island south shore bays is translating into appreciable differences in hard clam condition and ultimately into reducing total reproductive effort.

COMMERCIAL IMPLEMENTATION OF HIGH PRESSURE PROCESSING (HPP) FOR PACIFIC OYSTERS. David H. Nisbet*, Nisbet Oyster Co., P.O. Box 338 Bay Center, WA 78527.

High Pressure Processing (HPP) was first used commercially on Pacific oysters, *Crassostrea gigas* by Nisbet Oyster Co., Inc. a cultivator, processor and packer of Pacific oysters on Willapa Bay in Washington State. Initial pilot scale experimentation was centered on the oyster shucking protocol for pressure and dwell time regimes. Physical material flow proved a major obstacle to resolve in the feed and outfeed of the equipment. An engineering study was commissioned to determine real-time throughput capabilities of commercially available HHP equipment. Building design, product flow and ergonomics were also researched as the company expanded its processing facility. When the commercial high-pressure equipment installation was completed, studies were undertaken in collaboration with the Oregon State University Seafood Laboratory and Seafood Consumer Center. Extended sensory analysis and *Vibrio* control studies were considered most impor-

tant, as well as the development of other possible value added product candidates. The commercial considerations for high pressure processing included specific end product related studies and building design features including product flow, throughput analysis, ergonomics, equipment maintenance, and cleanup. Additionally, the physical size of Pacific oysters relative to available hydrostatic chamber size capabilities constitutes special considerations for HPP commercial installations.

OPTIMAL PLANTING CONDITIONS FOR MAXIMUM REPRODUCTIVE OUTPUT OF CAGE-PLANTED SCALLOPS, *ARGOPECTEN IRRADIANS*, IN ANCLOTE, FLORIDA.

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As part of an ongoing effort to restore bay scallop populations on the west coast of Florida, we compared the growth, survivorship and gonadal development of bay scallops planted in cages at various densities and planting conditions in the Anclote estuary. To test density effects, scallops were planted in 0.6-m L × 0.6-m W cages, constructed from 12.7-mm-mesh, within a seagrass bed. Densities of 50, 150 and 300 scallops per cage were tested in triplicate. Growth, survivorship, and gonadal development were monitored every six weeks between July 1999 and July 2000. Planting at 150 scallops per cage resulted in the most live scallops available for fall spawning. To test the effect of planting condition, 50 scallops per cage were planted in triplicate in each of four treatment combinations including within and outside a seagrass bed and either directly on the substrate or raised 20 cm above the substrate. Growth and survivorship were monitored every six weeks between September 1999 and April 2000. Results indicate that growth and survivorship were significantly lower in the cages planted directly on the substrate within the seagrass bed, but no significant difference was detected among the remaining treatments.

WATER LOSSES, SEASONAL MASS LOADING, AND BEST MANAGEMENT PRACTICES FOR CRAWFISH PONDS.

Landon D. Parr*, Robert P. Romaire, and W. Ray McClain. Louisiana State University AgCenter, Aquaculture Research Station, 2410 Ben Hur Road, Baton Rouge, Louisiana 70820.

Some crawfish (*Procambarus clarkii* and *P. zonangulus*) ponds discharge into impaired water bodies in Louisiana. The objectives of this research were to develop water discharge models, determine seasonal mass loading of solids and nutrients, assess effluent quality during final drawdown (May through June), and identify best management practices for crawfish ponds. Average crawfish pond water loss during a production cycle was 228 cm and was partitioned among evapotranspiration (68%), precipitation overflow

(13%), final drawdown (13%), and infiltration (6%). Modeling indicated that 15-cm of water storage capacity reduced precipitation overflow by 28% in high precipitation years, 61% in average precipitation years, and 100% in low precipitation years. Predicted mass loading was greatest in the winter (precipitation overflow) and late spring through early summer (final drawdown). During final drawdown, total suspended solids (TSS) were high in the first 5% and last 20% of water discharged. During final drawdown, deep vegetated ditches provided the best TSS reduction compared to narrow, shallow, non-vegetated ditches. Slow draining from the water surface and avoiding drainage of the final 20% of the pond volume are recommended best management practices. The final 20% of the pond volume can be treated in deep vegetated ditches, settling basins, or constructed wetlands.

EFFECTS OF *KARENIA BREVIS* ON SHELLFISH: DOES STRAIN MATTER?

Susan E. Pate,* Jeffrey J. Springer, and JoAnn M. Burkholder, Center for Applied Aquatic Ecology, North Carolina State University, Raleigh, NC 27606; Sandra E. Shumway, Department of Marine Sciences, University of Connecticut, Groton, CT 06340.

Red tides are found in the Gulf of Mexico and the coast of Florida and consist primarily of the toxic dinoflagellate, *Karenia brevis* (Davis). Previous studies show lipid-soluble polyether toxins (brevetoxins, PbTx) can accumulate by several species of shellfish exposed to *K. brevis*. Bloom characteristics, shellfish grazing rates, and biotransformative processes influence shellfish toxin levels. Little is known regarding interactions between shellfish and varying strains of *K. brevis*.

Experiments were conducted involving three bivalve species (*Argopecten irradians, Crassostrea virginica, Mercenaria mercenaria*). The three *K. brevis* strains represent low, moderate, and high levels of brevetoxin production and were introduced at cellular concentrations during a bloom event. Behavioral response and grazing rates were determined for each species versus each *K. brevis* strain. In addition, we microscopically examined fecal material to determine whether cells remained intact and viable after passage through the digestive tract. Preliminary results indicate that some cells pass through the shellfish digestive tract intact.

ASSESSMENT OF THE EPIZOOTIOLOGY OF *PERKINSUS* SPP. ON THE ATLANTIC COAST OF USA USING GENUS-, SPECIES-, AND STRAIN-SPECIFIC MOLECULAR PROBES.

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P. marinus represents a major cause of mortality of the eastern oyster (*Crassostrea virginica*) along the Gulf of Mexico and Atlantic coasts of the USA. Based the fluid thioglycolate medium (FTM) assay. *Perkinsus* infections attributed to *P. marinus* have been reported as far north as Maine but although infection preva-

lence in Northeast regions may be high, it may not correlate with oyster mortality. In addition to the influence of environmental factors, the presence of other *Perkinsus* species/strains that exhibit reduced pathogenicity for *C. virginica* may explain these observations. Two recently described species, *P. chesapeaki* and *P. andrewsi* that test positive by the FTM assay, can also be present in clams and oysters, but their virulence remains unknown. Thus, the accurate prevalence assessment of *Perkinsus spp* is needed for the detailed understanding of epizootic events. To discriminate between *P. marinus*, *P. andrewsi* and other *Perkinsus* species our laboratory has developed species-specific PCR-based assays. We are applying these molecular probes to investigate the epizootiology of *Perkinsus* species and strains in oysters, hard clams, and other shellfish along the East Coast (from ME to VA). [Supported by ODRP, NOAA award NA06RG0101-5, through the MD Sea Grant College, to GRV].

ECOLOGICAL EFFECTS OF FISHING: BIOLOGICAL, PHYSICAL, AND SOCIOLOGICAL IMPACTS OF DERELICT AND ABANDONED CRAB TRAPS IN MISSISSIPPI.

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The wire crab trap dramatically changed the Gulf of Mexico Blue crab (*Callinectes sapidus* Rathbun) fishery. Crab traps were introduced in Louisiana and Texas as early as 1948 and by the mid-1950s were widely accepted throughout the Gulf. While adoption of the crab trap had a positive impact on fishing efficiency and harvest, proliferation of traps has resulted in an increase in the problems associated with lost or discarded traps. Derelict traps contribute to the mortality of blue crabs and other bycatch, exacerbate user group conflicts, create visual pollution, and may cause damage to sensitive habitats. Derelict traps result form abandonment of fishable traps by fishermen and the inadvertent loss of actively fished traps from: 1) weather/hydrological factors, 2) deterioration of buoys, lines, or knots, 3) negligence in assembling and maintaining gear, 4) use of plastic jugs/bottles as floats, 5) clipping of float lines by vessel propellers, and 6) intentional cutting of buoy lines by vandals. Conservative estimates of trap loss for the Gulf of Mexico approach 250,000 traps per year. Hundreds of traps litter coastal waters in eastern and western Mississippi Sound. Concern over the magnitude of the problem and the potential impacts to the blue crab resource prompted Mississippi to develop a program to remove these traps form near shore waters.

THE REGISTRY OF TUMORS IN LOWER ANIMALS: A RESOURCE FOR BIVALVE CULTURE HEALTH STUDIES.

Esther C. Peters*, Tetra Tech, Inc., Fairfax, VA 22030; **Marilyn J. Wolfe** and **Jeffrey C. Wolf**, Experimental Pathology Laboratories, Inc., Herndon, VA 20172-0474.

Neoplastic diseases have been recognized in several orders of bivalves. Of particular concern for culture efforts are hemopoietic neoplasms of mussels and soft-shell clams and gonadal neoplasms of quahogs. The etiologies of these diseases are unknown but studies suggest that factors which could be manipulated in culture, such as diet, genetics (hybridization or breeding for disease resistance and faster growth rate to market size), and environmental conditions (water quality, crowding) could influence the development of these and other cellular proliferative disorders. The Registry of Tumors in Lower Animals (RTLA) has been moved to Experimental Pathology Laboratories, Inc., under contract to the National Cancer Institute, and will continue to provide a global resource for investigators interested in bivalve diseases. The collection of contributed specimens and reprints will be expanded and Internet access to a searchable and illustrated database provided. The RTLA welcomes visitors (by appointment) and will offer diagnosis of bivalve diseases contributed for archiving and training in comparative histopathology.

USING CREATED OYSTER REEFS AS A SUSTAINABLE COASTAL PROTECTION AND RESTORATION TOOL.

Bryan Piazza*, John Plunket, John Supan and **Megan La Peyre**, U.S.G.S. Louisiana Fish and Wildlife Cooperative Research Unit, School of Renewable Natural Resources, Louisiana State University Agricultural Center, Baton Rouge, LA 70803.

Protection and restoration of coastal shorelines remains a priority worldwide. This study tested the viability of creating sustainable oyster reefs for use as a coastal protection and restoration tool in Caillou (Sister) Lake, Louisiana. Six oyster shell reefs (approximately 25 m × 2 m × 0.75 m) were created along the shoreline during June 2002 in two areas representing typical low and high-energy environments. Reefs were located approximately 3–5 m from shore (60 – 90 cm deep). Marsh vegetation was dominated by *Spartina alterniflora, Juncus roemerianus*, and *Distichlis spicata*. The value of reefs for protecting shorelines was determined by tracking shoreline position and adjacent marsh health (vegetation biomass, redox, sediment accretion) at paired cultched and non-cultched sites. Reef sustainability was determined by measuring recruitment and survival of oyster spat. Fisheries value of the reef was quantified by sampling nekton. Recruitment and survival of oyster spat increased throughout the spring and summer. Fish community usage of cultched and non-cultched sites was similar and dominated by *Anchoa mitchilli*. Shoreline retreat appears to be slightly higher in high energy, non-cultched sites. Minimal movement and reworking of shell through two tropical storm events showed that reefs were stable.

BLUE CRAB (*CALLINECTES SAPIDUS*) GENETIC STRUCTURE AND DIVERSITY. **Allen R. Place*, Colin R. Steven,** and **Xiaojun Feng,** Center of Marine Biotechnology Suite 236 701 E. Pratt Street Baltimore, MD 21202.

A responsible approach to marine stock enhancement requires that potential negative impacts upon the gene pools of wild populations be mitigated through the use of genetically sound breeding and release protocols. Studies over the past decade of patterns of genetic variation and divergence in a variety of pelagic marine organisms have demonstrated that high dispersal potential at any of several life-history stages does not necessarily indicate high levels of actual gene flow and uniformity in population structure. Three published studies describing the population genetics of *Callinectes sapidus* all indicate substantial gene flow, with values sufficiently high to infer panmixia between all blue crab populations from New York to Texas. Despite this high level of gene flow, two striking patterns of temporal and geographic differentiation occurred: genetic patchiness and clinal variation. These studies were done with protein polymorphisms (allozymes) which are less diagnostic of population substructure than the more variable genetic markers found in mitochondrial and nuclear DNA. To help distinguish hatchery-raised crabs from wild cohorts we have characterized the genetic variability in both the mitochondrial genome and nuclear genomes of *Callinectes sapidus*. The implications of these findings to the overall genetic structure of *Callinectes sapidus* will be addressed.

FIBER DIGESTION IN THE BLUE CRAB, *CALLINECTES SAPIDUS*. **Allen R. Place*, Andrea Findiesen,** and **Nilli Zmora,** Center of Marine Biotechnology 701 East Pratt St. Baltimore, MD 21202.

A wide range of digestive enzymes have been reported in crustacea indicative of the diverse dietary preferences of the different species. Two of the most important carbon containing compounds in the blue crab diet are chitin (an unbranched homopolymer of b 1-4 linked N-acetyl-D-glucosamine residues, NAG) and cellulose (an unbranched homopolymer of b 1-4 -D-glucose residues, Glc). The traditionally held view of chitin and cellulose digestion in higher animals and invertebrates has been that gut microbes confer the ability to degrade these two polymers. However, recently the genes for chitinase and cellulase have been detected in the genomes of crustacea. Accordingly, using degenerate primers designed from aligned sequences of chitinases and cellulases, we have begun screening a heptopancreas cDNA library of the blue crab. Currently, we have isolated a 479 bp fragment that is highly homologous to the vertebrate and insect chitinase and just starting to probe for crab cellulases. Given that these two polymers are the two most abundant and renewable energy resource on earth, effective utilization of these fibers especially in diets for aquaculture rearing will be an important key to improving production and feed conversion efficiency in the future.

A COMPARISON OF FINFISH ASSEMBLAGES ON SUB-TIDAL OYSTER SHELL (CULTCHED OYSTER LEASE) AND MUD BOTTOM IN BARATARIA BAY, LOUISIANA. **John Plunket*, Megan La Peyre,** U.S.G.S. Louisiana Fish and Wildlife Cooperative Research Unit, School of Renewable Natural Resources, Louisiana State University, Baton Rouge, LA 70803.

Recent research suggests that oyster reefs provide unique three-dimensional habitat for many fish species. Along the northern shore of the Gulf of Mexico, oyster shell bottoms are predominantly flat, subtidal and cultched, providing a very different habitat. In this study, we compared finfish assemblages and gut contents at subtidal oyster shell (cultched oyster lease) and mud bottoms in Barataria Bay, Louisiana. Three mud and three shell sites were sampled from October 2001 to October 2002, using gill nets with mesh ranging from 25.4-63.5 cm, and 60×50 cm substrate trays. Data from the gill nets were used to compare fish assemblages, and to document diets through gut content analysis. Data from the substrate trays were used to document benthic fish and invertebrate communities associated with the subtidal cultched oyster shell habitat. Finfish abundance was greater at shell ($N = 223$) versus mud ($N = 170$) bottoms, with higher numbers of sciaenid fishes over shell. Substrate trays collected a variety of benthic fish and invertebrates, primarily naked gobies (*Gobiosoma bosc*), skilletfish (*Gobiesox strumosis*), toadfish (*Opsanus beta*) and xanthid crabs. These results support the contention that shell bottoms support unique communities of fish, as compared to mud bottom habitats.

A COMPARISON OF NEKTON USAGE OF MUD BOTTOM, CREATED LIMESTONE, SHELL, AND NATURAL SHELL REEF HABITATS IN TERREBONNE BAY, LOUISIANA. **John Plunket*, Gary Peterson, Bryan Piazza** and **Megan La Peyre,** U.S.G.S. Louisiana Cooperative Fish and Wildlife Research Unit, School of Renewable Natural Resources, Louisiana State University Agricultural Center, Baton Rouge, LA 70803.

Restoration of coastal environments increasingly involves habitat creation for fisheries species. The creation of artificial reefs is based on the assumption that estuarine hard-bottom habitats support more diverse, complex communities than soft bottom habitats. In Louisiana, the creation of artificial reefs has recently become a focus of activity among recreational fisherman and coastal managers. In 2002, we compared finfish abundance on a natural shell reef, a created clam shell reef, a created limestone rubble reef, and a mud bottom site in lower Lake Pelto, Louisiana. The four sites were sampled over one year using 200′ experimental gill nets, an 8′ otter trawl and fish traps. On average, species diversity was two times higher on natural and created reefs ($N = 15$), as compared to mud bottom ($N = 7$). The created limestone and natural reef consistently supported the more diverse, as well as the more even (Pielou's J) communities throughout the year. Sorenson's commu-

nity similarity index indicates large dissimilarities between the created reefs and the mud bottom (S<0.23), and more similar communities between both created and natural reefs (S>0.5). The two artificial reefs support communities of greater diversity and evenness than mud bottom habitat, and are comparable to natural reefs in diversity, but vary in species composition.

CONSUMER PREFERENCES AND ATTITUDES TOWARD IRRADIATED OYSTERS.

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Consumer attitudes and preferences toward raw oysters in general, and irradiated oysters, in particular, were evaluated from results of consumer surveys conducted through personal and telephone interviews. Seventy five interviews were conducted at the MSU-Coastal Aquaculture Unit Open House in Gulfport, Mississippi on December 6, 2001. Another survey was conducted at the MSU-Coastal Research and Extension Center booth among 140 participants of the 2002 International Boston Seafood Show in Boston, Massachusetts on March 12-14, 2002. Telephone interviews with a simple random sample of adults living in the Baltimore and Houston MSAs in households with telephones were done by the Survey Research Unit (Social Science Research Center at Mississippi State University) in June of 2002. Households were selected using random digit dialing procedures. Of the eligible respondents contacted in the Baltimore Metropolitan Statistical Area (MSA), 610 completed the interview and 85 refused to participate. Of the eligible respondents contacted in the Houston MSA, 606 completed the interview and 67 refused to participate.

FORM AND FUNCTION IN OYSTER REEFS: INFLUENCE OF REEF MORPHOLOGY ON HABITAT FUNCTION AND OYSTER SURVIVAL.

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With the decline in natural oyster's reefs there is increasing interest in restoration of reef habitat for fishery and ecosystem functions. Oyster reefs provide important structural habitat and have significant ecosystem impacts. However, the function of oyster reefs varies with reef morphology, especially vertical complexity that may affect 3-dimensional characteristics of the reef surface, edge convolution that may affect encounter surfaces for intertidal reefs and reef fragmentation. We have begun a multi-year study examining the influence of vertical complexity, edge convolution and fragmentation on faunal use, ecosystem function, and oyster settlement and survival on intertidal created oyster reefs in southeastern North Carolina. Reefs have been established with blocked high and low vertical complexity and circular versus convoluted edge as well as small and large fragment reefs. We are

assessing sediment nutrient fluxes, benthic microalgae, infauna, epifauna, and nekton use of these reefs through a variety of sampling approaches to examine community responses to variations in landscape factors. Reefs were established in 2002 and initial results indicate strong effects of vertical complexity and fragmentation and weaker effects for edge characteristics. Efforts to restore oyster reefs should consider the potential influence of reef design on ultimate habitat function.

REPRODUCTION, BIOENERGETIC AND SUMMER MORTALITY *OF CRASSOSTREA GIGAS*: EXPERIMENTAL APPROACH.

Stéphane Pouvreau*, Martha Enriquez-Diaz, Pierrick Le Souchu, Jean Paul Connan, Bertrand Le Roy, Christian Mingant, Jeanne Moal, Maryse Delaporte, Jean René Le Coz, and Jean Francois Samain, *UMR PE2M \\\"Physiologie et Écophysiologie des Mollusques Marins\\\", Station Expérimentale d\\\'Argenton, 11 presqu\\\'île du vivier, 29840 Argenton (FRANCE).

As a part of the French MOREST program, we examine experimentally, in 2002, the relationships between food level, reproductive processes, bio-energetic status and mortality on 3 batches of the same hatchery oyster population, produced in 2001. Each lot underwent a same annual temperature cycle (from 8 to 20 °C), a same food composition (4 algae), but a different food level: low (~30 cell.μl-1), medium (~60 cell.μl-1) and high (~100 cell.μl-1). Each month, several parameters were followed: (1) somatic growth, storage, gametogenesis using quantitative histology; (2) clearance rate, absorption efficiency, oxygen consumption and scope for growth (SFG), (3) biochemical composition. Results demonstrated that oysters under high food availability showed an accelerated gametogenesis and the highest reproductive effort. At the maximum of gametogenesis development (i.e. July), these oysters exhibited also the highest oxygen consumption and consequently the lowest SFG values. Experimental infection (by *Vibrio lentus* as infectious agent) confirmed this relative weakness in relation with the reproductive effort.

As a conclusion, it appears that food level that controls the reproductive effort can generate a bioenergetic imbalance at high trophic conditions. Thus, summer mortalities in eutrophic areas could be partly explained by these processes.

A COMPARISION AND FEASIBILITY STUDY OF TWO DIFFERENT BIOMONITORING SYSTEMS USING THE BLUE MUSSEL, *MYTILUS EDULIS*, AND THE AMERICAN LOBSTER, *HOMARUS AMERICANUS*.

Heidi Pye*, Winsor H. Watson III, Christopher Rillahan, Rachel Hamilton, and Jennifer Wishinski, 46 College Rd Zoology Department-UNH Durham, NH 03820.

The advantages of biomonitoring in accordance with traditional techniques include: 1.behavioral and physiological responses are more sensitive indicators of contaminant-induced stress, 2. While

traditional instrumentation measures specific substances, organisms integrate all stressors to provide an indicator of overall water quality. 3. and if utilizing keystone species the information will help assess impact of the contamination at population and community levels. To effectively use a bioindicator it is necessary to characterize its response and sensitivity (detection threshold) to contaminants. Our goal was to compare the response and sensitivities of the American lobster, *Homarus americanus* and the blue mussel, *Mytilus edulis*, to four different heavy metals (CuCl, CrCl3, PbCl2, CdCl2) common in the Great Bay Estuary. In general, detection levels were lower for mussels (0.5ppm CuCl, <1ppm PbCl2, >30ppm CdCl2) than lobsters (1ppm CuCl, 50ppm CrCl3, > 50ppm PbCl2, CdCl2). Clear responsiveness was limited to CuCl which occurred close to lethal levels (for *H. americanus* 1ppm response, 2ppm LD50). Given these results we would recommend using mussels, due to their higher sensitivity and ease of use. The only drawback is that mussels are sensitive to a variety of other environmental perturbations that can make responses to heavy metals difficult to elucidate.

LARVAL ECOLOGY: MOLECULAR TOOLS FOR THE BLACK BOX? Paul D. Rawson, School of Marine Sciences 5751 Murray Hall, University of Maine Orono, ME 04469-5751.

Many marine invertebrates, including ecologically and commercially valuable shellfish, have biphasic life histories with a relatively long-lived and highly dispersive larval stage. Ecologists have recognized the role that larval supply and settlement play in population and community dynamics while geneticists have focused on the impact that larval dispersal has on the distribution of genetic variation. Dispersal and settlement, in turn, are dependent on the local abundance of larvae, which can be extremely variable in space and time. Traditional methods for identifying and enumerating larvae can be time consuming, and because of the morphological similarity between larvae of many species, requires specialized training. Thus, our understanding of the links between planktonic processes that generate larval patchiness and larval settlement can perhaps be represented by a black box. Molecular methodologies, in particular PCR-based methodologies, provide tools for peering into this black box by allowing the rapid, and perhaps quantitative, analysis of larval abundance. We will discuss the development of some of these methodologies, the advantages and pitfalls associated with them, as well as providing examples of their application from work currently being conducted in our lab.

STATUS OF *PERKINSUS MARINUS* IN GALVESTON BAY, TEXAS: RESULTS OF THE DERMOWATCH PROGRAM. Sammy M. Ray,* Department of Marine Biology, Texas A&M University at Galveston, Galveston, TX 77553; Thomas M. Soniat, Department of Biology, Nicholls State University.

DermoWatch is a web site (www.blueblee.com/dermo), a monitoring program and an online community for the management of the oyster parasite, *Perkinsus marinus.* The web site contains an

embedded model, which calculates a time to critical level of disease from an initial weighted incidence of disease and water temperature and salinity. Six public reefs and three private leases in Galveston Bay have been sampled monthly since December 1998. The web site displays the most recent data from each site on the home page and archives all data, such that an historical record is maintained. Historical data show high levels of disease during the drought years of 1999 and 2000. With the cessation of the drought in 2001 and heavy rains associated with tropical storm Allison in June of 2001, disease levels throughout the Bay have been depressed.

SEASONAL AND TEMPORAL VARIABILITY IN CONDITION INDEX AND TISSUE BIOCHEMISTRY OF *ELLIPTIO COMPLANATA*. Deborah Raksany*, Catherine M. Gatenby and Danielle A. Kreeger. The Academy of Natural Sciences 1900 Ben Franklin Pkwy Philadelphia, PA 19103;

Due to diminishing biodiversity and habitat, it is imperative that we better understand the biology and the ecological functioning of our existing freshwater mussel populations. Temporal variability in the condition and physiological status of marine shellfish has been well studied, but there remains a dearth of knowledge with respect to these trends in freshwater mussels. Our goal was to quantify variability in physiological condition of *Elliptio complanata*, a common freshwater mussel in the Atlantic drainage. Condition index and proximate tissue biochemistry (protein, lipid, and carbohydrate) were monitored in adults collected from a healthy population over a three-year period. Both parameters varied seasonally and among years for similar seasons. For example, condition index peaked in August of 2000, but reached its peak in October of the following year. These results reflect the reproductive and seasonal conditioning processes of these animals, which may be responsive to environmental cues. By understanding temporal shifts in the physiological status of these animals in nature, we will be better equipped to gauge their functional roles in freshwater ecosystems and formulate appropriate diets to sustain them in captivity.

NUCLEIC ACID-BASED AQUATIC PATHOGEN MOLECULAR DIAGNOSTICS FOR DETECTION, RESEARCH AND ENVIRONMENTAL MONITORING. Kimberly S. Reece, Virginia Institute of Marine Science, Gloucester Point, VA 23062.

Advances in molecular genetic technology have facilitated progress on many fronts of aquatic disease research including pathogen identification, detection, and studies examining transmission dynamics, epizootiology, virulence mechanisms and host/parasite/environment interactions. Probes for in situ hybridizations and primers for use in PCR are now available for many pathogens found in the aquatic environment. These nucleic acid-based mo-

lecular detection methods can improve sensitivity and efficiency of disease diagnoses and detection of organisms in environmental samples, especially where it is difficult and/or time-consuming to isolate and identify pathogens. Rapid and accurate molecular detection assays have been developed to facilitate both field monitoring programs and studies to examine the effects of various environmental parameters on growth and distribution of pathogens. Studies that employ different molecular detection techniques will be presented including those where real-time PCR assays are being used to quantify the number of pathogen cells in water samples for environmental monitoring programs and disease transmission studies. In situ hybridization assays have been developed for confirming the identity of parasites in host tissues and for detecting pathogenic organisms in the gut contents of bivalves that, because of their filter-feeding behavior, are natural integrators of the water column.

VALIDATION OF POST-HARVEST PROCESSING OF *VIBRO PARAHEMOLYTICUS* IN OYSTERS: SPEED BUMPS ON THE ROAD FROM THE RESEARCH LAB TO THE PROCESSING PLANT. P. W. Reno*, Y-C. Su, M. Morrissey, and D. Nisbet, HMSC 2030 S. Marine Science Dr. Newport, OR 97365-5296.

Small scale laboratory experiments were carried out to determine the efficacy of high pressure processing in inactivating *Vibrio parahemolyticus* (VP), particularly serotype O3:K6, with in Pacific oysters. Oysters were held at HMSC isolation facility with uv-irradiated, sand-filtered seawater that had no detectible VP in the incoming water or in oysters. The oysters were exposed via bath for 3 h in static seawater with between 103 and 106 cfu/mL of VP. Bacterial counts per gram of oyster meat approximated the VP count per mL water. Bacterial counts remained stable in oysters for at least 10 h at 10°C. The results of these tests indicated >105/g reduction in colony counts was achieved at 310mPa/2min in a 1.5-L pressure unit. Transfer of the technology from the small scale (1.5 L capacity) research laboratory to a pressure unit operating under commercial processing conditions was undertaken to validate the process to accede to anticipated FDA requirements. Using a commercial pressure unit of 42 L volume, a series of time/pressure combinations are currently under way to determine the efficiency of killing under commercial conditions on oysters exposed by the techniques used in the research laboratory. The process is still ongoing, but results appear promising.

COMPUTATIONAL FLOW MODELING OF AQUACULTURE SYSTEMS. John Richardson*, Alden Research Laboratory, 30 Shrewsbury St., Holden, MA 01520-1843; **Carter Newell,** Great Eastern Mussel Farms, P.O. Box 141, Tenants Harbor, Maine 04860.

The successful design of floating raft-culture systems requires knowledge of how water circulates through the raft-culture structures. In this research advanced Computational Fluid Dynamics (CFD) Techniques were used to model flow through raft-culture systems used to grow oysters and Blue Mussels. The basic modeling techniques are general, and they can also be used to model the flow through other types of aquaculture systems (marine or terrestrial). The analysis techniques used for this study are capable of accurately simulating the 3-dimensional flow of water through raft-culture structures located in areas with complex bathymetries. The analysis scheme can, additionally, be used to simulate the transport of nutrients and wastes through the floating rafts.

CHARACTERIZATION OF THE *CRASSOSTREA VIRGINICA* SLC11A GENE (FORMERLY NRAMP). José A. F. Robledo* and Gerardo R. Vasta, COMB, UMBI, University of Maryland, Baltimore, MD 21202, USA.

Perkinsus marinus has been associated to extensive damage to oyster populations, with catastrophic consequences for shellfisheries. Although, selective breeding approaches for development of disease-resistant oyster stocks are promising, the identification of genes that are directly linked to disease-resistance/susceptibility represents an attractive alternative. The Slc11a (former Nramp: natural resistance-associated macrophage protein) is a divalent cation transporter, demonstrated to be a determinant of resistance/susceptibility to intracellular pathogens. Most parasites have developed efficient mechanisms for iron acquisition from their hosts. Reciprocally, most hosts have developed mechanisms to prevent pathogens from acquiring iron. Iron sequestration from the pathogen is also a non-specific host response to infection (nutritional immunity), and Slc11a is a critical component in this response. We have already characterized the *P. marinus* Slc11 (PmSlc11a) and obtain partial sequence of the *C. virginica* Slc11a (CvSlc11a). Sequence information was used for screening a *C. virginica* genomic library resulting in several clones' positives for CvSlc11a. The characterization of CvSlc11a gene in both host and parasite will provide insight into their competition for iron, and yield information on the mechanisms underlying disease susceptibility [Grant No NA06RG0101-5 from ODRP, NOAA, through the Maryland Sea Grant College to GRV].

***PERKINSUS MARINUS* CELLULAR BIOLOGY USING EXPRESSION SEQUENCE TAGS (EST). José A. F. Robledo*, Eric J. Schott, and Gerardo R. Vasta** COMB, UMBI, University of Maryland, Baltimore, MD 21202, USA.

During the last five years virtually all fields of biology have benefited from the tremendous volume of information generated by genomic approaches. Embedded within genomic sequence data is information needed for identifying targets for drug development and for dissecting the biological aspects that may constitute the basis for infectivity and pathogenicity. *Perkinsus marinus* has been associated with mass mortalities of the eastern oyster, *Crassostrea virginica*, for more than 50 years and although substantial progress

in understanding the disease has been accomplished, effective prevention or treatment methods are still lacking. Previously, we presented a dataset consisting of 300 ESTs generated from two *P. marinus* cDNA libraries constructed using *P. marinus* propagated in standard culture medium and in medium supplemented with *C. virginica* serum. We now present the analysis of a more extensive EST dataset corresponding to both libraries, focusing on those *P. marinus* genes or metabolic pathways that may be unique to this parasite, or which have been targeted for intervention in other parasite species. Based on our increased knowledge of *P. marinus* genomics/biology, possible strategies to enhance anti-parasite responses in the oyster will be discussed [ODRP, NOAA award NA06RG0101-5, through the MD Sea Grant College, to GRV].

DEVELOPMENT OF AN INDIVIDUAL, ENERGY-BALANCE BASED, GROWTH MODEL FOR THE MANILA CLAM (*RUDITAPES PHILIPPINARUM*). J. Flye Sainte Marie*, S. E. Ford, E. Hofmann, F. Jean, J. Klinck, C. Paillard, E. Powell, LEMAR, Univ. Bretagne Occidentale Inst. Univ. Européen de la Mer, Place Copernic F-29280 PLOUZANE.

To study Brown Ring Disease in the Manila clam, *Ruditapes philippinarum*, caused by the bacterial pathogen *Vibrio tapetis*, an environment-host-pathogen interaction model is being developed. As a base upon which to build a population model, an individual growth model, which does not include the pathogen, was first developed. The aim of the present study was to calibrate, to validate and to do a sensitivity analysis on this model. The model simulates the length and weight increase of an average individual under forcing of two environmental variables: temperature and food. Model simulations approximate the scope for growth and spawning events observed in nature. On the other hand, the simulations showed that chlorophyll a concentrations are not an adequate substitute for food availability for this infaunal bivalve. Although additional data are needed to develop a relationship between growth and food availability in the field, sensitivity analysis showed that this model is responsive to the parameters that determine scope for growth.

THE ANTIOXIDANT PATHWAY OF *PERKINSUS MARINUS*: FUNCTIONAL ANALYSIS AND LOCALIZATION OF TWO IRON SUPEROXIDE DISMUTASES. Eric J. Schott*, José A. F. Robledo, Wolf T. Pecher, Florence Okafor, and Gerardo R. Vasta, Center of Marine Biology 701 E. Pratt St Baltimore, MD 21202.

The economic and environmental impacts of *Perkinsus marinus* epizootics make imperative the understanding of this parasite's virulence mechanisms. It has been proposed that viable *P. marinus* trophozoites rapidly suppress or detoxify reactive the oxygen burst characteristic of oyster hemocytes. We now report that cultured *P. marinus* trophozoites are remarkably insensitive to transient exposure to superoxide and hydrogen peroxide (H2O2), but not hypochlorite. These findings are consistent with two observations: (1) Viable trophozoites are able to destroy hydrogen peroxide in vitro; (2) extracts of *P. marinus* contain abundant iron-type superoxide dismutase (FeSOD) activity, as well as ascorbate dependent peroxidase (APX) activity. We previously described the cloning and characterization of two *P. marinus* FeSODs that have the potential to convert superoxide to H2O2 in vivo. Recombinant PmSOD1 and PmSOD2 proteins have been crystallized for structural analyses, and used to raise specific antisera for immunolocalizations. The APX activity appears to be a 35 kD protein. Continuing analysis of *P. marinus* SOD and APX functions will be presented. The unique characteristics of the *P. marinus* antioxidant system may provide the basis for disease prevention or therapy strategies [Supported by ODRP, NOAA award NA06RG0101-5, through the MD Sea Grant College, to GRV].

CORRELATION OF FLAT PEARL STUDIES WITH PEARL SAC FORMATION IN A FRESHWATER MUSSEL (*CYRTONAIAS TAMPICOENSIS*). Donald Shepherd*, Professional Pathology Laboratories, Ltd, P.O. Box 326, Tow, TX 78672.

Flat pearl studies can illustrate the process of biomineralization of molluscan shell, by placement of a flat material, between the mantle and inner shell of the mussel. Protein and calcium carbonate crystals can be evaluated by specific stains and light and polarization microscopy. The initial stage is secretion of a protein layer of glycoproteins on the insert as the nucleating protein sheet. After several days, secretion of calcium carbonate crystals begins from the epithelial cells of the mantle. These crystals are calcite, which form rhomboid crystals by 15 to 17 days. A second crystal forms on the calcite crystals; it is an isoform of calcium carbonate - aragonite. The switch from calcite to aragonite is accomplished by a change in acid protein secretion (Lustrin A). The aragonite forms small bricks as in a wall to form the mother-of-pearl nacre. Photos of natural pearls from Tampico pearly mussels will be presented to illustrate natural pearl formation.

CHARACTERISATION OF SUMMER MORTALITIES OF *C.GIGAS* OYSTER IN FRANCE IN RELATION TO ENVIRONMENTAL PARAMETERS. P. Soletchnik, M. Ropert, A. Huvet, J. Moal, L. Degremont, E. Bedier, J.F. Bouget, B. Dubois, JL. Martin, M. Enriquez Diaz, N. Faury, O. Le Moine, T. Renault, B. Gagnaire and J.F. Samain. Ifremer 17390 La Tremblade, France.

Field characterization of summer mortality was performed in France in the frame of the Morest project. Natural and hatchery spat were compared between three oyster production areas in France. Regardless of the natural or hatchery origin, oysters died during the reproduction period after temperature reaches 19°C.

Thus, in southern areas, temperature accelerated gametogenesis of small spat (10mm) as well as adults, and mortality appeared for the two age classes. In contrast, sexual maturation proceeded more slowly in northern where spat mortality was lower compared to 18 months old oysters. However, critical gametogenesis and temperature were not sufficient to induce mortalities, as observed in examples with stable environment. Alternatively sediment proximity in addition to oyster manipulations increased mortality during spring and summer, suggesting that some additional environmental stresses were necessary to reproduce the phenomenon. These interaction processes will be detailed in the other Morest contributions.

A COMPARISON OF TWO OYSTER (*CRASSOSTREA VIRGINICA*) STOCKS TO DETERMINE SUITABILITY FOR USE IN OYSTER REEF RESTORATION IN VIRGINIA. Laurie Carroll Sorabella* and Mark W. Luckenbach, Virginia Institute of Marine Science P.O. Box 1346 Gloucester Point, VA 23062.

Restoration efforts for eastern oysters (*Crassostrea virginica*) in Virginia have focused on constructing sanctuary reefs that are intended to serve as spawner sanctuaries. Frequently, these reefs are stocked with hatchery-produced oysters to enhance regional recruitment rates. An important unresolved issue is the suitability of specific oyster stocks to achieve maximal reproductive output on sanctuary reefs. The efficacy of using stocks selected for aquaculture verses wild stocks for oyster reef restoration is not well established. We compared the performance of two hatchery-reared oyster stocks, the CROSBreed selected stock and a wild-caught oyster stock (Lynnhaven), after deployment onto reefs in the Lafayette River (Chesapeake Bay). Performance was evaluated based on growth, survival, female fecundity, sex ratio, disease status and cumulative egg production. Results indicate that reproductive performance of the two stocks varied depending on which disease predominated. Where MSX disease pressure was high, the CROSBreed stock outperformed the Lynnhaven stock for cumulative egg production; where dermo disease pressure was high, the Lynnhaven stock outperformed the CROSBreed stock. This work suggests that to maximize reproductive output, broodstocks used in reef restoration should be selected based on knowledge of disease pressure in the region.

DECADAL SCALE CHANGES IN SEASONAL PATTERNS OF OYSTER RECRUITMENT IN THE VIRGINIA SUB ESTUARIES OF THE CHESAPEAKE BAY. Melissa Southworth* and Roger Mann, Virginia Institute of Marine Science P.O. Box 1346 Gloucester Point, VA 23062.

Reproductive periodicity of sessile estuarine invertebrates reflects local seasonality of both environmental (temperature, salinity) and biological (food) parameters. Estuaries are ephemeral features in geological time, but considered somewhat constant in the course of recent human history – a decadal time scale. Analysis of long term trends in oyster settlement periodicity since 1960 in three major sub estuaries (James, Piankatank and Great Wicomico Rivers) of the Chesapeake Bay show marked changes in this periodicity within the 40 year time frame with the 50th percentile of cumulative recruitment occurring between day 194 and 250 of the year depending on year and location. Significant coherence in interannual variation is observed across a wide range of sites. These are discussed in relation to pre- and post-disease (both MSX and *Perkinsus*) events, periods characterized by high and low river flow, varying harvest pressure, and trends arguably associated with global warming.

FIRST REPORTED OCCURRENCE OF MSX IN CANADA. Mary F. Stephenson*, Sharon E. McGladdery, Michelle Maillet and Anne Veniot, Gulf Fisheries Centre, Department of Fisheries and Oceans, P.O. Box 5030, Moncton, New Brunswick, Canada E1C 9B6; Gary Meyer, Pacific Biological Station, Department of Fisheries and Oceans, 3190 Hammond Bay Road, Nanaimo, British Columbia, Canada V9T 6N7.

The first reported occurrence of MSX (*Haplosporidian nelsoni*) in American oysters (*Crassostrea virginica*) was observed on the Atlantic Coast of Canada in October 2002 associated with mortalities of >80% in adult oysters from St. Patrick's Channel, Bras d'Or Lakes, Nova Scotia. Histological examination revealed the plasmodial stage of MSX with confirmation using DNA probes received from the Office International des Epizooties (OIE) Reference Laboratory for Haplosporidiosis at the Virginia Institute for Marine Science. In collaboration with the Provinces, Industry, and First Nations, an extensive disease survey was conducted from October to December 2002 while affected areas were closed to the harvest of oysters. Heavy infections, adult oysters with plasmodia or spores, were contained within Bras d'or Lakes while light background levels were described from other areas. Stakeholders continue to work collaboratively on the development of MSX control strategies within Atlantic Canada.

A QUANTITATIVE, REAL-TIME PCR ASSAY TO DETECT THE PARASITIC DINOFLAGELLATE *HEMATODINIUM* SP. IN BLUE CRABS, *CALINECTES SAPIDUS*. Colin R. Steven*, Kristen Hunter-Cevera, Allen R. Place, Mike Sheppard, and Dick Lee, Center of Marine Biotechnology Suite 236 Baltimore, MD 21202.

Hematodinium sp. is a parasitic dinoflagellate that infects and kills several species of commercially valuable crustaceans, including the blue crab. This dinoflagellate is found in several different morphologies in the hemolymph and tissues of blue crabs. *Hematodinium* infections in the Chesapeake Bay show strong salinity and temperature dependencies during their seasonal fluctuations. We present our work towards the development of an ultra sensi-

tive, real-time, fluorescence-based, PCR assay for the detection and quantification of *Hematodinium* infection. This assay builds on a previously developed PCR-based diagnostic that relies on specific oligonucleotide primers designed against a section of the *Hematodinium* 18S rRNA gene (AF421184). Our quantitative, real-time assay incorporates a fluorescently-labeled, gene-specific probe as well as two gene-specific primers which allow us to accurately detect approximately 1.4 *Hematodinium* cells/ml hemolymph. This new diagnostic tool will allow investigators to quickly and easily monitor the extent and severity of *Hematodinium* infections in blue crabs, and ensure that infected crabs are not released from hatcheries.

THE MITOCHONDRIAL GENOME OF THE BLUE CRAB, *CALLINECTES SAPIDUS*. Colin R. Steven*, Xiaojun Feng, Allen R. Place, and Jeffrey L. Boore, Center of Marine Biotechnology Suite 236 701 E. Pratt Street Baltimore, MD 21202.

In animals, mtDNA is generally a small (15-20 kB) genome containing 37 genes that is maternally inherited. There is generally a single large non-coding region which, for a few animals, is known to contain controlling elements for replication and transcription. Animal mtDNA displays extensive intraspecific polymorphism (often in the non-coding control region) and often evolves faster than typical single-copy nuclear DNA. Most mtDNA variants involve nucleotide substitutions or small length changes; gene order is highly stable over short evolutionary time. No published studies using blue crab mitochondrial polymorphisms exist and the only crustacean mitochondrial genome deposited in GENBank is that for Artemia. Recently the DOE Joint Genome Institute has begun a Mitochondrial genomics program. We have initiated a collaborative project to sequence the entire *Callinectes sapidus* mitochondrial genome which will allow us to find variable regions for distinguishing the mothers of hatchery derived juveniles from those in the wild. Depending of the variability observed, these same markers would assist in defining the genetic substructure of blue crab in the Chesapeake Bay.

DEVELOPMENT OF MICROSATELLITE MARKERS IN THE BLUE CRAB, *CALLINECTES SAPIDUS*. Colin R. Steven, Johnathan Wilkes, Allen R. Place, Jessica Hill, and Brian Masters, Center of Marine Biotechnology Suite 236 701 E. Pratt Street Baltimore, MD 21202.

Current tagging methods for blue crabs, which include, fluorescent elastomers and coded wire tags can be expensive, labor-intensive and/or relatively short-lived. We have initiated the identification and characterization of genetic markers, or microsatellites, to augment current tagging methods. Microsatellites, or simple sequence repeats (SSRs), are tandemly repeated units of two to six nucleotides, located randomly throughout the genome of all organisms. The high variability among these loci has become a powerful and popular tool for ecology and population genetics.

Investigators use microsatellites to distinguish genetic subpopulations as well as individuals at the genetic level with a very high degree of certainty. We have isolated approximately two dozen dinucleotide and tetranucleotide microsatellite loci, and are in the process of screening these loci to determine their usefulness. Once validated, these microsatellite loci will be used to examine the genetic structure of the Chesapeake Bay blue crab fishery, and to determine the impact that restocking efforts would have on the natural fishery.

SETTLEMENT, SURVIVAL, AND PREDATION OF RED KING CRABS ON NATURAL AND ARTIFICIAL SUBSTRATA. Bradley G. Stevens*, NMFS/NOAA Kodiak Fisheries Research Center 301 Research Ct., Kodiak, AK 99615; and Kathy Swiney.

In tests with structurally complex live substrata, postlarval (glaucothoe) and juvenile red king crabs *Paralithodes camtschaticus* preferred hydroids and algae, over sand or worm colonies. Survival to stage C1 was highest for controls, least on sand, and intermediate on other substrata. Predators (larger crab) caused increased mortality of glaucothoe, but neither shelter presence or type, or predator size had any effect. Survival of juvenile crabs was significantly decreased by shelter absence, predator presence, and predator size. Density of juvenile crabs on shelters was higher than that of glaucothoe, and increased in the presence of larger predators, whereas that of glaucothoe did not. Despite active selection for complex substrata by settling glaucothoe, significant predation occurs there, and behavior of glaucothoe is not compensatory. In contrast, juvenile crabs modify their behavior to achieve higher densities in sheltered habitats, which dampens the effect of predation. These survival strategies have probably evolved to compensate for the much greater risk of predation in open habitats. Biogenic oases are important to settling larvae, and should be protected from disturbance by fishing activities. Knowledge of settlement behavior is essential prior to considering the potential of king crabs for stock enhancement or aquaculture.

USE OF LOG PILING STRUCTURES AS ARTIFICIAL HABITATS FOR RED KING CRABS *PARALITHODES CAMTSCHATICUS*. Bradley G. Stevens*, NMFS/NOAA Kodiak Fisheries Research Center 301 Research Ct.; J. Eric Munk, and Peter A. Cummiskey.

Juvenile king crabs use wooden dock pilings as habitats. We studied whether pilings could be used to mitigate for natural habitat lost during construction of a breakwater. Scuba divers counted organisms on six piling structures and adjacent seafloor areas at quarterly intervals. Site, season, and their interaction had significant effects on abundance. Abundance of juvenile (age 0 to1+) king crabs increased steadily from July 1997 through March, then declined in June 1998. Crab abundance was significantly higher on pilings than on the adjacent substratum, and at more exposed sites

than at sheltered sites. Red king crabs were associated with the presence of green urchins, decorator crabs, leather stars, and sculpins. Each site could be discriminated by their unique community of inhabitants. Why juvenile king crabs are attracted to pilings is unknown. Pilings are inefficient habitats that are not structurally complex, do not persist in the environment, and may not be the best structure for habitat enhancement. For these reasons, and because there is no evidence that RKC are habitat-limited, we do not recommend the use of pilings as artificial habitats to mitigate for the loss of natural habitat.

SUSTAINABLE COMMUNITY DEVELOPMENT VIA AN INSHORE MOLLUSCAN AQUACULTURE PARK: A CONCEPT FOR THE GULF OF MEXICO. John E. Supan*, La. Sea Grant College Program, LSU, Baton Rouge, LA 70803.

Industrial parks are areas permitted and/or zoned for the operation of prescribed businesses without the need for individual permitting. Such community programming is commonly used in the economic development of inner cities and rural areas across the nation. This same concept can be applied to coastal waters delineated and permitted for certain farming activities for economic development of coastal regions.

The concept of state aquaculture parks was proposed in March 1989 by the National Research Council's Committee on Assessment of Technology and Opportunities for Marine Aquaculture in the U.S as a means of fostering entrepreneurship through technology transfer and commercialization. A well-planned and administered aquaculture park can circumvent user conflicts, navigation, security, and liability issues that may otherwise hinder such use of coastal waters. A public entity could be the authority that selects the site, obtains public input, necessary permits, Coast Guard approval, and administers park operations, such as leasing areas to farmers, providing security.

The Gulf region's semitropical climate provides ideal conditions for sea farming. Oyster genetics research has created superior stocks, which can be coupled with technically advanced grow-out methods in a park setting to achieve their full economic potential.

HISTORY OF THE DEVELOPMENT, COMMERCIALIZATION AND SUCCESSFUL MARKETING OF THE FIRST HACCP-BASED POST-HARVEST PROCESS FOR THE REMEDIATION OF *VIBRIO* SP. IN RAW OYSTERS—THE AMERIPUREPROCESS®. John Tesvich* and Patrick Fahey, AmeriPure Processing Company, Inc., Franklin, LA.

The development of a hot water/cold shock treatment to remediate *Vibrio* sp. In raw oysters without removing the oyster from its natural shell was initiated in response to growing public health concerns and marketplace reaction to raw oyster-related *Vibrio* sp. Illnesses. This led to the patenting and commercialization of the first raw, yet dead (as a result of the PHT) in-shell oyster product, as well as the first HACCP-based PHT process to remediate *Vibrio*

sp. in raw oysters. The marketing of a value-added raw oyster with reduced risk of infection and excellent shelf life opened markets previously closed to raw oysters, particularly from the Gulf of Mexico. It also paved the way for other HACCP-based PHT processes and has sparked considerable interest among other oyster processors to license the AmeriPure Process®. The process is dependable, simple and economical. It is also adaptable to large and small operations with equipment that is easy to maintain and available from numerous manufacturers/fabricators.

SELECTION OF APPROPRIATE HABITATS/SITES FOR BAY SCALLOP RESTORATION. Stephen T. Tettelbach*, Christopher F. Smith, Peter Wenczel. Natural Science Division Southampton College of Long Island University, Southampton, NY 11968.

Strategies for restoration of bay scallop, *Argopecten irradians irradians*, stocks include collection of setting larvae in spat bags, direct seeding of juveniles or adults on the bottom ("free-planting"), or placement of broodstock in protective enclosures. Aggregations of the latter type of enclosures are often referred to as spawner sanctuaries. Larval collection is often attempted adjacent to spawner sanctuaries or, when data are available on tidal circulation patterns, in other areas where larvae are likely to be entrained. In Long Island, New York waters, we have evaluated potential sites for free-planting of scallops on the basis of several criteria, including: historical scallop productivity, anticipated larval dispersion, predator abundance, bottom characteristics (including sediment type and presence of SAV's), degree of exposure to prevailing NW winter winds (which can cause stranding of scallops on adjacent beaches), and the potential for scallop burial (in winter) by shifting sediments. Placement of net enclosures has been based on most of the above criteria, but additional factors of particular importance include water depth, potential hazard to navigation, and suitability for securing appropriate permits. The choice of appropriate strategies and habitats/sites must be considered simultaneously.

LINKING HARD CLAM (*MERCENARIA MERCENARIA*) REPRODUCTION TO PHYTOPLANKTON COMMUNITY STRUCTURE: I. CLAM GROWTH AND REPRODUCTIVE CYCLES. Stephen T. Tettelbach*, Natural Science Division, Southampton College of Long Island, Roger I.E. Newell, Christopher Gobler.

Hard clam, *Mercenaria mercenaria*, populations and fisheries have declined dramatically in the south shore bays of Long Island, New York since the mid-1970s. We hypothesized that this decline in recruitment was associated with variation in either the timing of gametogenic development or synchronicity of reproduction within the population due to changes in overall patterns of primary production. Quantitative histological techniques were used to assess the reproductive cycles of adult (>40 mm) female hard clams, from

October 2000 - October 2001, at five sites in south shore bays of Long Island. For comparison, we also sampled two sites in Raritan Bay, New Jersey where regular hard clam recruitment supports a large fishery. Timing of peak reproduction was nearly identical at the 5 south shore bay sites and was 1 to 2 weeks later in Raritan Bay. There were appreciable differences in reproductive effort between locations, with female clams from Bayshore and Patchogue showing the lowest and the two Raritan sites having the highest Gamete Volume fraction. Clam growth and condition index differed even more dramatically between sites, with poorest growth and condition also being exhibited at the Bayshore and Patchogue sites. University Southampton, NY 11968.

INFLUENCE OF FRESHWATER INPUT ON THE HABITAT VALUE OF OYSTER REEFS IN THREE SOUTHWEST FLORIDA ESTUARIES. S. Gregory Tolley*, Aswani K. Volety, Mike Savarese and James T. Winstead, Florida Gulf Coast University, 10501 FGCU Blvd S, Fort Myers, FL 33965.

In order to examine the influence of freshwater input on the habitat value of oyster reefs, a spatiotemporal comparison of reef-resident fishes and decapod crustaceans was conducted during three seasonally dry and three seasonally wet months in three Southwest Florida estuaries: the Caloosahatchee and Estero rivers, and the Faka-Union Canal. Lift nets containing 5 liters of oyster clusters were deployed monthly at three sites along the salinity gradient of each system. Salinities within each system varied both spatially and seasonally, with mean salinities being significantly higher downstream and significantly lower during wet months. Analysis of variance also indicated significant spatial and seasonal differences in the community metrics examined. Overall results suggested that abundance, biomass, and species richness of reef-resident organisms increased downstream where salinities were higher. Diversity (H') and richness were also greatest downstream in the Caloosahatchee, but diversity in the Faka-Union was highest upstream. In general, both biomass and diversity exhibited a significant positive correlation with salinity. Our results suggest that freshwater input (salinity) plays a significant role in structuring oyster-reef communities in southwest Florida estuaries. These results can be used to inform water management practices as well as efforts at oyster-reef restoration.

HISTOLOGICAL EVALUATION OF EARLY PEARL-SAC DEVELOPMENT IN THE TAMPICO PEARLY MUSSEL (*CYRTONAIAS TAMPICOENSIS*). Stephan Towers*, and Leonard DiMichele, Department of Wildlife and Fisheries Sciences, Texas A&M University, College Station, TX 77843; and Donald Shepherd, Professional Pathology Laboratories, Ltd, P.O. Box 326, Tow, TX 78672.

Pearl-sac development in the Tampico pearlymussel was evaluated histologically. Hemocytes massed at the wound entrance, sealing it off and staunching blood loss. Hemocytes also lined the incision track. Mucopolysaccharides formed an extracellular matrix important in wound healing, restructuring of blood sinuses, and development of a basal membrane. Epithelial cells originating from the graft began to proliferate onto the newly formed basal membrane. The pearl-sac was formed, and tall columnar cells began active secretions by day 30. Our results indicate that pearl-sac development is remarkably consistent across taxa and among implantation sites. The primary role of the host is seemingly to seal the wound, reconstruct blood sinuses, and provide a basal membrane. The role of the donor tissue is to provide epithelial seed cells. Both epithelia (lateral and medial) of the graft may proliferate, but only those from the lateral surface of the mantle appear to be involved with pearl formation.

MODELING INDIVIDUAL EASTERN OYSTER (*CRASSOSTREA VIRGINICA*) GROWTH IN THE MARYLAND PORTION OF THE CHESAPEAKE BAY. Jessica Vanisko*, Cooperative Oxford Laboratory, MDDNR, Oxford, MD 21654; and Thomas Miller, Chesapeake Biological Laboratory, UMCES, Solomons MD 20688.

Eastern oyster populations have declined dramatically in the Chesapeake Bay during the last century. A clear and quantitative description of oyster population dynamics is essential for the implementation of effective restoration efforts. Growth remains an important, but poorly understood component of these dynamics providing the link between spat (young-of-year oysters) and the reproductive and fishable stocks. Catch-at-length data collected at 55 sites from fishery-independent surveys were used in a length-based analysis of growth through modal decomposition, allowing the mean growth of individuals within a cohort to be followed through time over a maximum of 6 years. Initial sizes of spat were highly variable both temporally and spatially (mean = 24.03, CV = 32.81%). Maximum and minimum observed growth were 1.02–46.22 mm/yr. Growth rates declined with age class. Growth rates were also highly variable among sites due to site-specific differences such as salinity. These data were used to develop region-specific age-length relationships for oysters.

EVALUATION HACCP IN THE OYSTER ACTIVITY IN THE LAGOON SYSTEM ALVARADO, VERACRUZ; MEXICO. Itzel G. Villa*, Fabiola L. Reynoso, and Ma. del Refugio C. Chávez, km12 carr. Veracruz-cordoba, boca del río, Veracruz, Mexico cp 94290.

In the last 30 years the oyster production in the national environment has not been stable, presenting this in the State of Veracruz a descending behavior, diminishing of 40,569.4 t obtained in 1988 to 9,653.8 t for 1994, that which demonstrates that the oyster veracruzana's activity faces limitations, caused by the over exploitation, the contamination, the accumulation of sludge in the coastal lagoons, the climatological interferences, the ecological changes

and the sanitary problems; in some cases these alterations have caused serious problems of public health and even the exhaustion of the banks of oyster. Considering that the concept HACCP involves all the potential dangers of security of the foods (biological, chemical and physical), either that they happen in natural form, for environmental changes or that was generated by failure in the production process. The present project carried out a diagnose of the lagoon system of Alvarado using the HACCP with the aim to propose a handling plan for the exploitation of the American oyster (*Crassostrea virginica*), and this way to guarantee its sanitary quality as food for human consumption and to fulfill the regulations sanitary to product exportation.

REMOTE SENSING TO MAP AND ASSESS INTERTIDAL SHELLFISH RESOURCES IN THE SOUTHEASTERN USA.

Jeffrey S. Vincent, USC Dept. of Geography; Dwayne E. Porter, USC Baruch Inst. and School of Public Health, Loren Coen, SCDNR Marine Resource Research Inst.; Dave Bushek*, USC Baruch Inst.; and Steve Schill, GeoMetrics, Inc., Baruch Marine Field Laboratory/USC, PO Box 1630, Georgetown, SC 29442.

Oyster resources in the southeastern USA are predominantly intertidal. Water clarity and tidal stage limit the use of passive remote sensing systems while shallow water limits the ability of sonar to accurately map beds and reefs. Oysters can be observed directly during low tide exposure, but inaccessibility and other problems make mapping these intertidal oyster resources difficult and tedious with questionable accuracy. Currently, maps are produced via a lengthy process of ground surveys and manual interpretation of aerial photographs, both of which are time-consuming and prone to human error. This project is developing a library of hyperspectral imagery to identify spectral end members of shellfish from in situ and remotely sensed (HyMAP) imagery. Preliminary results indicate separation in hyperspectral characteristics of oyster resources compared to surrounding habitats. Furthermore, HyMAP spectral end members show reasonable separation and similarity with in-situ spectral end members. We will use these spectral characteristics to classify and map the distribution and condition of intertidal shellfish resources. If successful, we will develop an automated mapping technique in a GIS environment that can be used by resource managers to obtain more timely information on the changing condition of oyster resources and better direct enhancement/restoration efforts.

HISTORY OF THE COMMERCIAL APPLICATION OF HYDROSTATIC HIGH PRESSURE PROCESSING TO MOLLUSCAN SHELLFISH.

Mike Voisin, P.O. Box 3916 Houma, La. 70361-3916.

The history of the commercial application of Hydrostatic High Pressure to molluscan shellfish will be discussed by the CEO of the firm that developed the application. The challenges and op-

portunities during the development of this revolutionary process will be discussed. The process reduces certain (*Vibrio*) bacteria to non-detectable levels and shows potential to inactivate viruses in shellfish, at the same time the shellfish's muscle releases from the shell creating an easily processed product with reduced labor cost and increased yields.

ESTABLISHING MINIMUM FLOWS AND LEVELS OF FRESHWATER IN THE CALOOSAHATCHEE RIVER, FLORIDA, USING RESPONSES OF OYSTERS.

Aswani K. Volety*, S. Gregory Tolley and James T. Winstead, Florida Gulf Coast University, 10501 FGCU Blvd, Fort Myers, FL 33965.

Alterations in freshwater inflow resulting from watershed development and water management practices have impacted salinity and water quality and led to declines in oyster populations within southwest Florida estuaries. In the Caloosahatchee Estuary, Florida watershed management is typified by large freshwater releases during wet summer months and little or no releases during dry winter months. Effects of watershed management on oysters were investigated to provide guidelines for establishing minimum flows and levels of freshwater in the Caloosahatchee Estuary. Reproductive patterns, *Perkinsus marinus* disease, spat recruitment, and juvenile oyster growth, were investigated. Oysters in the Caloosahatchee Estuary spawn continuously from April-October. Upstream, sub-tidal locations exhibited good spat recruitment, low disease intensity, and higher juvenile growth rates compared to downstream, intertidal sites. High freshwater flows during summer flush out oyster larvae and spat from areas with suitable cultch and/or reduce salinities to unfavorable levels for spat settlement and survival. Limited freshwater releases during winter coupled with decreased releases in summer will result in suitable conditions for survival and enhancement of oyster reefs. Water quality targets that should sustain, enhance and restore oyster reefs have been both identified and communicated to water resource managers.

DECLINING INTERTIDAL OYSTER REEFS IN FLORIDA: DIRECT AND INDIRECT IMPACTS OF BOAT WAKES.

Linda Walters*, Paul Sacks, Lisa Wall, Jeffrey Grevert, Daniel LeJeune, Samantha Fischer, and Andrew Simpson, Department of Biology University of Central Florida Orlando, FL 32816.

Numerous intertidal reefs of the eastern oyster *Crassostrea virginica* have dramatically declined over the past 50 years along the east coast of central Florida. Many reefs are significantly smaller than in the past and have large dead margins on their seaward edges. It is hypothesized that these differences are due to increased recreational boating activity. To better understand the impact of boating on intertidal oyster reefs, we have begun to run replicated field trials in Mosquito Lagoon that include a motorboat passing a reef at one of three speeds (5, 10, 20 mph), one of three distances from shore (15, 30, 45 m) and one of two propeller

angles (45 and 90 degrees). On shore, observers have recorded dislodgment of shells, flow rates, wake height, wind speed, propagation time, and turbidity. With the present configuration, all 3 variables had a significant impact on the oyster reef.

CHROMOSOMAL MAPPING OF RIBOSOMAL RNA GENES AND TELOMERIC REPEATS IN ZHIKONG AND BAY SCALLOPS. Yongping Wang,*[1,2] and Ximing Guo.[1]

[1]Haskin Shellfish Research Laboratory, Rutgers University, 6959 Miller Avenue, Port Norris, NJ 08349, USA; [2]Experimental Marine Biology Laboratory, Institute of Oceanology CAS, Qingdao, Shandong 266071, PRC.

Chromosomal localization of major (18-5.8-28S) and minor (5S) ribosomal RNA genes, and the vertebrate telomeric repeat (TTAGGG)n were studied in two scallop species, zhikong scallop *Chlamys farreri* and bay scallop *Argopecten irradians*, using fluorescence in situ hybridization (FISH). Probes were made by PCR amplification, labeled with digoxigenin-11-dUTP and detected with fluorescein-tagged anti-digoxigenin antibodies. In zhikong scallop, the major and minor genes were mapped to two different regions of Chromosome 5. The major rRNA genes were located at the telomeric region of the short arm, while the 5S rRNA gene was located at an interstitial site on the long arm. In bay scallop, the major rRNA genes had two loci one on Chromosome 4 and the other on Chromosome 8, both at telomeric regions of the short arms. The 5S rRNA was found at an interstitial site of an acrocentric chromosome (Chromosome 10). In both species, the vertebrate telomeric repeat hybridized to telomeres of all chromosomes, and no interstitial sites were observed. The finding of major differences in the distribution of the rRNA genes between the two species suggests that chromosomal rearrangements may have played an important role in the evolution of scallops.

PRODUCTION OF TRANSPARENT EXOPOLYMER PARTICLES (TEP) BY BIVALVES. J. Evan Ward*, Kari B. Heinonen, Michael P. McKee, Bridget A. Holohan.

Department of Marine Sciences, University of Connecticut, Groton, CT 06340; Bruce A. MacDonald, Department of Biology, University of New Brunswick, Saint John, N.B., Canada, E2L 4L5.

In the marine environment, dissolved polysaccharide-rich organic matter coalesces to form transparent exopolymer particles (TEP). In turn, TEP has substantial impact on the flocculation of phytoplankton and other particles into aggregates (marine snow) which increase deposition of organic matter to the benthos. Previous studies have demonstrated that exudates and lysates from phytoplankton and bacteria contribute to the production of TEP. Little is known, however, about other sources of TEP precursors, especially in near-shore environments. The purpose of this study was to investigate production of TEP by several species of bivalves (*Mytilus edulis*, *Argopecten irradians*, *Crassostrea virginica*).

In laboratory studies, several individuals of one bivalve species were isolated in static or recirculating seawater chambers and allowed to feed for up to 9 hr. In the field, groups of oysters were isolated in flow-through, benthic chambers and allowed to feed for 1 to 2 hr. Water samples were taken periodically and analyzed for TEP, dissolved organic carbon (DOC), and bacterial numbers. TEP concentration was determined using an Alcian Blue staining technique and quantified using a spectrophotometer. Results indicated that TEP concentration in chambers with actively feeding bivalves was significantly higher than in control chambers without bivalves. No significant differences in bacterial numbers were found between control and experimental chambers suggesting that the effects of bacteria were similar in all treatments. Mixed results were obtained for DOC concentration. Our results indicate that bivalves do produce TEP, probably during feeding when large volumes of water pass over mucus-coated feeding structures. We suggest that bivalves may be an important source of TEP in near-shore waters.

ESTIMATING THE IMPACT OF BAY SCALLOP RESTORATION EFFORTS USING GENETIC DATA. Ami E. Wilbur,

Biological Sciences/CMS University of North Carolina-Wilmington 5600 Marvin K. Moss Lane Wilmington, NC 28409.

Shellfish populations in many areas are being augmented with hatchery-produced animals in an effort to counteract the effects of overfishing, habitat degradation and disease. While such efforts have the immediate effect of increasing local abundance, it is the expectation that the restorations will have a more dramatic effect on subsequent generations. Until recently, it has been difficult to evaluate the contribution made by such restorations because the offspring of hatchery-produced animals are not readily distinguishable from wild conspecifics. The constraints of hatchery methodologies, however, prevent the production of stocks that mimic natural populations with respect to genetic variation. These inevitable genetic differences between hatchery-produced and wild stocks can be used to differentiate individuals in the cohort following restoration. Recent efforts to assess the contribution of hatchery-produced bay scallops based on sequence analysis of mitochondrial DNA markers serve as a field test of this approach. Assessment of restoration efforts in Florida provided no genetic evidence of a contribution from the hatchery stock despite substantial increases in abundance following the restoration. In contrast, a substantial contribution from hatchery-produced scallops deployed in Chincoteague Bay was suggested by mtDNA analysis, indicating that the restoration effort was in part responsible for the increase in abundance.

COMPARATIVE SPERMATOZOON ULTRASTRUCTURE OF ARCIDAE BIVALVES *ARCA OLIVACEA* AND *SCAPHARCA BROUGHTONI.* Wan-Xi Yang*, School of Life Sciences, Zhejiang University, Hangzhou 310012, China; Jun-Quan Zhu, Department of Marine and Fisheries, Ningbo University, Ningbo 315211,China.

The ultrastructure of mature spermatozoon of two Arcidae family species *Arca olivacea* and *Scapharca broughtoni* was compared using transmission electron microscopy for the first time. The mature spermatozoon of both species consists of a head which is composed of a cone-shaped acrosome and a round nucleus and a tail region. Spermatozoon of both species has a round solid nucleus, which exhibits a triangular posterior invagination, housing the centriolar complex and proximal portion of the axoneme. The acrosome of *Scapharca broughtoni* is fat while that of *Arca olivacea* is very thin. In *Scapharca broughtoni*, the subacrosomal space contains an axial rod and a basal plate, while in *Arca olivacea*, no such structures were observed. Within the middle piece, the spermatozoon of *Scapharca broughtoni* has five spherical mitochondria, and in contrast, only four mitochondria were observed in *Arca olivacea*. Both species has long whip-like end portion, which is composed of an axoneme with the typical 9+2 structure.

MICROSCOPIC OBSERVATION OF TEGUMENT AND CEMENT GLAND DISTRIBUTION OF FEMALE PLEOPOD IN CHINESE MITTEN CRAB, *ERIOCHEIR SINENSIS.* Wan-Xi Yang*, College of Life Science, Zhejiang University, Hangzhou 310012, China; Antonina dos Santos, Inst. Nac. Inv. Agraria e das Pescas IPIMAR, Av. de Brasilia, s/n 1449-006 Lisboa, Portugal; Luis Narciso and Ricardo Calado, Laboratorio Maritimo da Guia-Faculdade de Ciencias da Universidade de Lisboa, Estrada do Guincho, 2750-642 Cascais, Portugal; Hong Zhou, Jian-Ping Lu and Nai-Cheng Jiang, College of Life Science, Zhejiang University, Hangzhou 310012, China; Xue-Ping Ying, Department of Biological and Environmental Science, Wenzhou Normal College, Wenzhou 325027, China.

Eriocheir sinensis is a vitally important economic species of China. In recent years, its production falling down partially because of egg-loss during larval aquaculture. To reveal possible causes of egg loss, we primarily studied the pleopod tegument structure and its cement gland distribution. The pleopod tegument consists of exoskeleton (subdivided into epicuticle, exocuticle and endocuticle) and epithelial cell layer, while the cement glands lie closely to the epithelial cells, with fine gland tubules come across the exoskeleton. We primarily consider that cement glands in the pleopod function in the egg attachment in *Eriocheir sinensis*.

IMMUNOLOGICAL STUDIES ON THE ORIGIN OF THE LAMELLAR COMPLEX (LCX) DURING THE SPERMIOGENESIS OF *MACROBRACHIUM NIPPONENSE* (DE HAAN). Wan-Xi Yang, School of Life Science, Zhejiang University, Hangzhou 310012, China.

Lamellar complex (LCX) is a transient organelle, which is believed to be derived from Golgi apparatus and lysosome during spermiogenesis of caridean shrimp *Macrobrachium nipponense* (de Haan). Conventional electron microscopical evidence shows that, in the round spermatid, no LCX observed surrounds the nucleus while saccules of Golgi apparatus begin to separate and move to the nucleus along with the condensation of cytoplasm. Typical LCX can be seen when nucleus of spermatid begins the sickle-shaping process, and it locates on the convex side of the nucleus. One important feature is that lysosomes merge into the Golgi saccules while the saccules open a cut or cuts. Most part of the LCX comes from Golgi apparatus. To prove this, we use GM130 monoclonal antibody to localize the Golgi apparatus. Immunofluorescence data show that GM130 exists mostly in the LCX, and immunocytochemistry results show that gold particles (representing GM130) distribute mainly on the LCX. All these evidence support that the idea that LCX originates mostly from Golgi apparatus.

INTERTIDAL OYSTER RESTORATION ALONG AN ERODING SHORELINE: AN ASSESSMENT OF SUBSTRATE TYPES FOR STABILIZATION AND PROPAGATION. Guy M. Yianopoulos, and William D. Anderson*, Marine Resources Division, South Carolina Department of Natural Resources, Charleston, South Carolina 29422.

Gulf coast *Crassostrea virginica* shell, South Carolina intertidal oyster shell, whelk shell (*Busycon spp.*) and intertidal seed oysters were established as cultch material along an eroding intertidal shoreline (1.83m mean tidal range) to compare the efficacy of substrate types for propagation to three-dimensional oyster populations. Four treatment areas were assessed for matrix accumulation, growth and recruitment over a three-year period. Shell treatments were covered with polypropylene netting (Cintoflex ®) mesh size of 3.175cm × 3.81 cm to provide stabilization. Recycled South Carolina intertidal oyster shell and whelk shell demonstrated the best matrix propagation, with whelk shell accumulating the most spat. Gulf coast shell recruited lower numbers, but grew larger spat. Transplanted intertidal seed oysters suffered mortalities during the three-year study but continued to recruit significant numbers of spat.

THE MORPHOLOGY AND ULTRASTRUCTURE OF SPERMATOZOON OF THE GASTROPOD *BULLACTA EXARATA*.

Xue-Ping Ying, Department of Biological and Environmental Science, Wenzhou Normal College, Wenzhou 325027, China; Wan-Xi Yang*, College of Life Science, Zhejiang University, Hangzhou 310012, China.

The morphology and ultrastructure of spermatozoon of mud snail *Bullacta exarata* are first described. It is composed of a head in which a simple cap-shaped acrosomal complex and a elongated nucleus are included and a tail containing middle piece, principle piece and end piece. The nucleus is cylindrical, tapering gradually towards the anterior tip. A posterior nuclear fossa is observed clearly. In the Middle piece, there is a ring consisting of 5 occasionally 6 mitochondria, which closely contacted the posterior portion of the head. The proximal centriole lies in posterior nucleus fossa and the distal one is in the center of the mitochondrial ring. The principal piece with 9+2 structure consists of axoneme and lateral fins. The end piece is short with relatively simple structure.

POPULATION GENETIC STRUCTURE OF THE SUMINOE OYSTER AS INFERRED FROM RESTRICTION FRAGMENT LENGTH POLYMORPHISM (RFLP) AND MICROSATELLITE MARKERS.

Qian Zhang*, Karen L. Hudson, Standish K. Allen Jr. and Kimberly S. Reece, Virginia Institute of Marine Science, College of William and Mary, Gloucester Point, VA 23062.

The Suminoe oyster, *Crassostrea ariakensis*, is being evaluated and considered as a non-endemic aquaculture species for Chesapeake Bay. To date, published reports on the taxonomic status and genetic characterization of this species have focused on interspecific relationships within the genus *Crassostrea*, and little is known about the population genetic structure of *C. ariakensis* in its native range. In this study, we used restriction fragment length polymorphism (RFLP) markers based on the mitochondrial cytochrome oxidase I (COI) gene and the first internal transcribed spacer (ITS-1) region of the nuclear ribosomal RNA gene region to examine the genetic variation within and among five geographically separated samples of *C. ariakensis* and hatchery stocks. RFLP data using nuclear and mitochondrial loci showed that the samples shared common haplotypes, but significant frequency differences were observed between the samples in the northern group (Northern China and Japan) and southern group (Southern China) indicative of population structure (P = 0.000). These results support previous phylogenetic analyses based on ITS1 and COI DNA sequences of 5-10 individuals from each sample. Microsatellite markers are currently being employed to further examine the population structure and to determine whether a bottleneck effect has occurred in hatchery stocks.

FINE STRUCTURAL ANALYSIS OF SPERMATOZOON OF THE BIVALVE *BARBATIA VIRESCENS* AND ITS EVOLUTIONARY CHARACTERISTICS.

Jun-Quan Zhu, Department of Marine and Fisheries, Ningbo University, Ningbo 315211, China; Wan-Xi Yang*, School of Life Sciences, Zhejiang University, Hangzhou 310012, China.

The ultrastructure of mature spermatozoon of *Barbatia virescens* was observed using transmission electron microscopy and its evolutionary significance was analyzed. The mature spermatozoon consists of a head and a tail. The head is composed of an apical, umbrella-shaped acrosome and cylindrical nucleus. In the longitudinal sections, striations can be seen clearly, which come across outer acrosomal membrane. The nipple-shaped subacrosomal space contains small granules. The nucleus has a U-shaped anterior invagination and an inverted V-shaped posterior one. The nucleus is highly condensed. The tail of the spermatozoon includes a middle piece surrounded by five or occasionally six spherical mitochondria and a long whip-like end piece with an axoneme with the typical 9+2 structure. A phylogenetic path can be traced by comparative study of sperm ultrastructure in the Family *Arcidae*. The spermatozoon of *B. virescens* has a very important role in the reproductive evolution of the Family *Arcidae*.

CHARACTERIZATION OF KEY CDNAS OF THE ENDOCRINE AXES REGULATING REPRODUCTION AND MOLTING IN THE BLUE CRAB, *CALLINECTES SAPIDUS*.

Nilli Zmora* and John M. Trant, 701 E. Pratt St. Baltimore, MD 21202.

For the first time in brachyurans, a number of cDNAs encoding key hormones, enzymes and receptors of the reproductive/molting endocrine axes were isolated from the blue crab. Using 5' and 3' RACE, O-methyltransferase, the major regulatory enzyme for methylfarnesoate (MF) production, was isolated from the mandibular organ. The deduced amino acid (AA) sequences are 74% identical to the *Metapenaeus ensis* enzyme. The enzyme that activates ecdysone, 20-hydroxylase (CYP4), was isolated from the Y-organ and is 59% identical to the *Cherax quadricarinatus* enzyme at the AA level. The ecdysone receptor (ECR) and vitellogenin (Vg) cDNAs were isolated from ovary. The AA sequence of ECR shares ~96% identity with the Fiddler crab and many insect ECRs. A 2 kb fragment of the 5'-terminus of a putative Vg transcript was isolated, however there is a low degree of homology when compared to crayfish and penaeid Vg sequences. Our attempts to isolate the mandibular-organ-inhibiting-hormone (MOIH) from the X-organ were unsuccessful. The above cDNAs, together with the published molt inhibiting hormone (MIH) sequence, will be used to develop the molecular assays for the investigation of the endocrine regulation of reproduction, molting and growth.

HATCHERY MASS PRODUCTION OF BLUE CRAB (*CALLINECTES SAPIDUS*) JUVENILES.

Yonathan Zohar*, **Oded Zmora, Andrea Findiesen, Emily Lipman, John Stubblefield, Anson H. Hines** and **Jana L.D. Davis**, Center of Marine Biotechnology, University of Maryland Biotechnology Institute 701 E. Pratt St. Baltimore, Maryland 21202. USA.

Responding to the rapidly declining abundance and harvests of the blue crab in the Chesapeake Bay, a multidisciplinary research program was established to study the blue crab basic biology, develop hatchery technologies for its mass production and examine the feasibility of its stock enhancement. This presentation will address the hatchery work. Exposing wild-caught, mated blue crab females to phase-shifted photo-thermal conditions resulted in out-of-season hatching of millions of zoeae 1. Larval rearing to the zoea 8/megalopa stage was conducted at densities of 40-110 individuals per liter based on a diet comprised of microalgae, rotifers and *Artemia nauplii*. Zoeae 8/megalopae were produced in an average 22 days, and survival rates of 41.5%. Maximal survival was 74%. Secondary growth of zoeae 8/megalopae to 20 mm juvenile crabs was conducted at lower densities of 2-40 individuals per liter. To reduce cannibalism, ample shelter structure was introduced and the crabs were graded by size. Diet was comprised of adult *Artemia*, shredded squid and artificial pellets. In large-scale conditions, 20 mm juvenile crabs were produced in 64 days at a survival rate of 46%. During summer/spring 2002, we produced 40,000 juvenile crabs, of which 25,000 were individually tagged and experimentally released to the Chesapeake Bay.

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    COVER PHOTO: Mussels, *Mytilus edulis*. Photo: S. E. Shumway.

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JOURNAL OF SHELLFISH RESEARCH

BIOCHEMICAL INDICATOR OF SEA SCALLOP (*PLACOPECTEN MAGELLANICUS*) QUALITY BASED ON LIPID CLASS COMPOSITION. PART I: BROODSTOCK CONDITIONING AND YOUNG LARVAL PERFORMANCE

FABRICE PERNET,[1,*] RÉJEAN TREMBLAY,[2] AND EDWIN BOURGET[1,3]

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ABSTRACT The aim of this study was to test the validity of a lipid based indicator of larval quality of sea scallop *Placopecten magellanicus*. Objectives were 2-fold: (1) to determine the link between lipid class content and reproductive state of adults in the field and in the laboratory and (2) to follow lipid class content, growth, and survival during embryonic and early larval development. Adult scallops were periodically sampled during gametogenesis for lipid class and histological analysis of the gonads in the field at two locations and in the laboratory after feeding three different diets. Females were induced to spawn and lipid class content, larval growth, and survival of five batches of eggs were followed for 8 days after fertilization. Site, diet, and time had significant effects on lipid class composition of male and female gonads and gametogenesis of females. Triacyglycerol accumulation during vitellogenesis was characteristic of female gonads and explained respectively 56.4% and 71.3% of the variability in maturity and egg size. When spawning was induced, no major effect of location or diet on lipid composition of gonad and subsequent eggs was detected. Nevertheless, the mean number of eggs produced by females increased with atresia level in gonad, suggesting that egg quantity was incompatible with egg quality. Lipid class composition during embryogenesis and young larval development showed a high demand for triacyglycerol.

KEY WORDS: broodstock nutrition, gametogenesis, hatching, larval growth, lipids, scallop, *Placopecten*

INTRODUCTION

The expansion of aquaculture has increased the demand for juveniles of a wide range of bivalve species. As a consequence, hatcheries need to produce large quantity of eggs and larvae of good quality. Larval production has to be considered in two phases: broodstock conditioning and larval rearing. Under northern temperate conditions, young larvae of most bivalves rely on endogenous sources of energy during embryogenesis before the transition to exogenous sources. The diet provided to adults can affect the biochemical composition of their gonads and their resulting eggs and larvae. For instance, the essential fatty acid composition of the microalgae fed to adult scallop *Pecten maximus* was reflected in the composition of gonads, eggs and larvae until 5 days after fertilization (Delaunay et al. 1992, Samain et al. 1992). Then, maturity and hatching success of eggs from adult *P. maximus* were improved with a diet based on *Isochrysis* sp. rich in essential fatty acids (Soudant et al. 1996a, Soudant et al. 1996b). Given the above, the performance of young larvae is directly linked to maternal nutrition.

Studies focusing on gametogenesis and early larval development highlight the primordial role of lipids and the relative importance of triacylglycerol (TAG). For example, gametogenesis in the scallop *Argopecten purpuratus* was associated with an increase in the ovary lipid content (Barber & Blake 1981). The sea scallop *Placopecten magellanicus* stores large quantities of TAG in the gonads before spawning (Napolitano & Ackman 1992). Then, lipid reserves accumulated in the eggs of the scallop *Crassadoma gigantea* cover 47.6% of energetic needs during embryogenesis (Whyte et al. 1991). TAG were preferentially catabolized during egg development of the clam *Mercenaria mercenaria* and the oys-

ter *Crassostrea virginica* (Gallager et al. 1986). Finally, the mass of lipid in the eggs is correlated with the hatching success of *P. maximus* larvae (Dorange 1989, Devauchelle & Mingant 1991).

The reproductive cycle of the sea scallop, like other marine bivalves, includes five distinct periods: vegetative, cytoplasmic growth, vitellogenesis, spawning and, finally, resorption of non-released gametes (see Barber & Blake 1991, Eckman 1996). Spawning is synchronous among individuals at a particular site, and most populations display a single annual spawning period extending over 1 or 2 mo between July and October, depending on latitude. In other scallop species, site-specific variation in gametogenic cycles has been observed (e.g., Bricelj et al. 1987).

Histological preparation of gonadal tissue provides the means to assigned numerical values to the developmental stages. For instance, mean egg diameter is indicative of the stage of the gametogenic cycle. Eggs gradually increase in size during gametogenesis, reaching a maximum size prior to spawning and decrease sharply after spawning as mature eggs are released (Barber & Blake 1981, Barber et al. 1988, Paulet & Boucher 1991). Histology allows quantification of the fraction of the gonad occupied by developing, mature, and resorbing (atresic) gametes (Beninger 1987, MacDonald & Bourne 1987).

The aim of this study was to test the validity of lipid class composition of gonad, egg, and larvae as a predictor of quality of sea scallop *P. magellanicus*. In the present article, "quality" refers to a set of physiological variables (lipid composition) that could explain the variability of reproductive state of adult (maturity, atresia and egg size) and larval performance (survival and growth). For example, the mass of lipid in bivalve larvae is a good indicator of quality because it has been positively correlated with growth and survival (Gallager et al. 1986). To our knowledge, gonad quality has never been assessed using lipid composition. We designed our study with two objectives in mind: 1) to examine the link between lipid class variation in the gonads and reproductive state of adults, in the field and in the laboratory and 2) to examine

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the link between lipid class composition of gonads and eggs with performance during early development (number of eggs released, growth, and survival).

MATERIAL AND METHODS

Animal Maintenance

This study was conducted at the experimental hatchery of Ministère de l'Agriculture, des Pêcheries et de l'Alimentation du Québec at Grande-Rivière (Gaspe coast, Quebec, Canada). Male and female adult scallops of comparable size (110 cm ± 10 cm) were harvested by SCUBA diving at the end of May 2001 at two sites on Gaspe coast: Percé (S1) at a depth of 30 m and Pointe Saint-Pierre (S2) at a depth of 20 m (48 °30′N; 65 °15′O). Laboratory held animals were maintained in a flow-through sea water system (28 ppt) and fed continuously with living microalgae. The latter were produced semicontinuously in the f/2 nutrient mixture (Guillard 1975). Temperature was maintained at 8 °C from the beginning of the experiment until July 9th and temperature fluctuated between 10 and 13 °C until spawning. Photoperiod was set at a constant cycle 16:8 (light/dark). Spawning was induced by thermal rise to 16 °C and mechanical shock using air-lift systems. Fertilization was made with a mixture of sperm at ca. 10 sperm to each oocyte. The fertilized eggs were left undisturbed in 1000 L Xactic® tanks (one tank per treatment) at 12–14 °C. Swimming embryos were then siphoned 24 h later into another tank, where they were maintained in suspension with a light bubbling. Four days after fertilization, as D-veligers emerged, water was poured through a 20-μm pore mesh, and the tank was cleaned. Larvae were reared until day 8 at a density of 1.5 larvae per mL.

Experimental Design

Adult Conditioning and Field Sampling

Adult scallops collected in the bay of Percé were fed from the beginning of June until mid-July 2001 with three artificial diets

(Fig. 1). The daily dry mass of algal ration was adjusted to 4% of scallop dry mass for each adult diet. Based on preliminary measurements, mean dry mass was assumed to be ca. 25 pg cell^{-1} for *Isochrysis* sp., *Chaetoceros muelleri*, and *Pavlova lutheri* and 150 pg cell^{-1} for *Skeletonema costatum*. Adult diet A was a mix of *Isochrysis* sp. (clone T-iso), *P. lutheri*, *S. costatum* (40/40/20 cells), adult diet B consisted of the standard mix of *Isochrysis* sp., *P. lutheri*, *S. costatum*, and *C. muelleri* (25/25/25/25 cells) and, finally, adult diet C was made of *Isochrysis* sp. and *C. muelleri* (25/75 cells). The adult scallops harvested at Pointe Saint-Pierre were conditioned from mid-July to mid-August with the standard diet B (treatment S2B, Fig. 1). Microalgae were harvested every 3 to 4 days for lipid class (n = 15) and fatty acid (n = 5) analyses during the entire feeding period. Adults maintained in the laboratory on the three artificial diets were periodically sampled in triplicate and their gonads analyzed from the beginning of the experiment (early June, t_0) during vitellogenesis (early July, t_1) until spawning (mid-July, t_2). Adults living in the wild at the two sites were sampled at t_0, t_1, and t_2 but also later during the reproductive cycle (mid-August, t_3 and mid-September, t_4; Fig. 1). Three pieces of ca. 100 mg of gonad were collected to perform lipid class (both sex) and histological analyses (females only).

Spawning Induction and Larval Rearing

Females from S1-fed diets A, B, C, and females from S2 were induced to spawn in separate buckets in mid-July and females from treatment S2B were induced to spawn in mid-August (Fig. 1). Wild animals were induced to spawn right after arrival in the laboratory. Number of spawning females varied from two to five per treatment. Eggs of each female were counted and sampled separately for lipid class analysis and size measurement. Egg fertilization was conducted with a mixture of spermatozoa from three to five males per treatment. Individual spawnings were pooled in one tank per treatment for fecundation and embryonic develop-
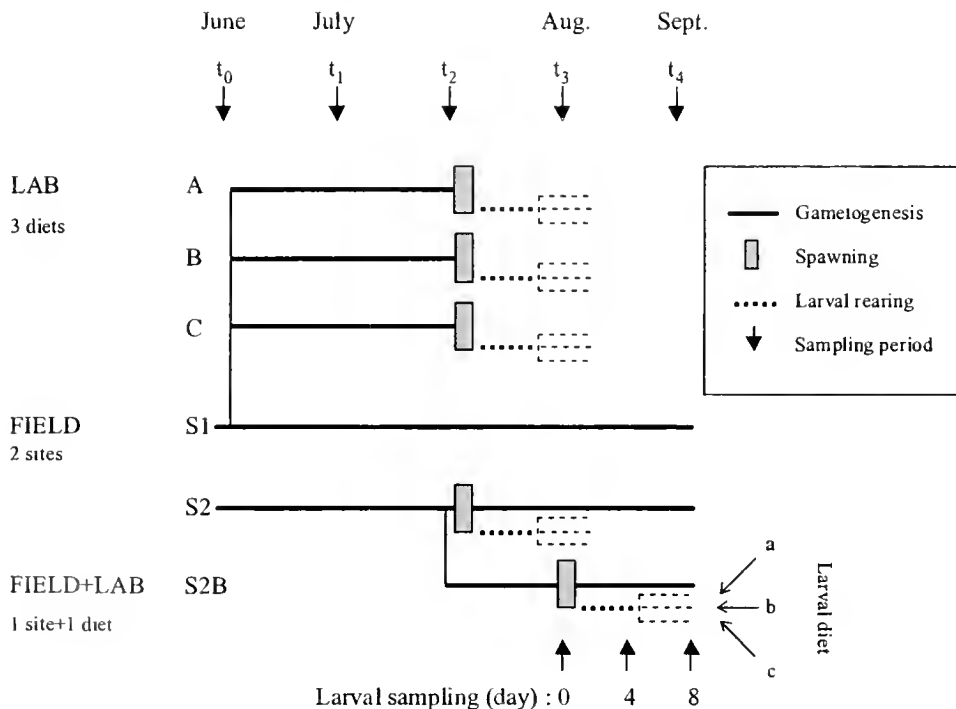
Figure 1. Experimental design.

ment (no treatment replication). Each group of 4-day-old larvae was separated among three tanks of ca. 200 to 400 L and fed different diets: a) *Isochrysis* sp. and *Pavlova lutheri* (50/50 cells), b) *Isochrysis* sp. and *Chaetoceros muelleri* (50/50 cells), and c) *Isochrysis* sp. with *C. muelleri* grown under silicate deprivation to enhance TAG accumulation (50/50 cells). Larvae were sampled for lipid analysis, density and growth measurements on day 4 and 8 after fertilization.

Laboratory Analysis

Sample Collection

Samples of 10 mL microalgal culture, 10,000 eggs, and 5000 larvae were filtered on prebaked GF/C filters at 450 °C and stored in 1 mL of dichloromethane in amber glass vials with Teflon liner caps under nitrogen at −20 °C until lipid extraction. Gonad samples for lipid analysis (ca. 100 mg) were directly stored in dichloromethane. Gonad samples for histologic analyses were stored at room temperature in Helly's fixative. Finally, samples of eggs and larvae used for size measurements were stored in 10% formaldehyde.

Lipid Extraction

Lipids were extracted after a 4-day to 1-mo storage period. Microalgae, egg, and larvae samples were first sonicated three times in 1.5 mL of CH_2Cl_2-MeOH (2:1; v/v) in an ice bath to remove the organisms from the filter. Gonads were ground in 6 mL of CH_2Cl_2-MeOH (2:1; v/v). KCl (0.88%) was added to the previous solution to obtain CH_2Cl_2-MeOH-KCl (2:1:0.6; v/v/v; Folch et al. 1957). The homogenates were mixed and centrifuged at 4000 rpm for 2 min to obtain a biphasic system. The lipid fraction (lower phase) was removed and transferred to a clean tube. The solvent was evaporated under a nitrogen flow and lipids suspended in 0.05, 0.1, or 1 mL CH_2Cl_2 for eggs and larvae, microalgae, or gonads, respectively. Lipid extracts of microalgae were fractionated in two aliquots to analyze lipid classes and fatty acid. Manipulations were carried out on ice and under nitrogen whenever possible.

Lipid Class Composition

Lipids (0.5% to 10% of total extraction depending on sample tissue) were spotted onto the S-III Chromarods (Iatron Laboratories Inc., Tokyo, Japan) using a Hamilton syringe. Four different solvent systems were used to obtain three chromatograms per rod according to Parrish (1987). This method separates aliphatic hydrocarbons (HCs), ketones (KETs), TAGs, free fatty acids (FFAs), free fatty alcohol (ALCs), free sterols (STs), diglycerides (DGs), acetone mobile polar lipids (AMPLs), and phospholipids (PL). Between each development, Chromarods were scanned by the flame ionization detection system of the analyser Iatroscan Mark-V (Iatron Laboratories Inc., Tokyo, Japan). Lipid classes were identified and quantified with the use of standard calibration curves obtained for each lipid class. The load applied to the rod ranged from 0.05 to 5.9 μg. Within each set of rods, one was used for the lipid standard and another one for extraction blank.

Fatty Acid Composition

Lipid extract of microalgae was analyzed by gas chromatography. Fatty acid methyl ester (FAME) were prepared from about 0.2 mg of the total lipids following the method of the American Oil Chemists' Society using BF_3/CH_3OH (12%; AOCS, 1989). FAME were suspended in 40 μL of hexane, and a 2-μL aliquot was injected with a 1:37 split in a Perkin Elmer Sigma 300 capillary chromatograph, equipped with a Supelco Omegawax™ 320 fused-silica capillary column 30 m × 0.32 mm × 0.25 μm ID. The following chromatographic conditions were used: 190 °C for 20 min, followed by an increase of 4 °C min⁻¹ to 210 °C for 25 min, followed by an increase of 5 °C min⁻¹ to 240 °C for 5 min. Helium was the carrier gas at a flow rate of 2 mL min⁻¹. The gas chromatograph was equipped with flame ionization detectors and the integrator software Varian Star Chromatography Workstation 5.51. FAME were identified by their retention times compared with standard (Supelco 37 component FAME Mix, Menhaden Fish Oil and PUFA-3, Supelco Bellefonte, PA) and quantified with tricosanoic acid (c23:0) as an internal standard. Notation used in fatty acid identification is L:BnX where L is the chain length, B is the number of double bonds and nX is the position of the double bond closest to the terminal methyl group.

Histology

Female gonads were dissected and stored in Helly's fixative. After rinsing, the samples were dehydrated through an ascending alcohol series, cleaned in toluene and embedded in paraffin. Specimens were sectioned (6 μm in thickness) and stained with Harris hematein and eosin. Examination of gonad sections was made using a compound microscope at a magnification of 40× with an image capture kit CoolSNAP-Pro cf Digital Kit™ 4.1. Percentage area of gonads occupied by mature and atresic eggs and size distribution of eggs for each sampling date were measured with Image-pro plus® 4.1.0 package software. Eggs were considered mature when stalked or ripe. Eggs with a much deformed appearance (jigsaw-puzzle shapes) were considered as atresic (Dorange 1989). Three counts were made for each tissue section and one tissue section was examined per individual.

Growth Measurements

Shell size was calculated as the average of the length (anterior–posterior distance) and height (dorsal-ventral distance) of larvae. Larvae were measured using a compound microscope (magnification of 40×) with an image capture kit CoolSNAP-Pro cf Digital Kit™ 4.1.

Data Analysis

Diet composition, in terms of lipid class and fatty acids, was submitted to one-way multiple analysis of variance (MANOVA). Fatty acids were grouped as saturated (SFA), monounsaturated (MUFA), and polyunsaturated fatty acids (PUFA). Among PUFA, 20:5n3 (eicosapentaenoic acid, EPA), 22:6n3 (docosahexaenoic acid, DHA), total n3 and n6 and finally n3-n6 ratios were distinguished. As the *n* value was smaller than the number of fatty acids (independent variables), there were not enough degrees of freedom to apply MANOVA without data grouping.

Lipid class composition and histology of gonads depending on treatment (diets A, B, and C and sites S1, S2) and time (from early June, t_0 to mid-July, t_2), were investigated using two-way MANOVA by sex. The total sum of squares was partitioned because of the asymmetric experimental design (Underwood 1997). At t_0, there were only two treatments (S1, in which A, B, and C were confounded and S2) whereas at t_1 and t_2, there were five distinct treatments. Consequently, contrasts were carried out between control and experimental treatments (t_0 vs. t_1) and then a

two-way MANOVA was run among experimental treatments at t_1 and t_2. Site-specific effects on the independent variables (lipid class composition and histology of gonads) from t_1 to t_4 were investigated using a two-way MANOVA by sex. It was not possible to perform an overall analysis due to the fact that animals in the laboratory were not sampled from t_2 to t_4, then leading to an asymmetric design (Fig. 1).

One-way MANOVA was used to investigate treatment effects on lipid class content and histological data of ovaries prior to spawning. Each individual was considered as an experimental unit ($n = 3$ per treatment).

One-way MANOVA was used to investigate treatment effects on lipid class content, size and quantity of eggs produced by adults. Each batch of eggs produced per female was considered as an experimental unit ($n = 2$ to 5 per treatment). The number of replicates among adult feeding regimes differed.

Age effects on lipid class content and growth from early embryogenesis until day 8 were analyzed using one-way MANOVA. As previously mentioned, individual spawnings were pooled in one tank for fecundation and embryonic development. The pooled eggs and larvae from each treatment were considered as experimental units ($n = 5$). Values of lipid class content and size of the pooled eggs used for fecundation were obtained by weighing the contribution of each female to the total number of eggs in the group (individual fecundity). This is a means to assess the initial composition of the pooled eggs, allowing their inclusion in the data analysis.

Finally, lipid class composition, quality, size and survival of 8-day-old larvae were subjected to one-way MANOVA to determine larval diet effects ($n = 3$).

When overall differences were detected, Least-square means multiple comparison tests (LSMean, SAS Institute Inc. 1999–2000, Cary, NC) were used to determine which means were significantly different. Probability levels were divided by the number of degrees of freedom of the tested factor (Bonferroni correction). Homoscedasticity was tested using Levene's test and was confirmed by graphical examination of the residuals (Sherrer 1984).

A stepwise multiple regression was used to examine the relationships between histological data as response variable (% area of gonads occupied by both mature and atresic eggs and egg diameter) and lipid class content as explanatory variable ($n = 49$). Another model was used to assess the relation between spawning performance as response variable (number and size of eggs at release and hatching success) and gonad histological data (egg maturity, atresia and size) and lipid composition as explanatory variable. Finally, a stepwise multiple regression was also used to examine the relationship between larval performance as response variable (growth and survival) and egg lipid composition as explanatory variable ($n = 5$). A significant threshold of 0.05 was adopted for all statistical tests. All statistical analyses were carried out using SAS 8.01 (SAS institute Inc. 1999–2000).

RESULTS

Lipid Class and Fatty Acid Composition of Diet

Diets A, B, and C fed to giant scallop contained respectively 88.31, 74.67, and 129.88 mg of lipid per g of algal dry mass ($P < 0.001$). Lipid class composition of diets A and B differed from diet C particularly in TAG ($P < 0.001$, Fig. 2). TAG content of diet C was 100 and 10 times higher than that of diets A and B respectively. Diet C contained significantly higher level of MUFA
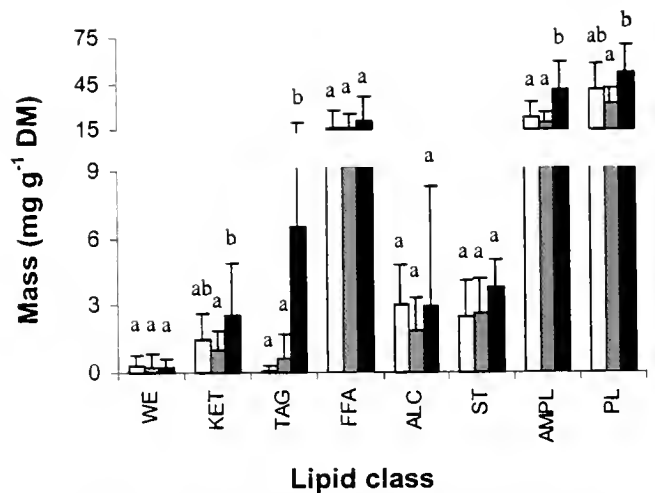
Figure 2. Mass of each lipid class (±SD), $n = 14$ expressed in mg per g dry mass in diet A (□), B (▨), and C (■) (For compositions of diet, see text). Lipid classes detected were wax estser (WE), ketone (KET), triacylglycerol (TAG), free fatty acid (FFA), fatty alcohol (ALC), cholesterol (ST), acetone mobile polar lipid (AMPL), and phospholipid (PL).

and PUFA than diets A and B, and differences of PUFA were mainly attributed to n6 fatty acid (Table 1). Values of 20:5n3 and 22:6n3 did not differ among diets ($P = 0.061$ and $P = 0.082$, respectively).

Gonad Lipid Class Content and Maturation

General Pattern

We examined the influence of three diets in the laboratory and two sites in the field on ovarian lipids and histology from vitellogenesis until the resorption period. Based on the percentage of mature eggs and egg diameter measurements, the first major spawning probably occurred at the beginning of July (t_1) and lasted until the beginning of August (t_3, Fig. 3). Egg size increased significantly from t_0 to t_1 and reached a maximum in mid-July (t_2). Thereafter, egg maturity and size gradually decreased in gonads until end of September (t_4). Adults reared in the laboratory displayed partial spawning at t_1 and were induced to spawn at t_2 (July 11, 12, and 15 for diets B, A, and C, respectively).

Total lipid level (TL) of female gonad varied from 6–16% dry mass during the experimental period (Fig. 4f). The highest level of TL was observed at the end of vitellogenesis (t_1), followed immediately by a sharp decrease during spawning (t_1 to t_2, $P = 0.008$, Fig. 4f). TL remained low until the end of the experiment. In male gonad, TL increased from t_0 to t_1 (Fig. 4l). The gonad lipid fraction varied between 4.5 to 7.5% dry mass. This suggests that, during gametogenesis, lipids were highly solicited in females but only moderately in males. TAG explained 84% of the variability of TL in female gonads, whereas PL explained 65% of the variability of TL in male gonad during the course of experiment.

Time, Treatment, and Site-Specific Effects on Ovaries

Mature eggs in the female gonads varied in time depending on the diet. There was a highly significant time effect from t_0 (end of May) to t_1 (beginning July, $P = 0.003$) and a significant interaction of treatment and time [from t_1 to t_2 (mid-July)] ($P < 0.001$,

TABLE 1.

Fatty acid mass (mg g^{-1} dry mass) and % (relative to the sum of fatty acid mass) in adult diets A (*Isochrysis* sp., *Pavlova lutheri*, and *Skeletonema costatum* 40/40/20), B (*Isochrysis* sp., *P. lutheri*, *S. costatum*, and *Chaetoceros muelleri* 25/25/25/25), and C. (*Isochrysis* sp. and *C. muelleri* 25/75), n = 5.

Fatty Acid	Diet A		Diet B		Diet C	
	Mass	%	Mass	%	Mass	%
12:0	0.05	0.12	0.09	0.23	0.05	0.06
14:0	5.63	14.40	4.66	12.54	10.80	13.73
14.1n5	0.19	0.49	0.18	0.48	0.38	0.48
15:0	0.16	0.40	0.17	0.47	0.44	0.56
16:0	3.22	8.22	3.10	8.34	8.01	10.18
16:1n7	3.22	8.24	5.21	14.01	15.36	19.52
16:2n4	0.91	2.34	1.14	3.06	2.21	2.80
16:3n4	3.68	9.40	4.56	12.27	7.40	9.41
18:0	0.24	0.62	0.31	0.84	0.67	0.86
18:1n9	2.30	5.89	1.55	4.17	3.11	3.95
18:1n7	0.73	1.85	0.59	1.60	1.39	1.76
18:2n6	3.13	8.00	2.02	5.43	4.03	5.13
18:3n6	0.23	0.58	0.26	0.71	0.81	1.03
18:3n3	1.67	4.27	1.03	2.77	2.01	2.56
18:4n3	2.66	6.80	1.75	4.70	3.05	3.88
20:0	0.02	0.06	0.00	0.00	0.08	0.10
20:1n9	0.00	0.00	0.00	0.00	0.07	0.08
21:0	0.00	0.00	0.00	0.00	0.00	0.00
20:4n6	0.21	0.54	0.46	1.23	1.73	2.19
20:4n3	0.01	0.02	0.01	0.02	0.02	0.03
20:5n3	6.80	17.40	7.13	19.18	12.65	16.07
22:0	0.06	0.15	0.07	0.18	0.07	0.09
22:1n9	0.00	0.00	0.00	0.00	0.02	0.02
21:5n3	0.00	0.00	0.00	0.00	0.00	0.00
22:5n3	0.00	0.00	0.02	0.06	0.05	0.06
24:0	0.00	0.00	0.00	0.00	0.01	0.02
22:6n3	3.99	10.20	2.86	7.70	4.27	5.43
ΣSFA	9.38	23.98	8.40	22.61	20.14	25.60
ΣMUFA	6.44	16.47	7.53	20.26	20.31	25.82
ΣPUFA	23.29	59.55	21.24	57.14	38.22	48.58
Σn3	15.13	38.68	12.80	34.43	22.05	28.03
Σn6	3.57	9.12	2.74	7.37	6.56	8.35
Σn3/Σn6	4.91		5.27		3.33	

Fig. 3). At t_1, animals fed diet C showed a higher maturity than those fed diets A and B. Scallops in the field showed maturity levels between those fed diets A and B. At t_2, the maturity of field scallops and scallops fed diet B dropped to values observed at t_0, the maturity of those fed diet C remained high whereas that of scallops fed diet A increased significantly to reach the level of those fed diet B at t_1. Thus, scallops maintained in the laboratory initiated vitellogenesis faster than those in the field, and those fed diet C matured more quickly and completely than those fed diets A and B. Thus, scallops fed diet B and adults maintained in the field started to spawn at t_1, earlier than adults fed diets A or C with lower maturity levels. Levels of atresia in female gonads showed significant time and treatment effects. In fact, percentage area of gonads occupied by atresic eggs increased from t_0 to t_1 ($P = 0.019$) and decreased markedly between t_1 and t_2 ($P = 0.023$), except for scallops from Pointe Saint-Pierre (S2). The lowest level of atresia was observed in scallops fed diet C. There was no site effect on maturity and atresia levels in female gonads. Scallops
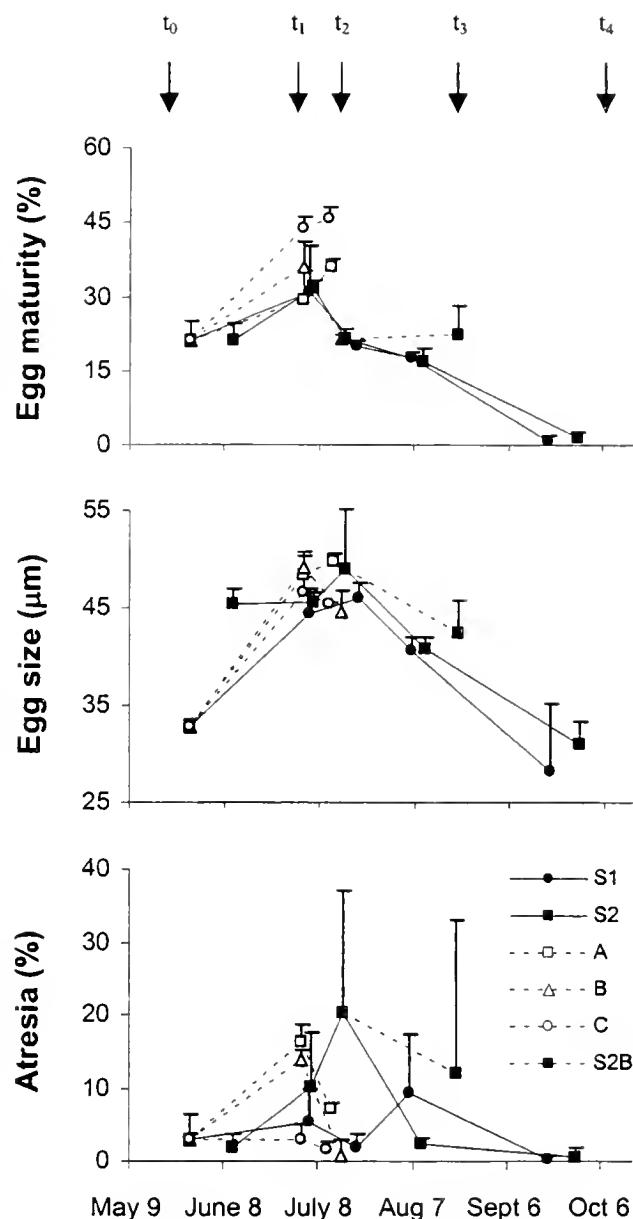
Figure 3. Female giant scallop maturity, egg size (mean diameter), and atresia as a function of time and treatment (±SD, $n = 3$). Broodstock in the field (normal line) at Perce (S1, ●), Pointe Saint-Pierre (S2, ■), and in the laboratory (dashed line) fed with diet A (□), B (open triangle), and C (○) and Pointe Saint-Pierre-fed diet B since mid-July (S2B, ■).

from Percé (S1) had smaller eggs than those from Pointe Saint-Pierre (S2: Fig. 3).

In ovaries, TAG levels sharply increased at the end of vitellogenesis, between t_0 to t_1 ($P = 0.005$), and dropped sharply during the spawning period, from t_1 to t_2 ($P = 0.016$, Fig. 4a). TAG levels gradually decreased from t_3 to t_4 ($P < 0.001$). Levels of TAG were highly variable depending on treatment and time. From t_1 to t_2, scallops from Percé (S1), and those fed diets A and C had significantly higher TAG content than those fed diet B, scallops from Pointe Saint-Pierre (S2) exhibiting intermediate levels. Finally, ovarian TAG levels were influenced by location since there were significant interactions of site × time ($P = 0.03$). In fact,
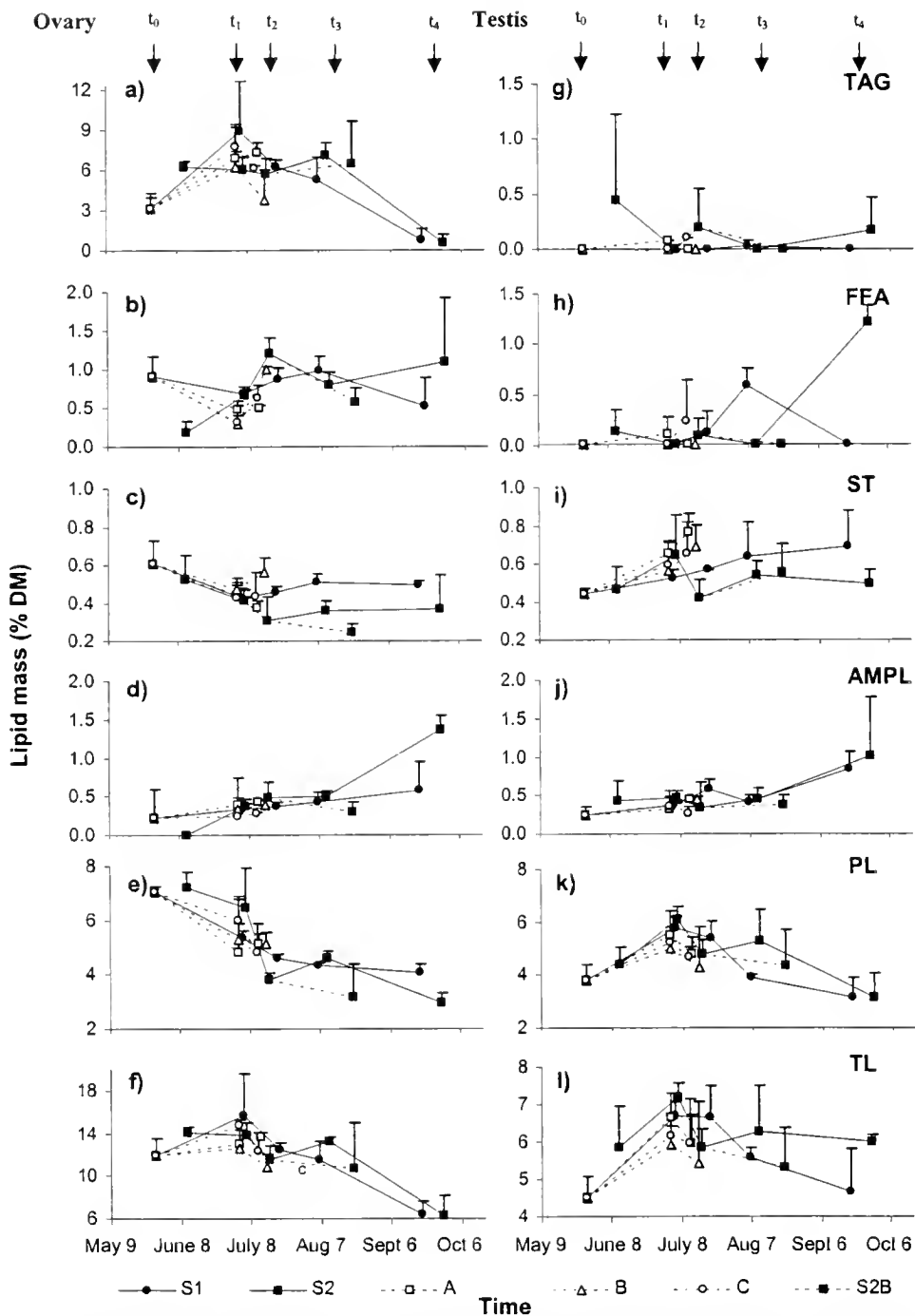
Figure 4. Mass of each lipid class expressed in mg per 100 mg dry mass (%) in ovary (left) and testis (right) as a function of time and treatment (±SD, $n = 3$). Broodstock in the field (normal line) at Perce (S1, ●), Pointe Saint-Pierre (S2, ■), and in the laboratory (dashed line) fed with diet A (□), B (open triangle), and C (○) and Pointe Saint-Pierre-fed diet B since mid-July (S2B, ■). Lipid classes detected were TAG, FFA, ST, AMPL, and PL. TL were obtained by summation of each lipid class.

scallops from Pointe Saint-Pierre (S2) did not exhibit a significant gonadal TAG increment as observed in scallops from Percé (S1) or those reared in the laboratory. TAG maxima occurred at different times depending on site (t_1 for S1 and t_3 for S2).

FFAs were a minor lipid class in female gonads accounting for ca. 6% of total lipid (Fig. 4b). In fact, there was no accumulation or depletion of FFAs from t_0 to t_1 but FFAs markedly increased from t_1 to t_2 in all the treatments. Thereafter, FFA levels gradually decreased toward initial values until t_4 except for scallops from S1.

ST content decreased from t_0 to t_1 ($P = 0.003$), reaching the lowest value at t_2 (Fig. 4c). Values of ST measured at t_3 and t_4 were intermediate between t_2 and t_0, suggesting a slow recovery after spawning. ST were affected by treatment in such a way that scallops fed diet B exhibited the highest level, those fed diets A and C and from Percé (S1) were intermediate and scallops from Pointe Saint-Pierre (S2) were the lowest. Moreover, a site effect was detected on ST because levels in scallop gonads from S1 were lower than those from S2 ($P = 0.012$).

AMPL, mainly glycolipids, pigments and remaining neutral lipids, gradually increased during the period of study ($P < 0.001$, Fig. 4d). There was no significant effect of treatment but a significant interaction of time $\times$ site ($P = 0.008$). PL content of gonads gradually decreased during the study as a function of treatment and site (Fig. 4e). Particularly, scallops from Pointe Saint-Pierre (S2) showed a pronounced drop from t_1 to t_2 ($P = 0.014$). TL decreased throughout the spawning period, from t_1 to t_4 (Fig. 4f).

Time, Treatment, and Site-Specific Effects on Testes

Lipid class composition of male gonad consisted mainly of structural lipids, such as ST and PL (ca. 88% of TL). TAG were consistently low (Fig. 4g). FFA were a minor lipid class in testes and accounted for ca. 2% of total lipids (Fig. 4h). There was a significant interaction of time and site on FFA content ($P < 0.001$), but no clear pattern emerged. ST content increased from t_0 to t_1 ($P = 0.028$) but subsequently was not affected by time, diet, or site (Fig. 4i). AMPL gradually increased all over the period of study with no significant effect of diet or site (Fig. 4j). The AMPL pattern observed in testes was similar to that in ovaries (student t-test, $P = 0.487$). PL increased before spawning from t_0 to t_1 ($P < 0.001$) and decreased markedly from t_1 to t_4 (Fig. 4k). There was no significant difference in PL in the male and female gonads (student t test, $P = 0.198$) except that PL content in testes increased from t_0 to t_1. Finally, TL content in testes increased from t_0 to t_1 ($P < 0.001$) and gradually decreased until t_4 as for PL (Fig. 4l). TL was also influenced by site since values in scallops from Percé (S1) were lower than those from Pointe Saint-Pierre (S2, $P = 0.041$).

Lipid Class Composition and Histological Analyses

A stepwise multiple regression was conducted to examine the relationship between lipid class and female maturity (Table 2). The model, including TAG and FFA, explained 61.7% of the variability of maturity. Based on this model, increasing ovarian TAG increases maturity level. TAG^2 only explained 16.7% of the variability of maturity, but it indicates that TAG values above a certain threshold are linked with lower maturity. TAG alone explained 56.4% of the variability of maturity. Variability of atresia in female gonads was weakly linked with ST ($r^2 = 0.12$). The model suggests that atresia increases as ST decreases. Finally, egg size was related to TAG and ST. The model suggests that egg size increases with TAG until a threshold (quadratic effect) and also increases as ST decreases. However, the influence of ST (partial $r^2 = 0.03$) was negligible compared with that of TAG (partial $r^2 = 0.68$).

Lipid Class Content, Growth, and Survival During Embryonic and Early Larval Development

Females fed diets A, B, C, and originating from Pointe Saint-Pierre (S2) were induced to spawn in mid-July (t_2) and those from S2B in mid-August (t_3). Lipid composition and maturity of gonads collected prior to spawning differed markedly in relation to treatment. Significant effects were detected in FFA ($P < 0.001$), ST ($p=0.015$), and PL ($P = 0.035$). Individual fed diet C showed the highest maturity, followed by those fed diet A and finally those from S2, S2B and B ($P < 0.001$). It seemed that broodstock conditioning just after the first major spawning (S2B) maintained but did not increase maturity (Fig. 3).

TABLE 2.

Stepwise multiple regression analysis using lipid class composition of female gonads as explanatory variables and maturity, atresia, and size of eggs in gonad as response variables, $n = 49$.

Maturity		
Regression Equation Y = 4.43 + 7.89 × TAG − 0.47 × TAG^2 − 8.04 × FFA, $r^2 = 0.617$, $P < 0.000$		

Regression Step No.	Source of Variance	Partial r^2	P Value
1	TAG	0.396	<.000
2	TAG^2	0.167	<.000
3	FFA	0.052	0.015

Atresia		
Regression Equation Y = 18.39 − 27.08 × ST, $r^2 = 0.120$, $P = 0.014$		

Regression Step No.	Source of Variance	Partial r^2	P Value
1	ST	0.120	0.014

Egg Size		
Regression Equation Y = 31.70 + 4.87 × TAG − 0.29 × TAG^2 − 10.34 × ST, $r^2 = 0.713$, $P < 0.000$		

Regression Step No.	Source of Variance	Partial r^2	P Value
1	TAG	0.473	<.000
2	TAG^2	0.210	<.000
3	ST	0.030	0.034

There was no effect of the treatment on lipid composition of eggs (except in FFA, $P < 0.001$, Fig. 5) and on the number of eggs released (Table 3, $P = 0.366$). In contrast, egg size varied according to the treatment ($P = 0.004$) and was correlated with size of 8-day-old larvae ($r^2 = 0.82$, $P = 0.035$). Eggs released by fe-
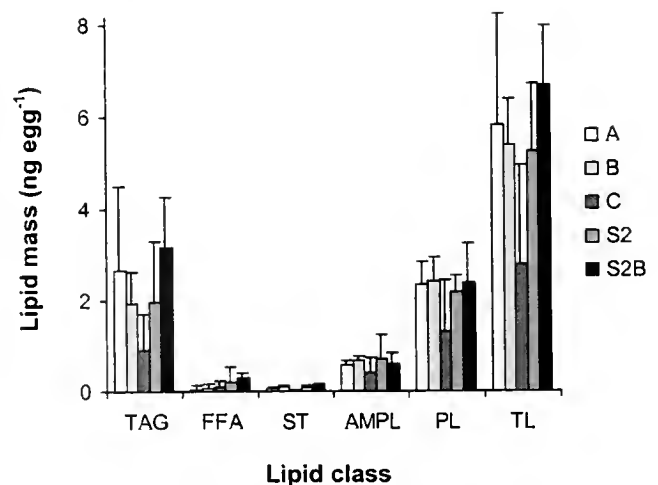
Figure 5. Lipid class profile of eggs as a function of treatment ($\pm$SD, $n = 2$ to 5). Broodstock were harvested in the field at Pointe Saint-Pierre (S2) and in the laboratory fed with diets A, B, C and Pointe Saint-Pierre-fed diet B since mid-July (S2B). Lipid classes detected were TAG, FFA, ST, AMPL, and PL. TL were obtained by summation of the lipid classes.

TABLE 3.

Effect of broodstock treatment (diet A, B, C, site S2 and site S2 fed diet B since mid-July [S2B]) on number of eggs and survival
(% determined from day 0 to 4 and 4 to 8) and size of egg and larvae (±SD when replicated).

Treatment	A	B	C	S2	S2B
No. of female spawning	4	5	2	2	3
Number of eggs $(10^6)^a$	6.42 ± 4.39	2.78 ± 1.94	3.28 ± 3.65	19.3 ± 11.35	15.05 ± 20.8
Survival (%)					
4-day old[b]	2.44	1.58	5.94	11.50	2.09
8-day old[c]	39.95	91.67	67.09	61.60	90.57
Size (μm)					
Egg[a]	79.0 ± 1.16	75.0 ± 1.31	79.0 ± 0.46	85.2 ± 2.63	82.2 ± 2.55
4-day old[b]	93.63	91.81	97.05	103.38	100.57
8-day old[c]	106.08	103.16	111.71	118.14	111.07

[a] The average value: female were individually induced to spawn by thermal shock.

[b] Calculated on the pooled eggs; for each treatment, individual spawnings were pooled in one tank for fecundation and embryonic development.

[c] The average value: each group of four day old larvae was placed in three tanks fed a different diet.

males originating from S2 were significantly larger than those from other treatments.

A stepwise multiple regression was conducted to examine the relation between gonad histology prior to spawning and spawning performance (number and size of eggs at release and hatching success). The model showed that number and size of eggs released increased with atresia (Fig. 6). Hatching success was not correlated with any variable.

From the time of spawning to 8 days of age, TAG levels decreased whereas FFA, ST, AMPL, and PL content increased sig-

nificantly (Fig. 7). No effect of larval age on TL could be detected ($P = 0.256$).

In the larval feeding experiment, diet-specific effects were observed in TAG content. Larvae fed diets B and C exhibited ca. twice as much TAG as larvae fed diet A (Table 4). However, such differences were apparently not sufficient to produce a significant effect on larval size and survival.

DISCUSSION

Gonad Lipid Class Content and Maturation

Gametogenesis of female giant scallop relies on the accumulation of neutral lipid in the gonad, particularly TAGs (Fig. 4a). In contrast, male gonads did not accumulate lipids during the experiment, and slight variations in TL were partly explained by variations of structural lipid content. The lipid composition of male and

Figure 6. Significant regression obtained after the stepwise procedure using ovarian histology before spawning as explanatory variables (egg maturity, astresia, and size) and number and size of eggs released and hatching success as responsible variables. The number and size of eggs were considered per female spawning ($n = 16$) whereas hatching success was measured on the group of eggs ($n = 5$).

Figure 7. Lipid class profile of eggs and young larvae as a function of treatment (±SD, $n = 5$). Broodstock were harvested in the field at Pointe Saint-Pierre (S2) and in the laboratory fed with diets A, B, C and Pointe Saint-Pierre-fed diet B since mid-July (S2B). Lipid classes detected were TAG, FFA, ST, AMPL, and PL. TL were obtained by summation of the lipid classes. Groups with different letters are significantly different ($P < 0.05$).

TABLE 4.

(a) Results of lipid class composition (ng larvae^{-1}), size (µm), and survival (% determined from day 4 to 8) of larvae aged 8 days depending on diet (a, b, and c); (b) summary of MANOVA; and (c) multiple comparisons.

Source of Variance	Lipid Class						Others	
	TAG	FFA	ST	AMPL	PL	TL	Size	Survival
(a)								
a	0.21	0.52	0.16	1.07	2.59	4.55	110.89	65.37
b	0.47	0.71	0.18	1.17	3.67	6.19	109.92	65.20
c	0.42	0.64	0.15	1.56	3.55	6.31	109.28	79.96
(b)								
P	**0.002**	0.514	0.770	0.295	0.127	0.081	0.917	0.590
(c)	A͟ ͟C͟ B	NS	NS	NS	NS	NS	NS	NS

Results are arranged in increasing order of estimated means from left to right. Groups underlined are not significantly different. Significant probabilities are in bold ($P < 0.05$).
NS, nonsignificant.

female gonads of giant scallop *P. magellanicus* was consistent with previous results for this species (Napolitano & Ackman 1992, Napolitano et al. 1993) and for the Chilean scallop *Argopecten purpuratus* (Caers et al. 1999), showing that the lipid level in testis was significantly lower than in ovaries. In fact, eggs accumulate large amounts of TAG during gametogenic development presumably to provide energy during embryogenesis. Spermatozoa contain little lipid, except PL and ST in cell membrane (Soudant et al. 1996a). Consequently, ovarian lipid metabolism during gametogenesis principally involved deposition of lipid reserves, whereas testis lipid metabolism was mainly governed by structural lipid dynamics.

TAG content of female gonad increased from the beginning of the experiment to reach a peak at the beginning of the spawning period and decreased with spawning (Fig. 4a). The decrease of total lipid in female gonad was previously attributed to the loss of eggs rich in TAGs during spawning in the scallop *Pecten maximus* (Pazos et al. 1997) and two clam species *Tapes decussatus* and *T. philippinarum* (Beninger 1984). Variations of ovarian maturity and egg size during gametogenesis were linked with TAG levels. A positive correlation between total lipid level and female gonad index ($r^2 = 0.779$) and mean egg diameter ($r^2 = 0.418$) has been reported in the literature (Pazos et al., 1997). Thus, TAG mass appears to be a good indicator of ovarian maturity.

Our study revealed that FFA levels sharply increased after the first major spawning (Fig. 4b). These molecules in a nonesterified form are usually observed in minor amounts in marine organisms. The presence of large quantities of FFAs in animal tissue is an indication of lipid degradation (Parrish 1999). FFA might result from the spawning, which leads to tissue breakdown and produces dead cells. Testis did not accumulate FFA during the spawning suggesting that the pattern of FFA is sex specific and closely related to TAG metabolism.

PLs were also a major lipid class constituent of ovaries and exhibited a slight continuous decline over the study (Fig. 4e). Maxima of PL in two clam species were observed during active gametogenesis, while the subsequent decrease coincided with gamete release (Beninger 1984). In our study, PL declined at t_0, before spawning. PL might be catabolized before spawning to sustain energetic needs to complete gametogenesis or to synthesize

TAG. Thus, PL may constitute an energy reserve catabolized before spawning in ovaries. In testis, PL increased before spawning and then sharply decreased. This pattern is consistent with previous reports on clam species (Beninger 1984).

The effect of feeding regimen during broodstock conditioning has been extensively studied (see Utting & Millican 1998). The originality of our study is that the three diets tested in this experiment differed in terms of lipid class and fatty acid profiles. The lipid rich diet C (*Isochrysis* sp. and *C. muelleri*, 25:75) promoted a greater and faster maturity and lower atresia than diets A and B (Fig. 3). In a previous study, *Isochrysis galbana* (Clone T-ISO) enhanced the rate of vitellogenesis and lowered atresia more than the other two diets during conditioning of *Pecten maximus* (Soudant et al. 1996a). This result was related to the high level of essential fatty acid 22:6n3 in T-ISO. In contrast with this result, diet C in our experiment had the same level of 22:6n3 as diets A and B. The higher and faster maturity and the lower atresia obtained with diet C could be attributable to the high KETs, TAGs, AMPL, or PL content or total MUFA or PUFA levels. It was not possible to attribute the observed differences in gonad lipid composition and histology to a specific lipid class or fatty acid because several components varied simultaneously.

Lipid Class Content, Growth, and Survival During Embryonic and Early Larval Development

Our study showed a positive relation between the number of eggs released and the level of atresia in the female gonad (Fig. 6). Similarly, a negative correlation between fecundity of *P. maximus* and the number of D-larvae was found and was attributed to the atresia of ovaries (Le Pennec et al., 1998). In spite of the lack of correlation between atresia in gonad and subsequent hatching success of eggs produced, our results suggest that the released of a high number of eggs is incompatible with high egg quality.

Lipid composition of gonad and egg failed in predicting spawning (number and size of eggs released) and larval (survival and growth) performances. In contrast, previous studies showed that hatching success of *P. maximus* is a function of egg lipid reserves (Dorange 1989, Devauchelle & Mingant 1991). The absence of relationship in our study might be due to the similarity of lipid composition between egg groups.

Lipid class composition during embryonic and larval development showed a strong TAG depletion (Fig. 7). In fact, 52% of egg TAG reserves were consumed during embryogenesis (egg to 4 days) and 32% during early larval development (4 to 8 days). Consequently, despite the fact that larvae feed on exogenous phytoplancton at day 4, TAGs continued to decrease until day 8. Similarly, scallop larvae of *Patinopecten yeoenssis* consumed 54% of the initial reserves of neutral lipids during embryogenesis (Whyte et al. 1991) and scallop larvae of *P. maximus* started lipid accumulation 4 days after the first feeding (Delaunay et al. 1992). Our study confirms the high TAG demand during embryogenesis and the effect on larval lipid content.

Concomitantly, increases of STs and PLs during embryonic and larval development have been observed (Fig. 7). This underlines the changes from eggs, full of energy reserves, to young larvae, rich in structure. Thus, structural lipids, and particularly PL seem to be synthesized at the expense of TAG. It has been suggested that TAG play a double role in oyster *Ostrea edulis* larvae by storing large amounts of saturated fatty acids for energy purposes while acting as temporary reservoir of PUFA transferred to the structural

lipids (Napolitano et al. 1988). However, PL decrease during the early development of *P. maximus* (Delaunay et al. 1992) and fish larvae (Tocher et al. 1985, Fraser et al. 1988), suggesting an energetic role of PL. A more detailed experimental design is needed to interpret interactions between egg origin and age of larvae on lipid dynamic.

A significant increase of FFA from trace amounts in eggs to ca. 11% of TL in 8-day-old larvae was observed (Fig. 7). This result is in agreement with previous studies (Napolitano et al. 1988). As mentioned earlier, FFA is generally considered a degradation product and may be attributed to the increasing number of moribund larvae in samples. However, Napolitano et al. (1988) discussed the possible physiologic role of FFA and concluded that FFA probably remain adsorbed to specific proteins rather than being freely circulating, toxic lipids. Thus, FFA may not necessarily reflect inadequate storage or extraction conditions.

AMPL content increased during larval development from day 4 to 8 (Fig. 7). AMPL containing pigments (Parrish 1987) likely reflected ingestion of microalgae by larvae. This would also explain the lack of an increase of AMPL during embryogenesis when larvae are not able to feed from exogenous sources.

Surprisingly, our experiment did not show a significant effect of development on TL content (Fig. 7). In contrast, other studies have reported a marked decline of TL during embryogenesis of scallop larvae (Gallager et al. 1986, Whyte et al. 1991, Lu et al. 1999). Graphical examination of TL pattern during embryogenesis suggests a specific egg group effect (Fig. 6), hence this interpretation need to be taken with caution.

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BIOCHEMICAL INDICATOR OF SEA SCALLOP (*PLACOPECTEN MAGELLANICUS*) QUALITY BASED ON LIPID CLASS COMPOSITION. PART II : LARVAL GROWTH, COMPETENCY AND SETTLEMENT

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ABSTRACT The purpose of this study was to examine the lipid class content of larval stages of the sea scallop *Placopecten magellanicus* during development and to examine the potential effects of varying feeding regimes on larval lipid content, growth, survival, settlement behavior, and survival of postlarval stage. The potential of lipid class ratios to forecast larval growth, survival, settlement behavior, and success was examined. At the start of exogenous feeding (day 4) three diets, which differed in triacylglycerol (TAG) content, were applied. Diet A consisted of *Isochrysis* sp. and *Pavlova lutheri*, diet B was a mix of *Isochrysis* sp. and *Chaetoceros muelleri*, and diet C consisted of the same two species, but *C. muelleri* was grown under silicate deprivation to enhance TAG accumulation. Larvae were periodically sampled for lipid class analysis, growth measurement and survival assessment. Behavior of pediveliger larvae for each diet was recorded using an endoscopic camera during settlement. Experiments were replicated twice and repeated 1 mo later. Our study shows that TAG level in larval food was positively correlated with growth rate, larval TAG content and, as a consequence, larval "quality," as measured by 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 was found, suggesting that survival at competency was partly explained by the recovery efficiency of energetic reserves as TAG after embryogenesis. Higher growth rates obtained with the diet enriched with TAG reflect its high caloric content and the presence of sufficient essential fatty acids. TAG-ST ratio of competent larvae was negatively correlated with settlement success (day 40). High quality larvae explore the same period of time whatever their age, whereas low quality larvae decrease exploration time with age. Consequently, the low settlement success observed in our experiments with high quality larvae might reflect delayed metamorphosis in response to poor environmental conditions.

KEY WORDS: behavior; larval nutrition; lipid; microalgae; *Placopecten magellanicus*; silicate deprivation, scallop

INTRODUCTION

Success of bivalve larval culture depends to a large extent on larval energy reserves to support embryogenesis and metamorphosis. Energy reserves depend on the nutritional value of microalgal diets supplied to the larvae (Whyte et al. 1989). Webb and Chu (1983) reviewed the role of chemical constituents in phytoplankton and concluded that lipids were the most important constituent of the algal diet for larval rearing, particularly polyunsaturated fatty acids (PUFAs). Since then, attention has been paid to understanding the nutritional role of these essential components for bivalve larvae (Whyte et al. 1989, Delaunay et al. 1993, Soudant et al. 1996).

To optimize larval quality, here referring to physiological characteristics that could explain variability of growth, survival, and success of metamorphosis, total lipid or lipid composition would appear most appropriate. In fact, total lipid is a good indicator of larval quality because it has been correlated with growth and viability of bivalve larvae. When larvae are able to feed from exogenous sources, excess energy is stored mainly as triacylglycerol (TAG), the major storage lipid (Gallager et al. 1986). During embryogenesis, the larvae of scallop *Patinopecten yessoensis* depend on endogenous reserves, and lipid accounts for 47.6% of their energetic needs (Whyte et al. 1991). During metamorphosis, neutral lipids, particularly TAG, are the primary energy reserve of the oyster *Ostrea edulis* (Holland 1978, Gallager et al. 1986) proteins being used later. Thus, lipids and proteins account for approximately 95% of total energy requirements of oyster larvae (Holland & Spencer 1973).

Given the above, quality assessment has used ratios of lipid classes. TAG content can be related to larval quality, but as TAG content is directly dependent on larval size, it must be normalized. The use of TAG-sterol (ST) ratio takes into account the size dependency of TAG content because there is a positive correlation between ST content and larval mass (Fraser 1989). In a study of stressed larvae of marine bivalves, crustacea and fish, TAG-ST ratio reflects the quality of the larvae (Fraser 1989). This ratio has been applied to fishery experiments in the field (Hakanson 1989, Hakanson et al. 1994, Lochmann et al. 1995, Ouellet et al. 1995) and in the laboratory (Delaunay et al. 1992, Ouellet & Taggart 1992, Miron et al. 1999, Miron et al. 2000). These studies showed that TAG-based ratios are linked with larval growth, survival, and habitat selection during settlement.

The larval cycle of sea scallop, *Placopecten magellanicus* is typical of bivalves. Once gametes are released in the water column and fertilization has occurred, embryogenesis proceeds toward development of veliger larvae, a process completed after ca. 4 days. Then, swimming larvae spend 4 wk or more feeding in the water column until they reach a length of ca. 220 μm (day 28), where they become pediveligers, with eyespots and a foot. At this stage, larvae explore the bottom to find a suitable substratum to settle and undergo metamorphosis (Culliney 1974).

In this study, growth, survival, settlement, and lipid class content of sea scallop larvae reared under a variety of feeding regimes are reported to verify the validity of a TAG based indicator of larval quality. We designed this experiment with several objectives: (1) to monitor the lipid class content of larvae over the entire larval cycle, (2) to verify the effect of feeding regimen on larval lipid content, growth, survival and settlement, and (3) to examine the effect of larval quality, as determined by a TAG-based ratio, on growth, survival, and settlement.

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MATERIALS AND METHODS

Rearing Procedures

This study was conducted at the experimental hatchery of Ministère de l'Agriculture, des Pêcheries et de l'Alimentation du Québec at Grande-Rivière. Male and female adult scallops were harvested by SCUBA diving at Pointe Saint-Pierre at a depth of 20 m on July 15, 2001. Spawning induction, fertilization and rearing of young larvae was as previously described in Pernet et al. (2003). Briefly, five females were induced to spawn separately right after arrival in the laboratory. Individual spawnings were pooled in one tank for fecundation and embryos development. Fertilization occurred with a mix of spermatozoa provided by five males. Larvae were reared in 500-L tanks with aeration, at 13°C, at an initial density of 1.5 individual per mL. They were fed at ca. 15 000 algal cells mL^{-1}. Microalgae were produced by a semicontinuous method, grown in the f/2 nutrient mixture (Guillard 1975), and harvested every 3 to 4 days for diet characterization (lipid class and fatty acid composition) during the experimental period (40 days). Water renewal in the settling tanks followed the method of Bourne et al. (1989).

Experimental Design

Larvae were split into six batches to apply three diets in duplicate 4 days after fertilization, as D-veliger emerged. Diet A consisted a standard mixture of *Isochrysis* sp. and *Pavlova lutheri* (50/50 cells), diet B was a mixture of *Isochrysis* sp. and *Chaetoceros muelleri* (50/50 cells), and diet C was the same as diet B, but the diatom *C. muelleri* was grown under silicate deprivation. This process slow cell division and enhances TAG accumulation (Lombardi & Wangersky 1991), whereby the energy normally allocated to silicate uptake and deposition is diverted to lipid production (Coombs et al. 1967). At day 28, collectors were added to culture tanks since larvae reached competency (>50% larvae had developed visible eyes). Larvae were allowed to settle until day 40.

Swimming larvae were harvested at day 4, 8, 12, 20, 28, 32, 36, and 40 after fertilization for growth, survival, and lipid class analysis whereas settled larvae were sampled at the end of the experiment (day 40). Behavior of individual pediveliger larvae on day 36 and 40 for each diet treatment and replicate culture was recorded on videotape using an endoscopic camera (see below). Endoscopic recorded behavior were categorized as follows: (1) larvae actively exploring or (2) not moving, remaining attached to the screen, (3) actively swimming, or (4) passive in the water column. Based on these observations, a time budget (relative time spent by each larvae exhibiting a specific behavior) was determined. Exploration distance and exploration rate (distance.time^{-1}) were measured for each larvae as well.

The complete experiment was repeated from mid-August to September, without the behavioral aspects. However, the second experiment was conducted without replicates because of the poor hatching success of the eggs.

Laboratory Analysis

Shell size was average of length (anterior–posterior distance) and height (dorsal–ventral distance) of at least 30 larvae. Larvae were measured using a compound microscope at a magnification of 40× with image capture kit CoolSNAP-Pro cf Digital Kit™ 4.1.

Lipid analysis was conducted as previously described (Pernet et al. 2003). Briefly, 5000 veliger larvae or 500 pediveligers were filtered on prebaked GF/C filters and stored in dichloromethane at −20°C until lipid extraction. Samples were sonicated and lipids were extracted according to Folch et al. (1957). Then, lipids were spotted onto the S-III Chromarods (Iatron Laboratories Inc., Tokyo, Japan) and separated according to Parrish (1987). Lipid classes were quantified with the analyser Iatroscan Mark-V (Iatron Laboratories Inc., Tokyo, Japan). Fatty acid composition of microalgae was analyzed by gas chromatography. Fatty acid methyl ester (FAME) were prepared following the method of the American Oil Chemists' Society (AOCS 1989) and injected in a Perkin Elmer Sigma 300 capillary chromatograph, equipped with a Supelco Omegawax™ 320 fused-silica capillary column. FAMEs
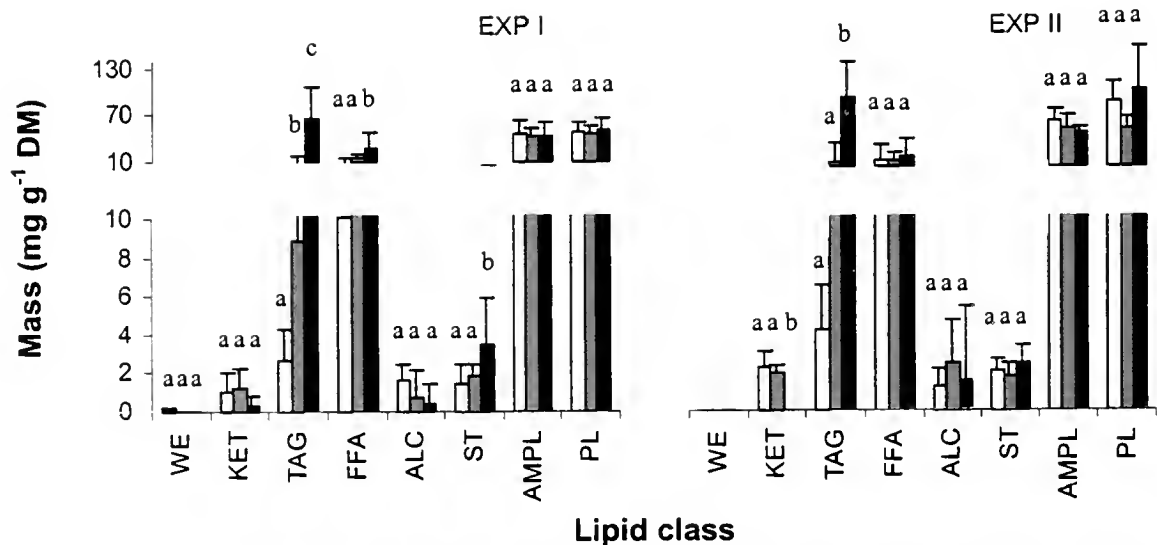
Figure 1. Mass of each lipid class (±SD, n = 9 and *n* = 6 for experiments I and II, respectively) expressed in mg per g dry mass in diet A (*Isochrysis* sp. + *Pavlova lutheri*, □), B (*Isochrysis* sp. + *Chaetoceros muelleri*, ▨), and C (*Isochrysis* sp. + *Chaetoceros muelleri* with silicate deprivation, ■) during experiment I and II. Lipid classes detected were wax ester (WE), ketone (KET), triacylglycerol (TAG), free fatty acid (FFA), fatty alcohol (ALC), cholesterol (ST), acetone mobile polar lipid (AMPL), and phospholipid (PL).

were identified by their retention times compared with standard (Supelco 37 component FAME Mix, Menhaden Fish Oil and PUFA-3, Supelco, Bellefonte, PA) and quantified with tricosanoic acid (c23:0) as an internal standard.

The methodology used to examine exploratory behavior of competent larvae was previously described (Walters et al. 1999). The equipment used was an Olympus K17-18-90 endoscope (1.7 mm diameter, 186 mm length) attached to a video camera fixed to a micromanipulator arm, allowing two-dimensional movement. Video output was sent to a video 8 mm integrated to a monitor (Optiscan 1US-1). An Olympus 250 W high-intensity xenon light source (model ILV-2) provided cold light to the extremity of the endoscope. Behavioral observations were performed on swimming larvae sampled in settling tank during water renewal on day 36 and 40 for diets B and C. These larvae were carefully transferred to circular tank type "Plankton Kreisel." A two-dimensional (23 × 16.5 cm) polypropylene collector (200-μm pore mesh) was placed perpendicular to the flow (ca. 1 cm s^{-1}). Observation of larval behavior in each trial lasted until the larvae had stopped movement for 5 min or was out of the field covered by the camera (5.5 × 3.5 cm). Between each recording, collector was shaken and rinsed to detach larvae and new larvae were injected into the system. Behavioral observations lasted for average 6.3 min, at least 1 min to a maximum of 33 min.

Data Analysis

The lipid class ($n = 15$) and fatty acid compositions ($n = 5$) of the diets were compared by one-way multiple analysis of variance (MANOVA). Fatty acid were grouped in saturated (SFA), monounsaturated (MUFA), and polyunsaturated fatty acids (PUFAs). Among PUFAs, 20:5n3 (eicosapentaenoic acid, EPA), 22:6n3 (docosahexaenoic acid, DHA), total n3 and n6 and finally n3-n6 ratio were distinguished (see Pernet et al. 2003).

Shell size, lipid class composition, and quality of swimming larvae depending on diet and age were investigated by two separate two-way MANOVA. Groups of larvae fed diet A were lost at day 28, leading to an unbalanced design. Then, the first analyzes included data from day 4 to 28 whereas the second analyses included data from day 28 to 40. Where differences were detected, Least Square Mean multiple comparison tests (LSMean) were used to determine which means were significantly different with probability levels divided by the number of degrees of freedom of the tested factor (Bonferroni correction). Shell size, lipid class composition, and quality of larvae depending on diet, age and behavior were investigated by one-way MANOVA. Treatments consisted of different combinations of factors: diets B or C at days 28 and 40 and, at day 40, larvae settled or swimming. Contrasts were performed to verify a posteriori particular effect.

Exploration rate and exploration distance of larvae depending on diet and age were submitted to two-way analysis of variance. Homoscedasticity was tested by running Levene's test and was confirmed by graphical examination of the residuals (Sherrer 1984).

Finally, to compare survivorship according to diet from day 4 to 28, Life Test procedures were used. When differences were detected, the χ^2 comparison tests were applied to determine which treatments differed significantly. A significant threshold of 0.05 was adopted for all statistical tests. All statistical analyses were run in SAS 8.01 (SAS Institute Inc., 1999–2000, Cary, NC).

RESULTS

Lipid Class and Fatty Acid Composition of Larval Diet

As expected, lipid class content differed among larval diets ($P = 0.0221$). TAG levels were highly different among diets (Fig. 1). In experiment I, diet C had higher TAG content than diets A ($P < 0.0001$) and B ($P < 0.0001$) and diets A and B also differed ($P = 0.0333$). In fact, diets A, B and C contained 2.68, 8.92 and 65.11 mg.g^{-1} algal dry mass of TAG which represent 2.32, 8.31, and 31.19% of lipid class composition respectively. Free fatty acids (FFA), cholesterol (ST), and total lipids (TL) levels were also different among diets (Fig. 1). During the course of experiment II, microalgae lipid class composition revealed nearly the same differences of TAG and total lipid content among diets. However, FFA and ST contents were the same whereas ketone levels were different ($P = 0.0001$). In short, the lipid composition of the diatom *Chaetoceros muelleri* was altered by varying the silicate level in the culture medium. The TAG content of cells

TABLE 1.

Fatty acid mass (mg g^{-1} dry mass) and % (relative to the sum of fatty acid mass) in larval diet A (*Isochrysis* sp. + *Pavlova lutheri*), B (*Isochrysis* sp. + *Chaetoceros muelleri*), and C (*Isochrysis* sp. + *Chaetoceros muelleri* with silicate deprivation) during experiment I and II ($n = 5$).

Fatty Acid	A Mass	A %	B Mass	B %	C Mass	C %
12:0	0.07	0.09	0.07	0.08	0.20	0.09
14:0	13.29	17.24	12.01	14.80	28.80	13.16
14:1n5	0.58	0.75	0.52	0.64	0.53	0.24
15:0	0.23	0.30	0.40	0.49	1.79	0.82
16:0	7.56	9.81	9.15	11.27	53.73	24.55
16:1n7	6.06	7.87	11.18	13.77	48.37	22.10
16:2n4	0.86	1.12	1.28	1.57	2.46	1.13
16:3n4	0.42	0.55	4.44	5.46	4.42	2.02
18:0	0.24	0.32	0.98	1.21	3.67	1.68
18:1n9	4.55	5.91	4.32	5.32	6.32	2.89
18:1n7	1.20	1.56	1.38	1.70	1.77	0.81
18:2n6	3.50	4.54	4.76	5.87	5.45	2.49
18:3n6	0.72	0.94	0.81	0.99	6.55	2.99
18:3n3	4.33	5.61	3.44	4.24	3.48	1.59
18:4n3	11.56	15.00	8.33	10.26	13.48	6.16
20:0	0.02	0.02	0.09	0.11	0.09	0.04
20:1n9	0.23	0.30	0.29	0.36	0.09	0.04
21:0	0.02	0.02	0.00	0.00	0.56	0.25
20:4n6	0.28	0.36	0.90	1.11	7.58	3.46
20:4n3	0.07	0.09	1.43	1.76	0.65	0.30
20:5n3	10.43	13.53	8.26	10.17	19.47	8.89
22:0	0.06	0.08	0.16	0.19	0.38	0.17
22:1n9	0.04	0.06	0.07	0.09	0.06	0.03
21:5n3	0.00	0.00	0.00	0.00	0.00	0.00
22:5n3	0.02	0.02	0.03	0.03	0.27	0.12
24:0	0.02	0.03	0.02	0.02	0.00	0.00
22:6n3	10.71	13.89	6.88	8.47	8.68	3.97
ΣSFA	21.51	27.90	22.87	28.17	89.21	40.76
ΣMUFA	12.68	16.45	17.77	21.88	57.14	26.11
ΣPUFA	42.90	55.65	40.55	49.95	72.49	33.13
Σn3	37.11	48.14	28.37	34.94	46.03	21.03
Σn6	4.51	5.84	6.47	7.97	19.58	8.95
Σn3/Σn6	8.24		5.18		2.42	
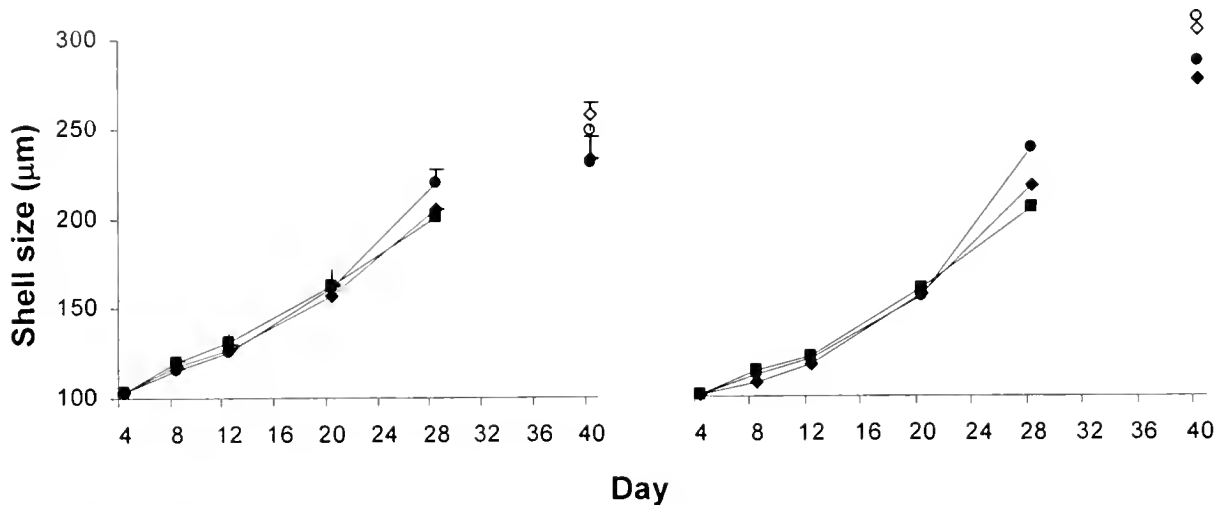
Figure 2. Growth of larvae fed diet A (■), B (♦), and C (●) depending on larval stage and experiment (I or II). Distinction between settled (open symbol) and plantonic (filled symbol) larvae was made at day 40 (±SD, $n = 2$ and $n = 1$ for experiments I and II, respectively).

cultured in the silicate deprived medium was higher than those in the complete medium.

Fatty acid analyses performed of diets A, B, and C during experiment I and II revealed that diets A and B did not differ (Table 1). However, silicate deprivation enhanced fatty acid accumulation in diet C with no distinction among SFAs, MUFAs, or PUFAs. Relative content of PUFAs from diets A and B (ca. 53%) differed from that of diet C (33%). As a consequence, diet C may be qualified as a high TAG-rich SFA and MUFA diet. The three diets had all essential fatty acid requirements, each of them containing eicosapentaenoic (EPA, 20:5n3) and docosahexaenoic acid (DHA, 22:6n3). Diet C was the richest in total n3 and 20:5n3 whereas diet A exhibited the highest level of 22:6n3. DHA-EPA ratios of diets A, B and C were respectively 1.03, 0.83 and 0.45.

Larval Growth and Survival

Diet and time interacted in their effects upon larval growth ($P = 0.032$). The difference of larval size among diets first appeared when larvae had reached competency at day 28 (Fig. 2). In fact, larvae fed diet C exhibited a higher mean size (220.09 μm) than larvae fed diet B (204.56 μm, $P = 0.0011$) and diet A (200.74 μm, $P < 0.0001$). There was no size difference between larvae fed diets A and B ($P = 0.3960$). Consequently, diet C increased larval growth by ca. 20% compared with diets A and B. However, these results were not maintained at day 40 because larvae fed diet B reached the same size as those fed diet C (respectively 257.16 and 248.96 μm, $P = 0.339$). Furthermore, settled larvae were larger than swimming ones (253.9 vs. 231 μm, $P = 0.004$). Larvae in experiment II showed the same tendency as in experiment I, but dietary effects seemed more pronounced (Fig. 2). In fact, mean size of larvae fed diets A, B and C at day 28 were respectively 199.29, 210.65 and 230.90 μm and discrepancies among size related to diets were maintained after settlement.

Larvae fed diet A suffered high mortality on day 28 in both experiments (Fig. 3). In experiment I, survivorship patterns were similar between larvae fed diets B and C but larvae fed diet B seemed to have a better settlement success than those fed diet C at day 40. In experiment II, larvae fed diet C seemed to promote higher survival than those reared on diets B and A at competency,
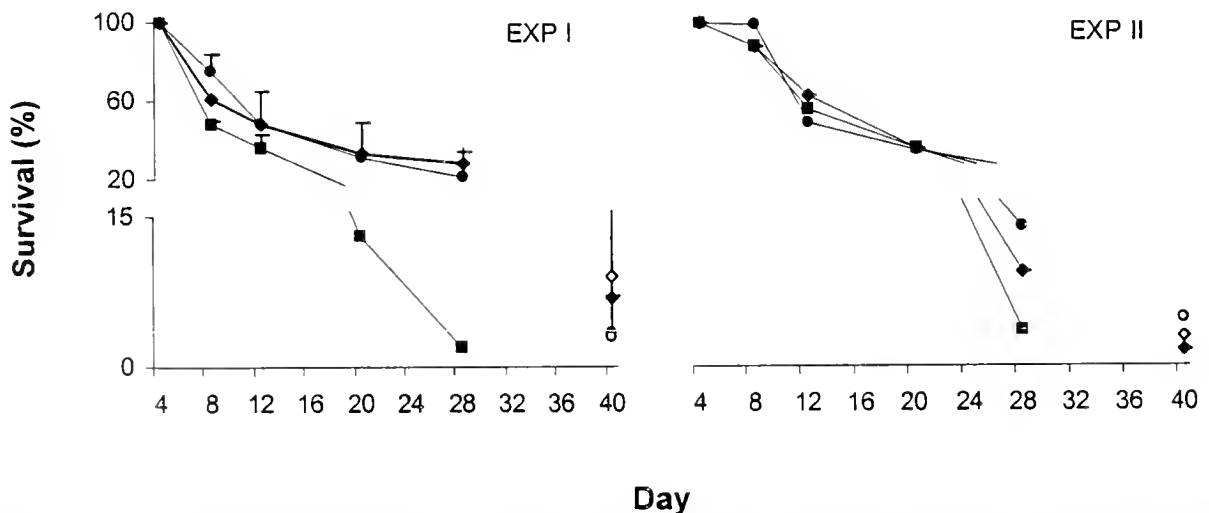
Figure 3. Survival of larvae fed diet A (■), B (♦), and C (●) depending on larval stage and experiment (I or II). The distinction between settled (open symbol) and planktonic (filled symbol) larvae was made at day 40 (±SD, $n = 2$ and $n = 1$ for experiments I and II, respectively).

and a higher settlement success than larvae fed diet B (respectively 4.6% vs. 2.7%, Fig. 3).

Lipid Class Composition During Larval Cycle

From D-Veligers to Competency

Age and diet interacted in their effects upon lipid class composition of sea scallop larvae ($P = 0.0001$), particularly, TAG, ST, PL and, as a consequence, total lipid TL (Figs. 4 and 5). FFA and acetone mobile polar lipids (AMPL) were accumulated during larval development without any diet effect.

From the beginning of the experiment to day 20, TAG content of larvae was constant with no difference attributable to diet. TAG content of 28 days old larvae fed diets B and C rose by factors of 15 and 70 respectively, while it remained constant for larvae fed diet A (Figs. 4 and 5). Consequently, the high TAG level in diet C was correlated with a better accumulation of TAG in larvae prior to metamorphosis compared with larvae fed diet A ($P < 0.0001$) and B ($P = 0.012$). Moreover, diet B allowed a greater accumulation of TAG than diet A ($P = 0.0007$).

The two structural lipids, ST and PL followed the same trend (Fig. 4 and 5). From the beginning of larval development until day 20, ST and PL were gently rising, independently of diet. After day 20, these lipids were accumulated at different rates according to diet. PL and ST contents of larvae fed diets B and C showed a marked rise, whereas they remained low for larvae fed diet A.
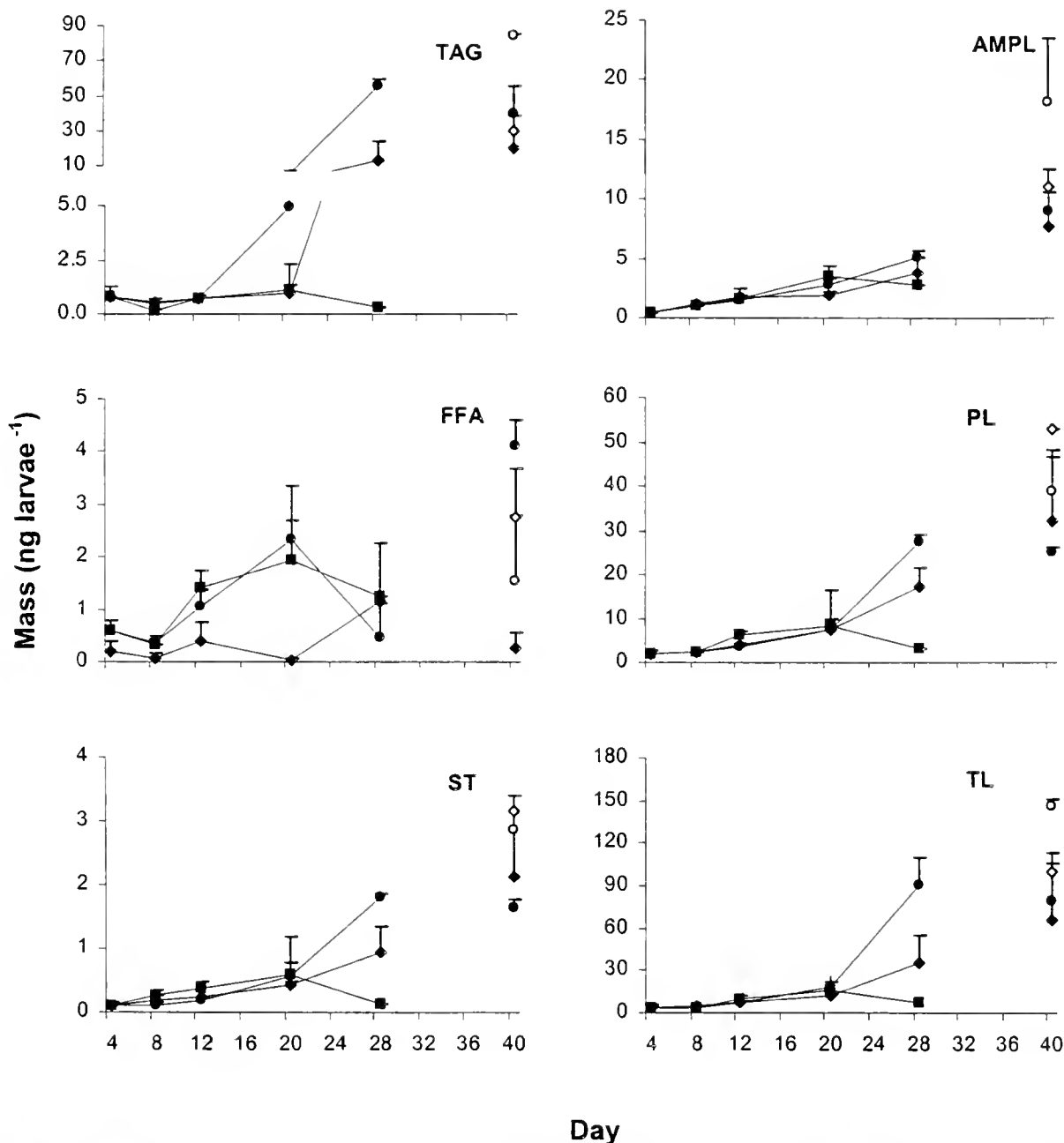
Figure 4. Lipid class profile of larvae of experiment 1 fed diet A (■), B (♦), and C (●) depending on larval stage. Distinction between settled (open symbol) and planktonic (filled symbol) larvae was made at day 40. Lipid classes detected were TAG, FFA, ST, AMPL, and PL. TL was obtained by summation of each lipid class (±SD, $n = 2$).
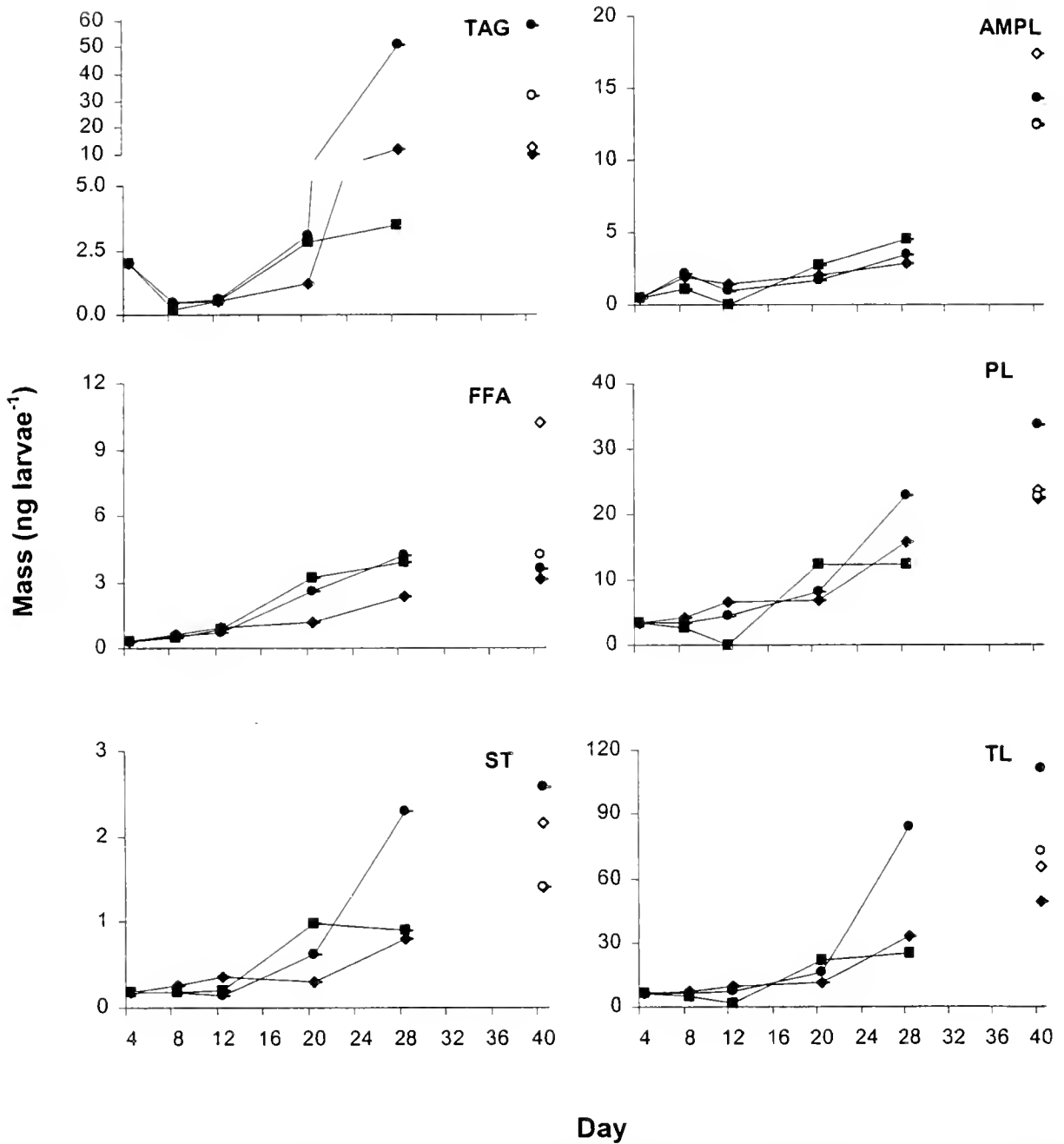
Figure 5. Lipid class profile of larvae of experiment II fed dies A (■), B (♦), and C (●) depending on larval stage (no replication). Distinction between settled (open symbol) and planktonic (filled symbol) larvae was made at day 40. Lipid classes detected were TAG, FFA, ST, AMPL, and PL. TL was obtained by summation of each lipid class.

FFA displayed distinct patterns compared with the other lipid class. Despite a significant time effect on FFA content, FFA were not accumulated during larval development in experiment I (Fig. 4). In fact, the level of FFA in young larvae at day 8 was the same as that of pre-competent larvae at day 28 ($P = 0.16$). Average FFA content was 9.01%. High levels of FFA in sample may be attributed to the presence of moribund larvae. The presence of large amounts of FFA in animal tissues (more than 10%) is usually an indication of lipid degradation and decreases in the amounts of TAG and PL.

Finally, AMPL content of larvae increased gradually from day 4 to competency without any measurable effect of the feeding

regimen ($P = 0.543$, Figs. 4 and 5). AMPL consist principally of pigments and may reflect ingestion of microalgae.

From Competency to Settlement

In experiment I, TL level in larvae fed with diet B increased significantly from day 28 to 40 ($P = 0.041$). This effect was mainly attributable to the augmentation of PL level during this period ($P = 0.009$, Fig. 4). This pattern was not observed in larvae fed diet C. Levels of TAG in larvae fed diet C were higher than those fed diet B ($P = 0.005$). Settled larvae had higher TAG and TL content than planktonic ones ($P = 0.018$ and $P = 0.015$

respectively). However, data of experiment II showed inverse result since settled larvae fed diet C had a lower TAG and TL contents than planktonic larvae (Fig. 5).

Larval Quality

ST and PL were highly correlated with larval size, confirming the adequacy of these structural lipids as weighting factors for the size dependency of TAG levels (Fig. 6). Thus, TAG-ST and TAG-PL ratios were used as indicators of larval quality.

From D-Veligers to Competency

Age and diet interacted in their effects upon TAG-ST and TAG-PL ratios ($P < 0.001$, Fig. 7). Between day 4 and day 20, there was no difference in either TAG-ST or TAG-PL ratios. At day 20, larvae fed diet C showed higher ratios of TAG-ST and TAG-PL than those fed diets A and B. From day 20 to day 28, just before settlement, a sharp rise of both ratios was observed for larvae fed diets B and C in both experiments. High TAG level in diet C suggests higher larval quality prior to metamorphosis compared with diets A and B.

Correlation analysis showed a significant positive relation between size of 28-day-old larvae and their TAG-ST and TAG-PL ratios at day 20 (Table 2). There was also a positive relation between survival of 28-day-old larvae and TAG-ST and TAG-PL ratios at day 8. The strength of this relationship gradually decreased with age of larvae (Table 2).

From Competency to Settlement

TAG-ST and TAG-PL ratios of planktonic larvae did not vary significantly during settlement ($P = 0.258$ and $P = 0.559$ respectively, Fig. 8). Planktonic larvae fed diet B showed lower levels of TAG-ST and TAG-PL ratios ($P = 0.001$ and $P < 0.001$, respectively).

The level of ratios of settled larvae remained constant compared with those observed at competency (Fig. 7). However, there was a significant effect of diet on TAG-ST and TAG-PL ratios of settled larvae. Larvae fed diet C maintained higher TAG-ST and TAG-PL ratios than larvae fed diet B. Settled and swimming larvae at day 40 had the same ratio values, despite different TAG levels.

A negative correlation between larval quality (TAG-ST ratio) of 28-day-old individuals and settlement rate at day 40 was apparent (Fig. 9). The higher the TAG-ST ratio, the lower was the settlement rate. However, this relation was not significant for TAG-PL ratio.

Larval Behavior During Settlement

Larval behavior time budgets (relative time spent by each larvae exhibiting a particular behavior) at day 36 showed no evident effect of feeding regimen or larval quality. Larvae fed diets B (low TAG-ST ratio) and C (high TAG-ST ratio) spent most of their time swimming in the water column. Active exploration of the collector consisted in ca. 16% of observation time whereas immobility on
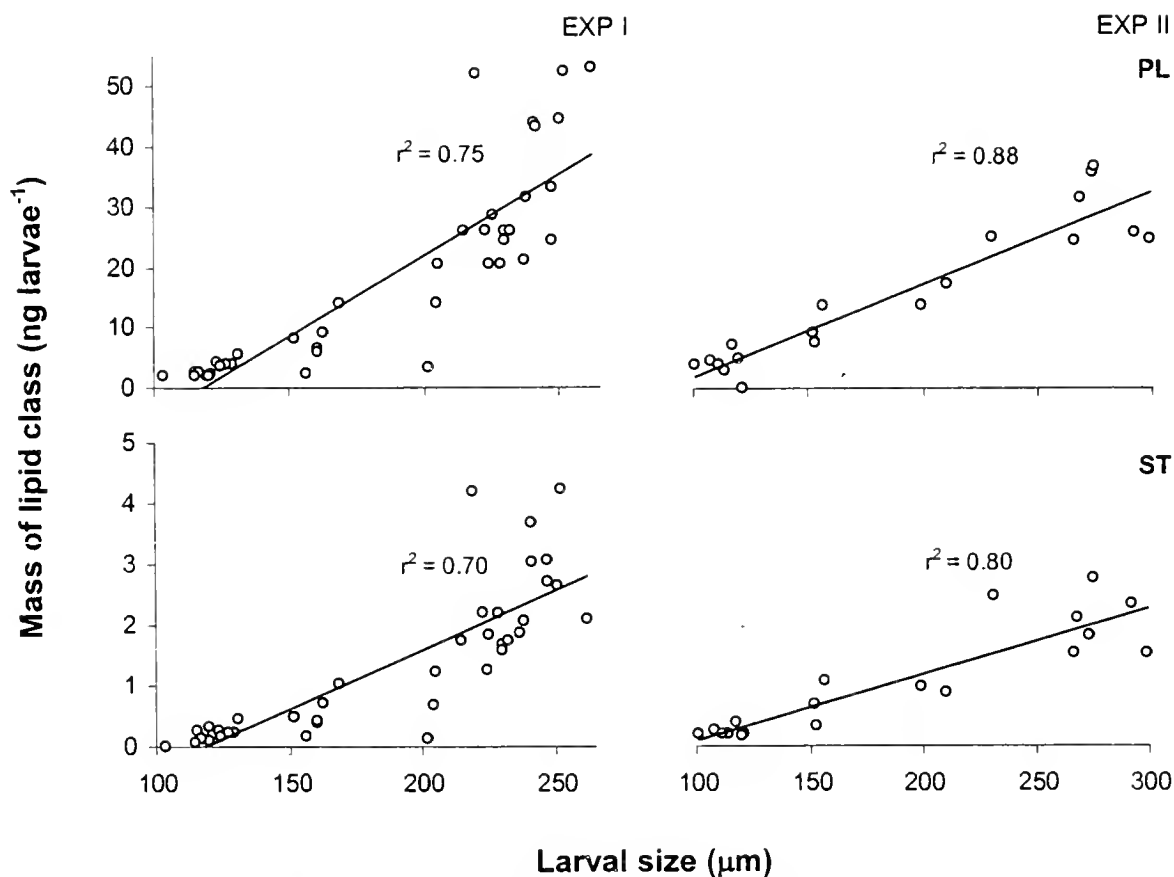
Figure 6. Relation between structural lipid as cholesterol (ST) and phospholipids (PL), and larval size in experiments I and II ($n = 39$ and $n = 19$, respectively).
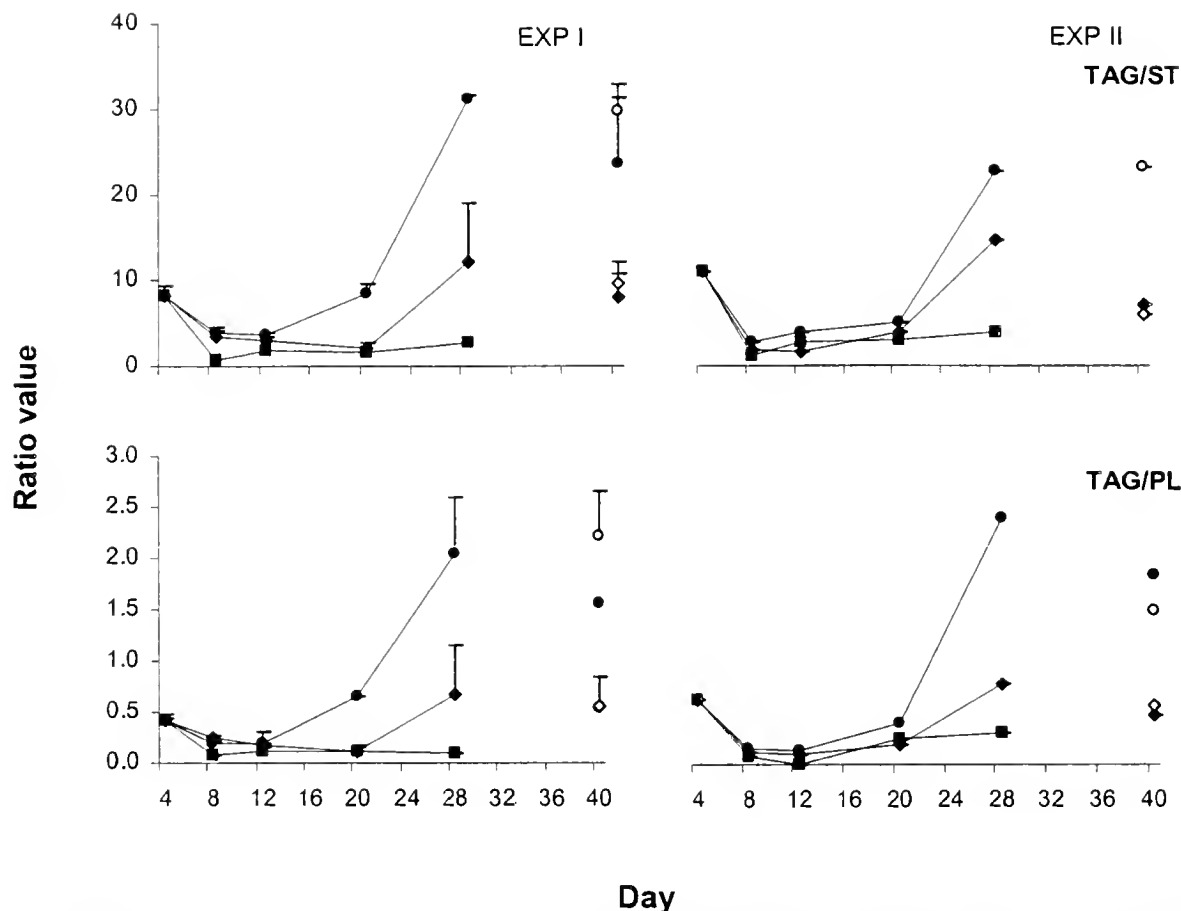
Figure 7. Triacylglycerol (TAG)-cholesterol (ST) or TAG-phospholipid (PL) ratios of larvae-fed diet A (■), B (♦), and C (●) depending on larval stage in experiment I and II. Distinction between settled (open symbol) and planktonic (filled symbol) larvae was made at day 40 (±SD, *n* = 2 and *n* = 1 for experiments I and II, respectively).

the screen accounted for ca. 34% of observation time of each larvae (Fig. 10). However, larvae fed diet B or C exhibited different time budgets at the end of the experiment (day 40). In fact, larvae fed diet C showed active exploration of the collector for 9% of the observation time whereas larvae fed diet B exhibited little exploration behavior (<2%). The relative time spent immobile on

the screen by larvae was higher at day 40 compared with day 36, and, at day 40, it was higher for larvae fed diet B than those fed diet C. Exploration rate of 40 day old larvae seemed to be higher than observed for 36-day-old larvae fed diet B ($P < 0.05$, Fig. 11A). Finally, exploration distance was similar for larvae fed both diets (Fig. 11B).

DISCUSSION

Lipid Composition of Larvae

During the first 20 days, lipid reserves of the larvae of the sea scallop *Placopecten magellanicus* remained low (Figs. 4 and 5). A similar pattern for total lipids occurs in Japanese scallop *Patinopecten yessoensis* (Whyte et al. 1987). From late embryogenesis to 20 days, energy from food intake might be insufficient to sustain simultaneously larval growth and lipid accumulation. In contrast, a continuous increase of TAG from day 3 is normal in developing larvae of the great scallop *Pecten maximus* and suggests that food was efficiently assimilated (Delaunay et al. 1992). Thus, the observed pattern in sea scallop larvae might reflect a lag in the metabolic and digestive processes of food assimilation and storage.

From day 20 to 28, TAG and structural lipids accumulated as the larvae reached pre-metamorphic condition. During this period, dietary sources of energy were directed toward growth, develop-

TABLE 2.

Matrix of Pearson correlation coefficients between size or survival of 12-, 20-, and 28-day-old larvae with TAG-ST and TAG-PL ratios of 8-, 12-, and 20-day-old larvae.

Variable	Age (d)	TAG/ST			TAG/PL		
		8	12	20	8	12	20
Shell size (μm)	12	−0.031			0.327		
	20	−0.070	−0.061		−0.023	−0.062	
	28	0.519	0.667	**0.739**	0.070	−0.056	**0.734**
Survival (%)[a]	12	0.289			−0.015		
	20	0.449	0.427		0.459	0.210	
	28	**0.706**	0.543	0.313	**0.760**	0.476	0.309

[a] Based upon initial number of 4-day-old larvae.
Data of experiment I and II were pooled (*n* = 9 larval cultures).
Significant probabilities are in bold ($P < 0.05$).

Day
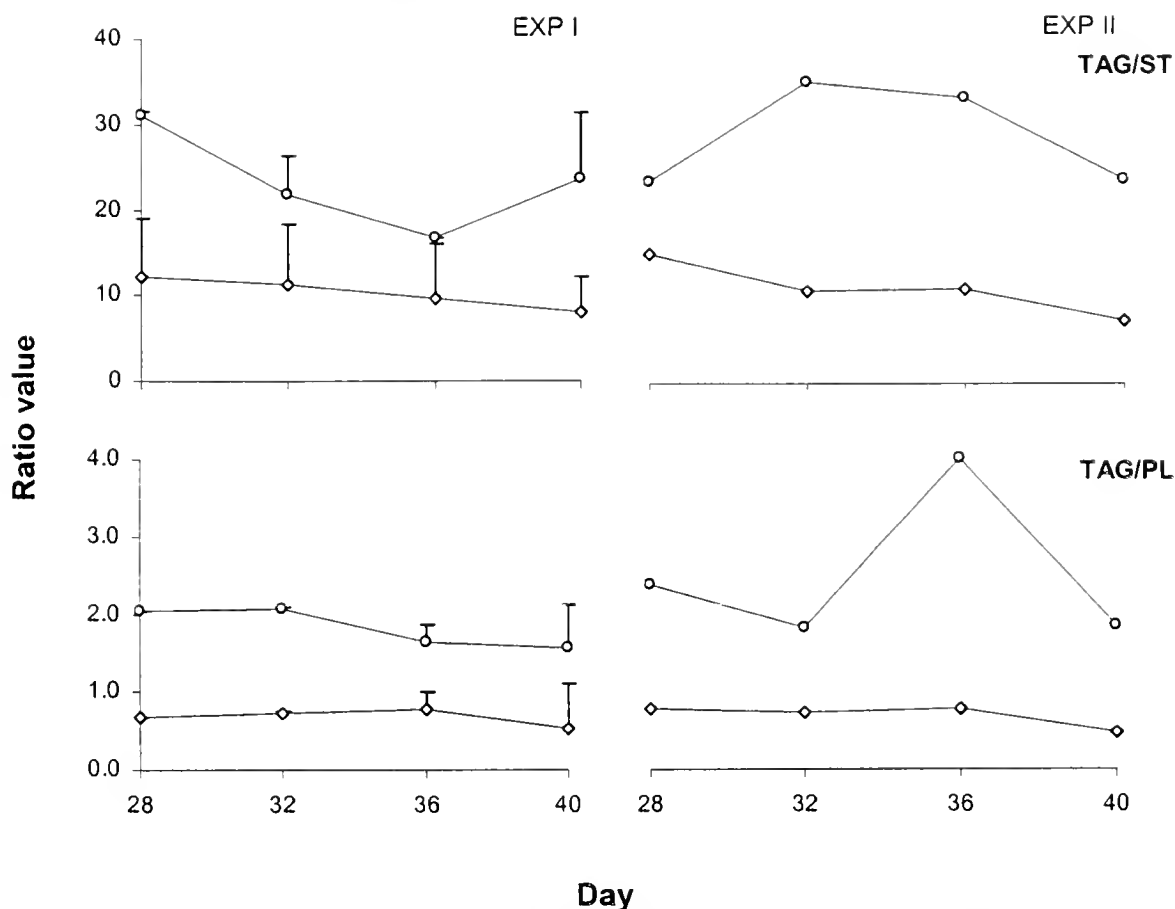
Figure 8. Triacylglycerol (TAG)-cholesterol (ST) and TAG-phospholipid (PL) ratios of competent planktonic larvae fed diet B (◊) and C (◯) depending on age in experiments I and II (±SD, $n = 2$ and $n = 1$ for experiments I and II, respectively).

ment of primary gill filaments and foot, as well as storage to meet the energy demand for metamorphosis (Whyte et al., 1987).

Finally, during settlement, from day 28 to 40, lipid levels remained stable. These results contrast with the low lipid levels following metamorphosis of oysters (Holland and Spencer, 1973; Labarta et al., 1999), scallops (Whyte et al. 1992) and barnacles

(Lucas et al. 1979). Three reasons might be evoked to explain this pattern. Firstly, a low level of lipid reserves following metamorphosis of marine invertebrates is still debated. For example, lipids provided 59.5% of energy needs during metamorphosis of *Ostrea edulis* (Holland and Spencer, 1973) whereas another study showed that metamorphosis of the same species was fuelled mainly by
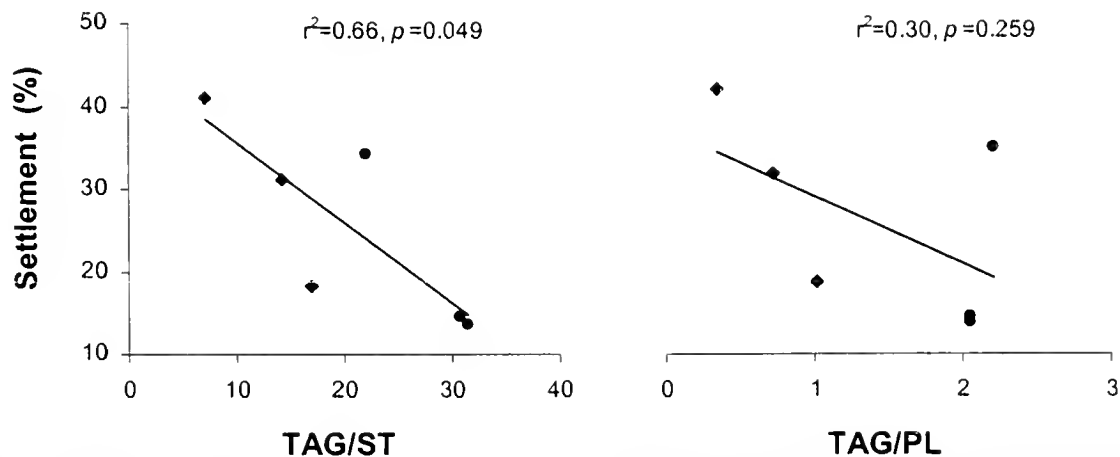
Figure 9. Triacylglycerol (TAG)-cholesterol (ST) and TAG-phospholipid (PL) ratios of larvae fed diet B (♦) and C (●) at competency (day 28) as a function of the settlement success. Settlement success is the percentage of settled larvae at day 40 based of the number of competent larvae at day 28. Data of experiments I and II were pooled ($n = 6$ larval culture).

Diet B   **Diet C**

Day 36

TAG/ST= 9.69
TAG/PL= 0.79
N=28
16%
36%
4%   44%

TAG/ST= 16 67
TAG/PL= 1.63
N=19
15%
32%
6%
47%

Day 40

TAG/ST= 8.04
TAG/PL=0 54
N=8
1% 15%
1%
83%

TAG/ST= 23.43
TAG/PL= 1 55
N=27
11%
53%   34%
2%
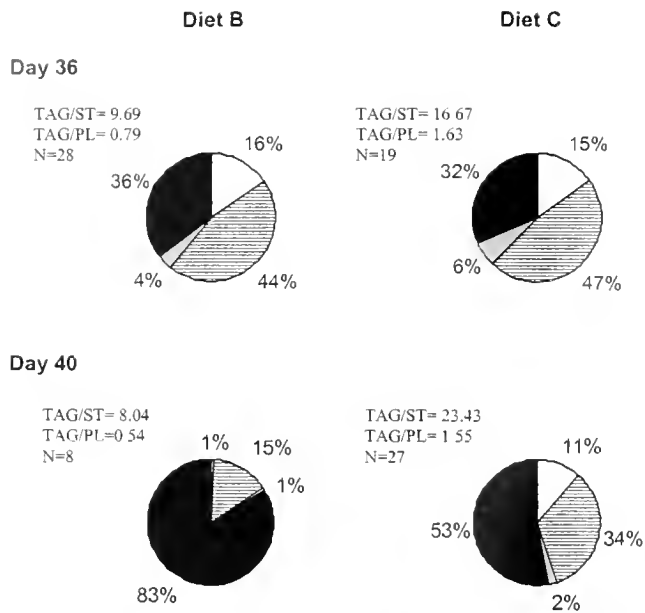
Figure 10. Time budgets for sea scallop larvae according to diet and time. Recorded behavior were larvae actively crawling (☐) or remaining fixed on the screen (■) swimming (▨) or passive in the water column (☐). Each pie chart represents the proportion of observation time spent by each larva exhibiting a specific behavior. (n:2 replicate culture)

proteins, lipids accounting for only 16.8% (Rodriguez et al. 1990). Secondly, low TAG levels have been observed in newly settled larvae (Holland & Spencer 1973). In our experiments, larvae may not have been as newly settled as necessary to observe a TAG depletion. Lipid analysis were performed on sample of ca. 5000 larvae containing settled larvae of different ages. Thus, some of them may have started recovering energy reserves and increasing the mean lipid level of the cohort. For instance, post-metamorphic larvae of *O. edulis* had recovered pre-metamorphic neutral lipid mass 4 to 11 days after settlement (Holland & Spencer 1973). Finally, sea scallop larvae may continue to feed on microalgae during settlement and counterbalance the lipid utilization. In fact, the oyster *Crassostrea virginica* has the ability to feed during settlement and metamorphosis (Baker & Mann 1994). The feeding hypothesis agrees with the maintenance of dietary differences in TAG levels during settlement.

Effects of Dietary Triglyceride Enrichment

The diet enriched in TAG promoted higher larval growth (Fig. 2). *Chaetoceros muelleri* grown under silicate limited conditions leads to the highest growth rate of juvenile oysters at low feeding rations whereas at higher feeding rations, the silicate limited culture was a poorer diet than the control culture (Enright et al. 1986). The high level of calorie-rich SFAs and MUFAs in the silicate-limited diet was evoked by the authors to explain higher growth rates of the oysters at the lowest feeding ration. At the higher feeding ration, the fatty acid composition, and particularly the relatively low content of 22:6n3 became a limiting factor and might explain the lower growth rate of oyster larvae fed with the silicate limited cells. In our experiments, high growth rates were obtained with a diet including silicate limited culture of *C. muelleri* (diet C) probably because SFA and MUFA were 4 times higher and PUFA were also twice higher than in diets A and B.

Abnormally high mortality was observed for larvae fed diet A in both experiments and was correlated with extremely low TAG and low total lipid levels in diets and larvae. This agrees with previous studies showing a positive relation between larval lipid content and survival (Gallager et al. 1986, Delaunay et al. 1992, Ouellet & Taggart 1992). It seems that low lipid or TAG level in the feeding regimen might have deleterious effect on survival, whereas there was no difference in survival for the other regimes.

TAG levels in competent larvae were correlated with TAG levels in diet (Figs. 4 and 5). A possible explanation is that the metabolic cost involved in TAG synthesis and storage could be lower when feeding upon microalgae rich in SFA and MUFA, TAG, or more generally, total lipid rather than with other biochemical sources such as protein or carbohydrate. However, conclusions based on these observations must be tempered by the possibility of effects as a result of variations of other unmeasured variables such as digestibility or palatability due to diatom cell wall properties or biochemical compounds such as amino-acids and vitamins (see Robert & Trintignac 1997, for review). In short, we cannot argue a direct causal relationship between diet and larval lipid composition.

Larval Quality

We used two indicators to assess larval quality: TAG-ST and TAG-PL ratios. During larval development prior to competency, both ratios have led to the same results and conclusions. As PL have an energetic role during early larval development or starvation period (Tocher et al. 1985, Fraser et al. 1988, Fraser 1989, Delaunay et al. 1992) the use of the more conservative TAG-ST ratio should be more appropriate to estimate larval quality. However, PL depletion was not observed in our study of sea scallop larvae, as reported for larvae of *Patinopecten yessoensis* (Whyte 1987). This allows us to use both indicators without distinction.

Based on these indicators, a positive relation between size of 28-day larvae and larval quality at day 20 was found (Table 2). It has previously been reported that poor growth was related to a low TAG-organic matter (OM) ratio in D-larvae, while higher levels of ratio were not necessarily reflected by growth rate (Delaunay et al. 1992). Consequently, it seemed that ST was a better denominator than OM. This might be due to the fact that OM values include both structural and storage molecules, thus decreasing accuracy of a storage-structure ratio.

A positive relationship between number of competent larvae produced and 8 d larval quality indices was found (Table 2). This is in accordance with results of previous studies. Indeed, risks of mortality of the shrimp *Pandalus borealis* were well correlated with the condition indices of the larval group as measured by the proportion of larvae exhibiting a TAG-wet mass ratio <0.2 (Ouellet & Taggart 1992). Moreover, high mortality of larvae of scallop *P. maximus* could be related to an abnormally low initial TAG-OM ratio (Delaunay et al. 1992). Our study suggests that survival at competency was partly explained by the efficiency of recovery of lipids after embryogenesis and, as discussed by Delaunay et al. (1992), reflects the difficulties of the transition to the D-veliger stage.

Finally, a negative correlation between the quality (TAG-ST ratio) of competent larvae (28-day-old) and settlement success (at 40 days) was found (Fig. 9). The better the quality, the lower the settlement success. In the barnacle *Semibalanus balanoides*, it appears that cyprids of high physiological condition, as measured by
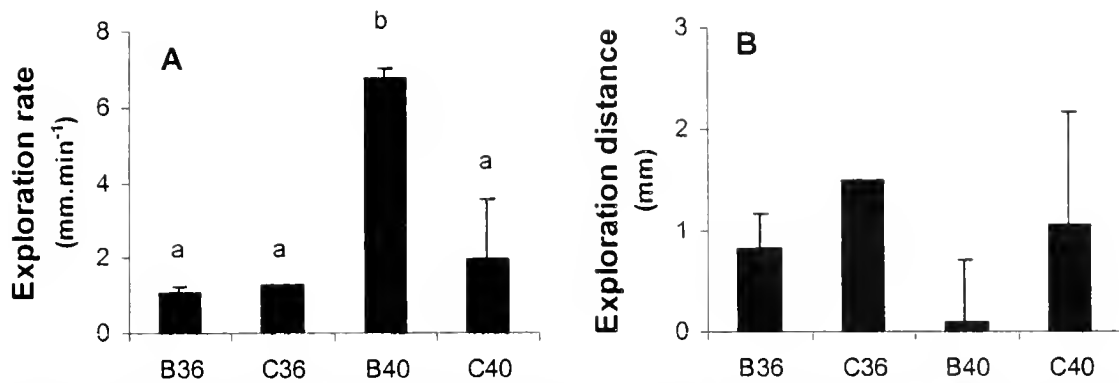
Figure 11. Exploration rate (A) and distance (B) as a function of diet (B or C) and age of larvae (36 or 40 days old). Exploration rate is the distance traveled by exploring larvae (*n* varied from 2 to 14 as a function of treatment combination) per minute. Exploration distance is the average value of the distance traveled by all the observed larvae (*n* varied from 8 to 28). Vertical bars represent standard errors between replicate cultures.

a TAG-ST ratio, settle in the best quality habitats compared with those in low condition (Miron et al. 1999). In fact, TAG-ST ratio of cyprids was highest at low intertidal level, the preferred attachment location site. This suggests that larvae fed with rich TAG diet C and having high TAG-ST ratio might delay metamorphosis to encounter better quality settlement sites, whereas larvae fed with poor TAG diet B and having low TAG-ST ratio would not have enough reserves to make to a meticulous selection of settling site and therefore would settle more rapidly. According to this scenario, larvae in good condition would delay metamorphosis until reaching a critical physiologic threshold where settlement would become urgent. In support of this hypothesis, behavioral observations showed that larvae fed diet C (high TAG-ST) spent 12–15% of observation time exploring the substratum whatever the age, whereas larvae fed diet B (low TAG-ST) did not explore the substratum at day 40 (Fig. 10). Thus, it seemed that larvae fed diet B lost selectivity with age as observed with the barnacle *Balanus amphitrite* (Rittschof et al. 1984, Pechenik et al. 1993, Qian & Pechenik 1998, Miron et al. 1999). Moreover, in our study, larvae spent 15% to 47% of observation time swimming in the water column. Such swimming activity in competent larvae has previously been reported for the bryozoan *Bugula neritina* (25% to 45% of observation time), which was attributed to the stillness of the water (Walters et al. 1999). Similarly, substrates became attractive to the cyprids of *Balanus amphitrite* in flow, whereas no exploration was observed in individuals from the same cohort placed in still water (Miron et al. 2000). Larvae of other invertebrate species also seem to avoid settling in low flow conditions (Mullineaux &

Butman 1991, Pawlik et al. 1991). For example, larvae of the polychaete *Phragmatopoma californica* tumbled along the bottom in the presence of fast flow, whereas they were swimming in the water column in slow flow (Pawlik et al. 1991). Thus, the low settlement success observed in larvae of high quality might be the effect of delayed metamorphosis because of inappropriate hydrodynamic conditions in our experiment. If this interpretation was correct, larval quality need to be considered jointly with hydrodynamics to fully understand the decision process of settling larvae.

ACKNOWLEDGMENTS

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A RAPID TEST FOR THE DETERMINATION OF THE SPAWNING STATUS OF THE BAY SCALLOP, *ARGOPECTEN IRRADIANS* (LAMARCK, 1819)

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ABSTRACT   The bay scallop, *Argopecten irradians irradians* (Lamarck, 1819), is a generally semelparous, commercially important marine bivalve found along the shores of the Northeast Atlantic from Cape Cod, Massachusetts to New Jersey. It can be found in areas of varying conditions, including current flow, nutrient levels, salinities, siltation levels, all factors that can affect its size when it becomes legally harvestable. A harvestable scallop is defined as having a visible growth ring signifying that it has completed its reproductive cycle. However, there are areas on the island of Nantucket, MA, that produce scallops that lack this classic growth ring, giving rise to disagreements between scallop fishermen and regulatory agencies concerning the legality of harvesting them or returning them to the water. A rapid, 10-min test has been developed to quickly determine whether scallops in a particular area have spawned. It was determined that bay scallops, at least those found in Nantucket waters, retain mature spermatozoa in their gonads throughout the scallop harvest season, which in Massachusetts, runs from October through March of the next year. Detection of their presence could be useful in determining their spawning status. A small piece of male gonad is removed, homogenized briefly, and stained to detect the presence of these residual spermatozoa. It is hoped that the implementation of this rapid test will help to settle some of these local disputes, which should help ensure the protection of seed scallops.

KEY WORDS: scallops, *Argopecten*, spawning, seed

INTRODUCTION

The bay scallop, *Argopecten irradians irradians* (Lamarck, 1819), found in shallow bays along the Northeastern United States coast from Cape Cod to New Jersey, is a hermaphroditic, generally semelparous bivalve that is sexually mature at the age of 1 yr (Belding 1910). The reproductive period may last from mid-June into September in the waters surrounding Cape Cod, depending on local conditions, after which time scallops may be harvested because most will not survive to complete a second reproductive season (Belding 1910, Marshall 1960, Taylor & Capuzzo 1983, Tettelbach et al. 1999).

The waters surrounding the island of Nantucket, Massachusetts, have yielded steadily declining scallop harvests from a high of 117,000 bushels in 1980 to a low of 6,800 bushels in 1998 (Curley 2002). It has been long recognized that seed scallops must be protected because they are the primary source of the next year's harvest (Belding 1910). Scallops resulting from late spawning tend to be much smaller the next year, although many may catch up in size with scallops spawned earlier in the year, again, depending on local conditions (Auster & Stewart 1984). However, many of these late-spawned scallops may lack the distinctive growth ring, generating confusion among fishermen and regulatory agencies as to whether these are seed scallops and should not be harvested (MacFarland 1991).

Massachusetts laws governing the taking of mature bay scallops require the presence of a "well defined raised annual growth ring" (MGL,c130,s.70). However, in some areas of Nantucket, varying conditions, such as water temperature, food supply, current flow, and heavy siltation, among others, can lead to scallops lacking this distinctive ring. In addition, several investigators have documented late spawning events that give rise to smaller scallops with very small growth rings very near the hinge line (McFarland 1991, Tettelbach et al. 1999, Tettelbach et al. 2001). Scallops spawned near the beginning of the spawning season, generally beginning around the middle of June when water temperatures reach 15°–16°C in Nantucket waters, in an area of good current flow and sufficient food, can often yield seed scallops that are as large or larger than the average adult by the time harvest season arrives (Kelley & Ceely 1980). During one very productive year, 1990, in Pleasant Bay on Cape Cod, MacFarland (1991), found that if scallops were harvested based on size alone, as was recommended by local fishermen, 66% of those harvested would have been large seed.

In 1999, one area, Madaket Harbor, was closed to scalloping because of the presence of a large number of seed, and the following year saw the initiation of much stricter enforcement of taking only scallops with the distinctive growth ring. This has led to controversy because Madaket Harbor has generally poorer growing conditions and often yields scallops without the classic annual ring. Fishermen point to a fine line, usually within a centimeter of the hinge, as the annual growth ring, giving rise to the local term "nub" scallop, i.e., a scallop that has spawned (1+ yr) but that demonstrates no normal growth ring. Others state that this is a first-year scallop that has not spawned (0+ yr) and therefore should be returned to the water. Heretofore, confirmation has relied on preserving scallops in formalin and sending the specimens to a laboratory to have histologic slides prepared and read, with the results often obtained weeks later. To help eliminate this confusion, a rapid yet definitive test was developed to aid local regulatory agencies in differentiating seed scallops from those that have spawned. This test is based on the observation by the author over several years that bay scallops, at least those found in Nantucket waters, retain residual mature sperm in their gonads into March and April of the following year, whereas residual eggs are generally resorbed quickly, most likely because of their high energy content and the scallop's need to store energy for the upcoming winter. The purpose of this investigation was 2-fold: to determine whether this sperm retention was a sporadic event or was generally found throughout the scallop population and secondarily, to develop a rapid test to detect the presence of the residual sperm.

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METHODS

Fifty bay scallops that displayed the classic annual growth ring (1+ yr) were collected each month from Nantucket Harbor from October 1998 through March 1999. Scallops were obtained from three sources, SCUBA diving, from commercial scallopers, and from upwellers and lantern nets maintained at Nantucket Marine Laboratory. Because the purpose of this study was to see whether scallops that had spawned retained sperm throughout the harvest season, only those scallops that had clearly spawned were used. In addition to the presence of the growth ring, the gonad was large and flaccid, the upper shell was generally encrusted with flora and fauna, and the bottom valve was distinctly of a greater curvature than the upper shell. Also, 25 known seed scallops (0+ yr) that had been obtained in July and August of 1998 from sets onto onion bags and subsequently maintained in upwellers and lantern nets were also tested each month.

Residual sperm in bay scallops were examined by clipping a small piece (1–2 mm^2 is sufficient) from any part of the male gonad (see Fig. 1) and placed in a disposable 1.5-mL plastic conical tube containing approximately 0.5 mL of 10% formalin (this amount is not critical).

The tissue was ground for 15–30 sec using a pellet pestle, and a drop of this mixture was placed onto a glass slide and spread. Once air-dried, the slide was dipped in methanol to fix the tissue, and stained for 10–30 sec in a Safranin: Wright-Giemza: Water (1:2:10) stain.

The slide was rinsed briefly in running tap water, air-dried and read at 1000× magnification under oil immersion. Results can be hastened by drying the slide at each step on a slide warmer or hot plate. This procedure also lends itself to sampling by needle biopsy if sacrificing of the scallop needs to be avoided (Schneider et al. 1997).

RESULTS

Sperm with characteristic bullet-shaped heads (S) were stained grayish–blue with Wright–Giemza, and the tails (T) were stained
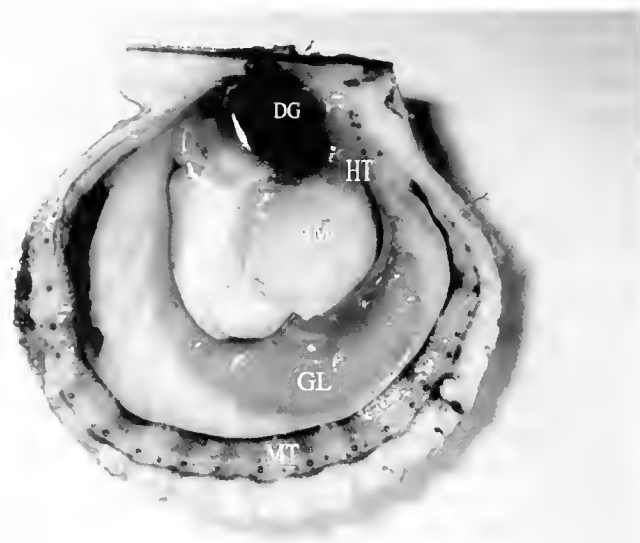
Figure 1. Anatomy of the bay scallop, *Argopecten irradians* (L.) seen with the upper or left valve removed; male gonad (MG); female gonad (FG); adductor muscle (AM); gill (GL); digestive gland (DG); heart (HT); mantle (MT).
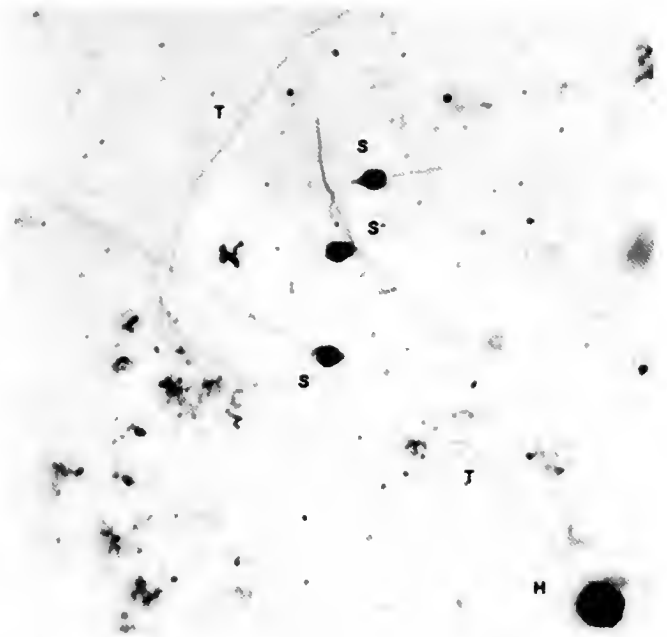
Figure 2. Residual sperm (1000×) seen in a representative bay scallop harvested in February 2001 confirming that it has previously spawned and is legally harvestable. Sperm heads (S); tails (T); hemocytes (H).

pink by the safranin (Fig. 2). These can be readily distinguished from the larger nucleated hemocytes (H) that tend to become more pervasive in the gonad as the season progresses, ostensibly phagocytizing the remaining sperm. Late in the scallop season, i.e., February and March, some scallops may demonstrate mostly tails (Fig. 2).

Results for 100% of the seed scallops (0+ yr) tested negative for the presence of spermatozoa. In the post spawning scallops (1+ yr), of 300 individuals tested from October through March, 241 displayed residual sperm, 55 demonstrated only tails, and the remaining 4 tested negative (these were from the scallops collected in March).

DISCUSSION

Because one of the primary concerns in maintaining a semelparous and commercially important species, such as *Argopecten irradians irradians*, is ensuring the survival of the seed scallops, it is important that they not be taken before their contributing to the following year's population.

Traditional methods of determining whether bay scallops have spawned, in addition to the presence of a distinct annual growth ring, include a larger gonad, grayish in color or with a whitish line or band as compared with a much smaller gonad, shiny black in color, as seen in reproductively immature scallops. Also, a distinctly curved lower or right shell as compared with the upper shell and a generally rougher appearance due to a greater longevity in the water with a concomitant accumulation of flora and fauna upon its upper shell help to separate the older scallop from its younger cohort (Belding 1910).

However, these differences are often subjective and can lead to controversy between fisherman and local regulatory agencies. In addition, the spawning season in Massachusetts waters, as described by Belding (1910) and Sastry (1963), generally has run from the middle of June through mid-August but now seems to be

extended to include September (Kelley & Sisson 1981, McFarland 1991). Tettelbach (1999) found similar results for some scallop populations in New York state.

In Nantucket, these late-spawned or nub scallops lack the distinctive growth ring seen in earlier-spawned scallops. These may spawn the next year, but in one experiment, 80% of these nub scallops held in cages survived yet an additional year (2+ yr) and 50% of those spawned (Conant, K, Assistant Town Biologist, Nantucket, MA, personal communication, 2002). However, many fishermen see a line, usually falling within 10 mm of the hinge, as the growth ring, and harvest these as adults. McFarland (1991) found that 9% of the scallop population had growth rings between 4 and 8 mm from the hinge line, of which 50% spawned the next year and the remaining 50% spawned the following year. It remains unclear as to the significance of the contribution of this small portion of the population, but it may play a role in the persistence of some scallop populations (Tettelbach et al. 1999). Although the significance of this secondary spawning remains unclear, it is absolutely clear as to the significance of taking scallops that have yet to spawn. It is hoped that the development of a rapid yet simple test to determine whether scallops in an local area have spawned or not will be useful in reassuring both regulatory agencies and scallop fishermen that only adult animals are being harvested.

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OPTIMIZATION OF SETTLEMENT OF LARVAL *ARGOPECTEN PURPURATUS* USING NATURAL DIATOM BIOFILMS

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ABSTRACT Larval settlement is a critical stage in the artificial production of *Argopecten purpuratus*. The study investigated the feasibility of improving post-larval settlement of this species using a substrate (cultch) that was pre-treated with a biofilm of native diatoms. Four species of diatoms were isolated from the surface of collectors that had high numbers of juvenile scallops (spat). These four species were able to attach themselves and grow on a polystyrene substrate. Scallop post-larval settlement was evaluated experimentally in two ways: (1) laboratory experiments in 10-L buckets; and (2) under natural condition by *in situ* experiments at the Marine Reserve "La Rinconada" (Antofagasta, Chile). Effects of biofilm treatments were examined using collectors that were coated with diatoms and collectors handled using normal culture methods (new netlon held in filtered seawater that did not have a biofilm). Results of the laboratory experiments showed a higher percentage of *A. purpuratus* post-larval settlement on collectors coated with *Fragilariopsis pseudonana* compared with control collectors ($P > 0.05$). Results comparing biofilms of the diatoms *F. pseudonana* and *Navicula veneta* showed higher settlement on collectors pretreated with *N. veneta* ($1,156 \pm 172$ spat per collector $^{-1}$). Statistical analysis showed that the addition of diatom biofilms enhanced spatfall and always produced larger settlement compared with untreated collectors. These results indicate that addition of cultured diatom biofilms improves scallop larval settlement.

KEY WORDS: *Argopecten purpuratus*, diatoms, biofilms, post-larval settlement

INTRODUCTION

The northern Chilean scallop, *Argopecten purpuratus* (Lamarck 1819), is the most important commercial bivalve species in Chile. Production in 1999 was 20,668 t, valued at $13 million (US) and the industry provided 3,600 direct jobs (Lozano 2000). The aquaculture production, however, is not sufficient to satisfy the international demand for this species. A major reason is the large variation in natural seed production (Navarro et al. 1991, Disalvo 1991, Avila et al. 1994, Riquelme et al. 1995, Avendaño et al. 2001) that supplies about 30% of the annual Chilean production (Farías et al. 1998).

A major problem with seed supply occurs at metamorphosis when larvae settle on a substrate (Keough & Downes 1982). Following attachment larvae undergo considerable morphologic and physiologic changes as they metamorphose from a pelagic to a benthic existence (Illanes 1990). There are generally a large number of larval mortalities (Tremblay 1988, Bourne et al. 1989, Castagna 1975). Ambrose et al. (1992) reported that there was little information concerning factors that influence scallop larval settlement and this led to numerous studies on the subject in the mid 1990s. A goal of these investigations was to increase settlement by improving substrates for settling larvae including color of the substrate, size, monofilament density and composition of the collector (Miron et al. 1995, Pouliot et al. 1995, Pearce & Bourget 1996). Further, the mechanism by which scallop larvae detect and settle on a particular substrate is still not understood (Harvey et al. 1997). Many studies showed that biologic, chemical and physical factors could induce larval settlement of marine invertebrates (Weiner et al. 1989; Bonar et al. 1986, Christensen 1989, Maki et al. 1990, Chevolot et al. 1991). Many of these studies showed that bacterial films were important for triggering larval settlement (Meadows & Campbell 1972, Kirchman et al. 1982, Weiner et al.

1989, Maki et al. 1990, Pearce & Bourget 1996). Bacterial communities were found associated with other microorganisms such as diatoms forming a multi-specific biofilm that was firmly attached to a substrate. These multi-specific biofilms emitted several types of signals, including: (1) peptic (Zimmer–Faust & Tamburri 1994) or associated fatty acids (Pawlik 1986) and (2) polysaccharides and glycoproteins (structure of a biofilm) (Hadfield 1986) that would stimulate marine invertebrate larvae to settle (Pawlik 1992, Keough & Raimondi 1995).

Harvey et al. (1995), using electron microscopy showed that biofilms were not only composed of bacteria but microalgae and detritus as well. The various organisms may have different effects on settlement of different species of scallops. Benthic diatoms that colonize substrates might not only be a source of nutrition for more advanced post-larval stages of marine invertebrates (Takami et al. 1997) but also may be necessary for the settlement of mollusc larvae, as shown in abalone culture (Seki 1980, Hahn 1989).

The purpose of this study was to isolate native diatoms from scallop collectors that had high levels of settled spat and evaluate the feasibility of improving post-larval scallop settlement by using of biofilms composed of specific diatom species. Results of laboratory and *in situ* field experimental work are reported here.

MATERIALS AND METHODS

The study was performed in three stages: (1) isolation of native diatom species; (2) laboratory experiments undertaken at the hatchery of the Facultad de Recursos del Mar de la Universidad de Antofagasta (FAREMAR, Faculty of Marine Resources at Antofagasta University); and (3) *in situ* field experiments undertaken in "San Jorge" bay at the Marine Reserve "La Rinconada" (27°03'24"S–70°51'30"W).

Isolation of Diatoms

Diatoms isolation was undertaken at the Cultivos Marinos Internacionales hatchery in "Inglesa" bay, Chile (27°03'24"S–

70°51′30″W). Netlon collectors with high levels of scallop-spat settlement were selected (more than 2,500 spat per collector⁻¹). Seventy pieces of netlon mesh were cut into 100 cm² sections. They were washed several times with a marine saline solution (SSM) (Austin 1988), and placed in Schott bottles with 50 ml of seawater filtered to 0.2 μm. Diatoms were removed from the mesh with an Ultrasonic Homogenizer (Cole–Parmer) for 60 sec. The resulting solution was diluted in test tubes with 9 mL of F/2 commercial Fritz Chemica Inc. supplemented with sodium metasilicate (F/2M, Guillard & Ryther 1962). These tubes were incubated for 7 d at $20 \pm 1°C$, photoperiod 12:12 and a light intensity of 100 $\mu mol\ m^{-2}s^{-1}$. Dominant diatom species were isolated using the microfishing technique described by Hoshaw and Rosowski (1979). Four species were identified after the method of Rodríguez (1998) as: *Navicula veneta* (Kutzing), *Navicula cryptocephala* (Hustedt), *Navicula menisculus* (Schuman) and *Fragilariopsis pseudonana* (Hasle). The species were then purified by exposure to a wide range of antibiotics (Hoshaw & Rosowski 1979).

Adherence of Diatoms to Polystyrene Substrates

Adherence assays to attach diatoms to the experimental substrate were performed using the method of Gawne et al. (1998). Polystyrene petri dishes with a diameter of 3 cm were filled with 5 mL of filtered seawater (0.2 μm) autoclaved for 15 min at 121°C and inoculated with either species of *N. veneta* (Nv), *N. cryto-cephala* (Nc), *N. menisculus* (Nm), and *F. pseudonana* (Fp) at a concentration of 5×10^5 cells $\times$ ml⁻¹ (3.5×10^5 cells $\times$ cm⁻²) in the pre-stationary phase. Three replicates of each culture were incubated in a controlled environment room at $20 \pm 1°C$ and a photoperiod of 12:12 for 48 h. Each petri dish was washed 5 times with 0.2 μm filtered seawater to insure that only those diatoms adhering to the bottom of the dishes were retained, non-adhering diatoms were thus eliminated. Diatom adherence at incubation times of 1, 6, 12, 24, and 48 h were recorded by direct count with an inverted microscope Olympus IX50 at a magnification of ×100 (Guillard 1973) The percentage of diatom adherence was calculated by comparing the concentration of inoculated diatoms with those observed on the bottom of the petri dish.

Growth of Diatoms on Polystyrene Substrates

Polystyrene petri dishes with a diameter of 3 cm were filled with 4 mL of F/2M solution autoclaved for 15 min at 121°C. Each dish was inoculated with one of the four species of diatoms from the pre-stationary phase at a concentration of 5×10^4 cells $\times$ ml⁻¹ (2.8×10^3 cells $\times$ cm⁻²). To assess the growth of diatoms, microalgal counts were performed every 48 h for a period of 144 h under identical conditions to those in the adherence experiments.

Stage II–Laboratory Experiments an Settlement of Post-Larval Scallops

The effect of native diatoms on the settlement of *A. purpuratus* post-larvae, was evaluated by conforming settlement among coated with biofilm of the four species of diatoms.

(a) Determination of post-larvae settlement substrate pre-treated with diatoms (according to the criteria of diatom adherence on substrate).

Bioassays were performed in buckets containing 10 L of 1 μm filtered seawater and no aeration. Each bucket was inoculated with strains of diatoms in the stationary phase (Fox 1983) at a concentration of 5×10^5 cells $\times$ ml⁻¹. After inoculation of the diatoms, a piece of netlon collector was placed in each bucket (length × width $= 30 \times 60$ cm) and incubated for 48 h. A set of collectors that were placed and kept in 10 μm-filtered seawater was pre-treated according to procedures done by commercial companies (natural). The control was new netlon that did not have a biofilm (Ct s/b).

At the end of the incubation period, "eyed" scallop larvae (>220 μm) were added to each bucket at a density of 1 larva $\times$ ml⁻¹, and maintained for a 7-day-period. During this time, the water in each bucket was changed daily, larvae were filtered on a 120 μm screen, washed on 205 μm screens, and returned to their respective buckets. Larvae were fed daily with a mixed diet of 7,500 cells $\times$ ml⁻¹ of *Chaetoceros calcitrans* and 10,000 cells $\times$ ml⁻¹ of *C. gracilis*. After seven days the netlon collectors were removed, cleaned with horsehair brush and the spat collected on a 205-μm screen. The number of attached spat was determined using an Olympus BH2 stereoscopic microscope. Results were expressed as "percent settlement" calculated by comparing the number of attached spat on collectors to the number of "eyed" larvae added to each bucket (Avendaño–Herrera et al. 2002).

$$Settlement\ (\%) = \frac{Number\ of\ attached\ post\text{-}larvae \times 100\%}{1 \times 10^4\ \text{"eyed"}\ larvae}$$

(b) Determination of post-larvae settlement on substrate pre-treated with diatom (according to the criteria of diatom growth on substrate).

Bioassays to assess diatom growth were performed in buckets containing 10 L of 1 μm filtered seawater using a constant 24-h photoperiod with a light intensity of 50 $\mu mol\ m^{-2}\ s^{-1}$ and aeration. Buckets were inoculated with diatoms at concentrations similar to the polystyrene substrate growth experiments. To stimulate growth during the incubation period, treatments and controls were enriched with the addition of F/2M. Netlon spat collectors measuring 30×60 cm that are typically used by commercial companies were placed in each bucket and incubated for a 96 h period.

The bioassays with larvae were carried out as previously described.

Stage III–in Situ Field Experiments of A. Purpuratus Attachment to Collectors Treated with Diatoms

When the effect of the four diatom species on settlement of scallop larvae was known from the laboratory experiments, strains of *F. pseudonana* and *N. veneta* were selected for further testing in the natural environment. Buckets with 20 L of 1-μm filtered seawater were inoculated with diatoms in the stationary phase at a concentration of 5×10^3 cells $\times$ ml⁻¹ and incubated with aeration and constant 24 h photoperiods at a light intensity of 50-$\mu mol\ m^{-2}\ s^{-1}$. A biofilm was established on one set of collectors using the method commonly used by commercial companies (Natural) and as control was used new the control used new netlon without biofilm (Ct s/b). Treatment and control buckets were enriched with the addition of F/2M and incubated for 10 days after inoculation of the diatom six netlon spat collector (30×60 cm) and placed in each bucket.

A collector from each experiment was sampled to determine the density of diatoms attached to the surface of the collectors at the end of the incubation period. Three pieces of netlon were cut into 25 cm² pieces, washed repeatedly with SSM and placed in 50-ml Schott bottles, and the diatoms attached to pieces of netlon were removed using an Ultrasonics Homogenizer for 60 sec. The number of diatoms attached to the monofilaments of each piece of

netlon was determined by direct counting using a Neubauer chamber and an Olympus BH-2 microscope. Results were extrapolated for the complete area of the collectors (1,800 cm²).

The five remaining collectors from each treatment and control were placed in 1 × 1 mm "onion" bags, labeled, and placed in the ocean at a depth of 16 m at the Marine Reserve Area for 38 days (January 15 to February 22, 2002). Prior to placing the collectors in the water, plankton-sampling method was used to assess *A. purpuratus* spatfall. Water temperature was recorded to evaluate larval and spat growth during the 38-d period (17°C ± 1°C).

After 38 days the collectors were removed from the ocean following the method of Wallace (1982) and taken to the Laboratorio de Ecologia Microbiana de la Universidad de Antofagasta (Microbial Ecology Laboratory of the University of Antofagasta) to assess spatfall. The effect of diatom biofilms on settlement of *A. purpuratus* was determined by counting the juveniles (spat) that were firmly attached to the monofilament of the treated collectors. Results were not affected by those spat that fell off collectors during transport because the interest was on spat that were firmly attached to the collectors. Each collector was removed from the onion bag, washed with circulating water for 5 min, and the attached material collected was deposited on a 205-μm-mesh screen. To avoid loss of spat, each collector was cleaned with a horsehair brush and the spat were preserved in (70%) ethanol for counting with a stereoscopic Olympus microscope.

Statistical Analysis

The growth rate of diatoms was calculated using Guillard's equation (Stein 1979), which describes mean microalgal duplication velocity:

$$K = [3.322/(t^2 - t^1)] \times (\log N^2/N^1)$$

Where K is the mean microalgal duplication velocity of the microalgal biomass, N^1 is the cellular density of the beginning of the experiment, N^2 is the cellular density at the end of the experiment, t^1 is the time at the beginning of the experiment and t^2 the time at the end. Results were tested by ANOVA to compare growth rates and maximum density values (Sokal & Rohlf 1980).

To evaluate the effect of diatoms on settlement of larvae in laboratory experiments, the results were tested by ANOVA with the statistical significance criteria (alfa = 0.05) and Multiple LSD Comparison Test (Sokal & Rohlf 1980). The influence of selected diatoms on settlement of larvae in the "*in situ*" field experiments was realized counting the collector naturally pre-treated (Natural) as one treatment. Results were submitted to the Dunnet test comparing results from the various treatments to those of the control (Zar 1984).

RESULTS

Isolation of Diatoms

Four species of diatoms were isolated from the microflora that was attached to the surface of scallop collectors. Only four species could be purified to an axenic condition using a mixture of chemotherapeutics and these were: *Navicula veneta* (Kutzing), *Navicula cryptocephala* (Hustedt), *Navicula menisculus* (Schumann), and *Fragilariopsis pseudonana* (Hasle).

Adherence and Growth of Diatoms on Polystyrene Substrates

N. veneta rapidly colonized plastic substrates without the addition of nutrients and 100% adherence was observed 48 h after

inoculation (Fig. 1). A similar situation was observed with *N. cryptocephala* and *N. menisculus*.

Two growth patterns were observed for the four diatom species when F/2M was added to the cultures, an accelerated growth for *N. veneta* and *N. menisculus* and slower growth for *N. cryptocephala* and *F. pseudonana*. Figure 2 illustrates that the four species were in the exponential phase of growth after 96 h of culture and a maximum cell production was observed after 144 h. When duplication velocity was compared, the rate for rapid growing species was (K = 1.57 ± 1 duplication × days⁻¹) and the slow growing species was (K = 1.38 ± 1 duplications × day⁻¹), the difference was significant ($P < 0.05$).

Stage II–Effect of Diatoms on Attachment of Scallop Larvae in Laboratory Studies

Results of experimental laboratory studies showed a higher percentage of post-larvae attached to collectors incubated with diatoms for 96 h in 1 μm filtered seawater enriched with the addition of F/2M (criteria of diatom growth on substrate) compared with collectors incubated with diatoms for 48 h in 1 μm filtered seawater (criteria of diatom adherence on substrate) (Fig. 3). Collectors treated with *F. pseudonana* had a larger number of spat attached to the collectors. The breakdown was 2,567 ± 205 and 7,727 ± 107 post larvae × collector⁻¹ under the criteria of diatom adherence and diatom growth on substrate, respectively. Collectors without biofilms had lower numbers of spat on them. Statistical analysis of settlement of larvae between collectors with and without diatoms films after 48 h incubation showed a significant difference between treatments with different diatom species and the control that had no film ($P < 0.05$). There was no statistical difference between settlement on the control and cultch that had been treated with 10 μ in filtered seawater.

The only significant difference in settlement on cultch treated with different species of diatoms was cultch treated with *F. pseudonana* ($P < 0.05$), incubated with diatoms for 96 h in 1 μm filtered seawater enriched with the addition of F/2M, and where settlement reached 77.27% of the total available post-larvae. Under laboratory conditions, collectors treated with 10 μm filtered seawater showed a significant increase in the number of settled larvae (33.84 ± 7.13%). This number was close to the average of those treated with the five species of diatoms (31.91 ± 2.21%).
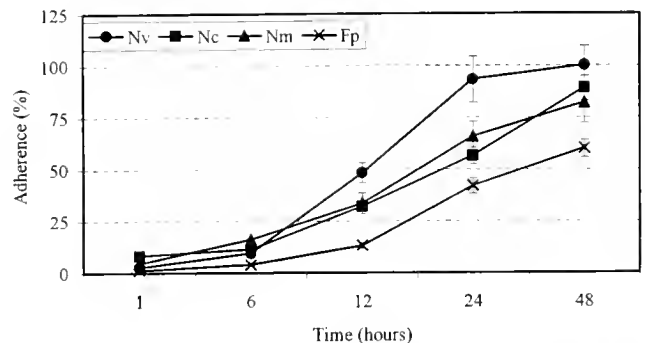
Figure 1. Percent attachment of the axenic diatoms *Navicula veneta Navícula* (Nv), *cryptocephala* (Nc), *Navícula menisculus* (Nm), and *Fragilariopsis pseudonana* (Fp) incubated for 48 h on polystyrene substrates. Vertical lines show standard deviation.
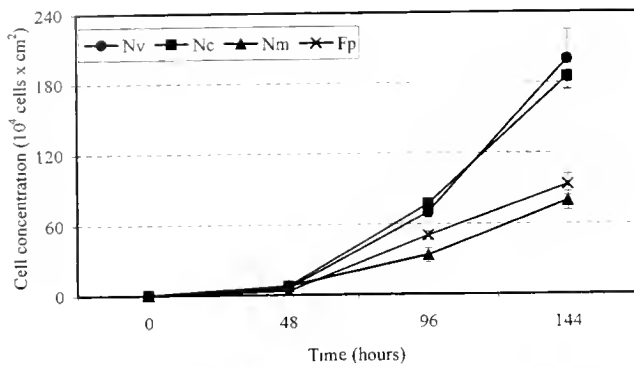
Figure 2. Growth curves of the axenic diatoms *Navícula veneta* (Nv), *Navícula cryptocephala* (Nc), *Navícula menisculus* (Nm) and *Fragilariopsis pseudonana* (Fp) grown with the addition of culture medium marine phytoplankton F/2M. Vertical lines show standard deviation

Figure 4. Number of *A. purpuratus* spat on collectors coated with different diatoms species in field experiments. Vertical lines show standard deviation.

Stage III-in Situ Field Experiments of Settlement of A. Purpuratus Larvae on Collectors Treated with Diatoms

The best results for stimulating settlement in scallop larvae were found with the diatoms *F. pseudonana* and *N. veneta* and these species were used in field experiments. After 10 days of incubation the concentration of these two diatoms on cultch was 73 ± 3.5 × 10^4 and 47 ± 3.7 × 10^4 cells × cm^{-2}, respectively. Plankton sampling showed a concentration of 8,197 larvae × m^{-3} with a mean size of 181.4 μm in water column in the Reserve. Recorded seawater temperatures during the 38 days of the experiment in the area of "Rinconada" did not show drastic changes.

Results of settlement on collectors after 38 days in the natural environment are shown in Figure 4. Collectors treated with the diatom *N. veneta* had a higher number of spat (1,156 ± 172 spat × $collector^{-1}$) compared with the controls and other treatments. Statistical analysis showed that spat settlement on collectors with diatom biofilms was always higher than controls ($P < 0.05$).

DISCUSSION

The presence of diatoms in the microflora of biofilms on substrates is a natural phenomenon and formation of such a microenvironment on a clean surface is inevitable (Cooksey & Wigglesworth-Cooksey 1995). Harvey et al. (1955) showed that the secondary surface colonizers after bacteria were a diverse species of

Figure 3. Percentage of *A. purpuratus* spat that settled on collectors pre-treated with native diatoms according to diatom adherence criterion (White rectangle = 48 h) and diatom growth criterion (Black rectangle = 96 h). Vertical lines show standard deviation.

benthic diatoms and these have been traditionally used as a settlement surface for abalone (Seki 1980, Hahn 1989). In this study, the diatoms *N. veneta* and *N. menisculus* adhered better and grew faster on the plastic substrate compared with *N. cryptocephala* and *F. pseudonana*. The diatoms *N. veneta* and *N. menisculus* are probably more adapted to adherence and formation of a primary biofilm on such substrate compared with the other two species (Characklis & Bryers 1990). Numerous investigators have stated that adherence and development of a biofilm are associated with the physical and chemical properties of the substrate (Wigglesworth-Cooksey & Cooksey 1992, Callow & Fletcher 1994). Structure of the diatoms has an important role in facilitating adherence as well as the production of extracellular polymers that interact with the substrate and may affect diatom adherence positively or negatively (Wetherbee et al. 1998).

Results of larval settlement in laboratory experiments showed variable rates of settlement for the four species of diatoms. The presence of spat was always greater when diatoms were present compared with clean substrates. Studies of the biology and culture of marine invertebrates indicate that before settling on a substrate, the larvae require biofilm capable of emitting to the environment chemical signals that stimulate their settlement (Kavouras & Maki 2000.).

Studies of the effect of microbial biofilms on attachment of pectinid larvae have shown variable results. In laboratory experiments, Hodgson and Bourne (1988) showed higher attachment of *Chlamys hastata* on surfaces that had biofilms compared with surfaces without biofilms and Parsons et al. (1993) reported similar results for *Placopecten magellanicus*. In this study, the higher percentage of spat attached to collectors that were exposed to 10 μm filtered seawater under controlled growth conditions (3,384 spat × $collector^{-1}$), may have resulted from stimulation by live organisms in the biofilm. Microbenthic components are considered to be among the principal diatom components. Cyanoficeas epibenthic and their associated bacteria (Meadows & Anderson 1968) in the presence of nutrients would increase their density, colonizing a higher percentage of the substrate that are used for adherence, favoring the settlement of scallop larvae. Because they colonize the substrate and cause higher spatfall, characteristics of biofilm growth or production of some unidentified substance may cause this higher spatfall. The composition of natural diatom biofilms and associated microflora that colonized the netlon are unknown but they could be variable and produce changes in composition and

structure of the community that could produce irregular settlement (Suzuki et al. 1987).

Butman et al. (1988) suggested that stimulation of invertebrate larval settlement is commonly enhanced by substances that enrich the substrate. In metamorphosis of scallops, it has been shown that if no stimulation is present then no settlement will occur thereby suggesting that specific stimuli may be necessary for different species (Padilla 1979).

There were significantly more spat on collectors incubated for 96 h with *F. pseudonana* compared with collectors with *N. veneta, N. cryptocephala* and *N. menisculus* that may indicate a selectivity of scallop spat for a specific species of diatoms. Studies of the ingestion of *A. purpuratus* larvae exposed to probiotic bacteria (11, 77 y C33) showed larvae selected two of the strains (Riquelme et al. 2000). Reasons for selection of biofilm surfaces for settlement are unknown but various theories exist. Bourne and Hodgson (1991) proposed that selection was due to differences in nutrition that occurred during transition among the ciliate velum of the planktonic phase and the filamentous gill of the young benthonic. Bivalve larvae may respond to colonized substrates of biofilms that serve as a bridge between planktonic feeding and filtering feeding, by using the foot for pedal feeding. Observations made under microscopy have allowed visualizing the gradual detachment caused by the movement of the food and the ingestion (Unpublished MS). Studies of settlement of abalone larvae showed that the success of settlement and density of juveniles on cultch depended on diatom species (Daume et al. 1999). Initial studies of attachment of five species of diatoms on polyethylene showed minor colonization of the *F. pseudonana* strain after 48 h of incubation but none by the other four species (Unpublished MS). Probably the majority of spat in the *F. pseudonana* treatment settled because of the formation of a primary biofilm that was favored by the incorporation of nutrients and also physical characteristics of the substrate surface (Characklis & Marshall 1990). The diatoms are not only a nutrient source for marine invertebrate larval stage, but they also have the capacity to liberate chemical stimuli or extracellular component into the environment (Wetherbee et al. 1998). This extracellular component could be absorbed by pectinid larvae, improving the larval survival in the substrate (Pearce & Bourget 1996, Kavouras & Maki 2000). It is also possible that the gradual biofilm detachment could be used as food (Unpublished MS). Some investigators report that the microbial biofilm gradually detached from the substrate, and the detachment

of cells from the biofilms is a nature process in the biofilm development (Stoodley et al. 2001). This detachment phenomenon shows that the gradual cell detachment from the biofilm could be used as food for the pectinid larvae. Studies on settlement of abalone larvae to biofilms with 18 species of diatoms showed better attachment of the larvae to biofilm with high density of diatom (Kawamura & Kikuchi 1992). Increase in density of colonizing diatoms on the substrate, static conditions in the experiment, and relative confinement of larvae probably facilitated detection of diatom biofilms by larvae.

Results of adherence of diatoms *F. pseudonana* and *N. veneta* in field experiments showed similar values (10^5 cells $\times$ cm^{-2}). Collectors incubated with *N. veneta* had the majority of spat and showed that pre-conditioning the surfaces with a diatom biofilm is a preferred substrate for scallop larvae. Pearce and Bourget (1996) proposed that larvae of the sea scallop, *Placopecten magellanicus*, were able to choose between different substrates for settlement, favoring monofilaments with a biofilm. Harvey et al. (1997) found a significant effect of natural biofilms on bivalve settlement (66%) and of *Pecten magellanicus* (35%) compared with cultch without a biofilm. Hence there is a preference of settlement substrates by some pectinid larvae, one criterion being determined by nutrition (Bourne & Hodgson 1991). The stimulus for settlement can be due to various factors including pectinid species, diatom composition, and density of the biofilm. Diatoms may be effective because of a particular microcosm with extracellular material that enhances settlement. Further, the constant supply of artificial substrates with specific and adecuated biofilms is the key to produce higher growth and survival (Hahn 1989, Takami et al. 1997).

In conclusion, diatom biofilms enhanced settlement of *A. purpuratus* larvae in laboratory and field experiments, spatfall reaching higher values than on collectors without biofilms or using traditional biofilms. This suggests that native diatom biofilms may be used to increase production of spat of other bivalves, including northern Chilean scallop *Argopecten purpuratus*.

ACKNOWLEDGMENTS

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ADHESIVES TO ATTACH JUVENILE BAY SCALLOPS TO PLASTIC NETTING IN AQUACULTURE

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ABSTRACT Farming the bay scallop, *Argopecten irradians irradians*, is a labor-intensive effort, primarily due to biofouling control on the netting of culture cages. We tested commercially available adhesives for possible application in a cageless scallop aquaculture methodology: attaching juvenile bay scallops via adhesives to polyethylene netting. The new culture method holds promise to minimize the culture structure surface area subject to biofouling and to facilitate harvesting. We present results for five adhesives.

KEY WORDS: bay scallop, *Argopecten irradians*, aquaculture

INTRODUCTION

Traditionally, bay scallops have been reared in floating cages or lantern nets in the United States. Biofouling of cage netting and a subsequent decrease in water flow and food availability is a major obstacle for growers of filter feeding shellfish. Physical removal of fouling organisms by brushing and power washing represents a labor-intensive expense. Cageless culture methods, the topic of this article eliminate the labor and expense of cleaning fouled netting. Methods used to attach seed shellfish to floating structures include, piercing the "ears" of scallops to attach a line or using adhesives to bond the shell to netting. A student group has tested adhesives for tagging marine mammals (private communication).

Ear hanging experiments with bay scallops ~10 mm diameter at the Martha's Vineyard Shellfish Group (MVSG) *ca.* 1990 were performed by piercing the ears with a Dremel® tool and stringing them on monofilament line. Although the shell surfaces were heavily covered with fouling organisms, the scallops grew at remarkable rates. Over the course of the growing season, the drilled ear failed to grow and eventually the weight of the growing scallops caused the ears to break.

In an attempt to replace ear hanging with an alternative cageless method, we report our recent study to find suitable adhesives and specialty cements to affix juvenile scallops to hanging structures in the water column. The ideal adhesive must be strong, adhere to damp surfaces, set up quickly, cure under water and not break down in seawater. Further, it must not injure the shellfish, interfere with their growth, or leave any toxic residue in the tissue. To be useful in aquaculture, the adhesive must be cost-effective both in the cost of the labor to affix the shellfish to netting and the cost of the adhesive.

METHODS

We attached bay scallops to high-density polyethylene netting (ADPI Enterprises, Inc. Durethene BOP-2L, mesh size 2.25-inch

by 2.25-inch). The animals were quickly blotted dry and the adhesive was applied in air. After a cure time of about 15 min, the scallop was immersed in seawater, either in a tank or off a dock at the MVSG facility. The adhesives are listed in Table 1.

Testing

Tests of adhesives on live scallops were "pass or fail" tests. If the adhesive held the scallop to the netting, it was graded pass. If the adhesive failed to hold and the scallop dropped off, the test was graded fail. We discovered that it was important to engulf the polyethylene netting in adhesive to form a good bond. Shells were about 3 cm in height.

Scallop shells were cleaned free of algal fouling. Live animals were scrubbed with a brush in buckets of seawater to remove algae, tubeworms, barnacles, and other fouling. Shells were blotted dry with paper towels. In addition to removal of biofouling, some tests were performed by touching each shell top with anhydrous ethanol or blowing dry with compressed air. No significant improvement in results was noted with these drying techniques, probably because of the high ambient humidity. When bonding shells to netting, it is easiest to place the animal on top of the netting with the adhesive sandwiched between the animal and the netting. However, the animal "drools", which keeps the adhesive wet, and "claps", which disturbs the bond as it is setting up. We tried both configurations (shells under netting and shells on top of netting) and found no significant differences in bonding.

Our results are shown in Table 2. The last column (#bonds) is the number of animals bonded to netting at the beginning of the study. The time that the animals were out of water is noted in the second column. In three cases, anhydrous alcohol flowed past the shell edge and the adhesive bonds were intact but several animals died. The number of bonds to empty shells (dead animals) is noted in parentheses.

TABLE 1.

List of adhesives

Adhesive	Manufacturer	Adhesive Type
Ceramicrete	Dr. Arun Wagh, Argonne Nat. Lab.	Ceramic cement +5% phosphoric acid
Bone & dental cement 51458	Stoelting co.	Cement/methacrylic acid ester/amine
PSI-326 (Smart Glue)	Polymeric systems Inc	Two-part epoxy
Fastcure epoxy 051135-08107	3M Company	Two-part epoxy
Prism 454	Loctite Co.	Single component

TABLE 2.

Results on bay scallops.

Adhesive	Air Time	Life Test Conditions	Results	# Bonds
PSI-326 (Smart Glue)	15 min	Seawater; 19 wk	3 out of 12 intact (2 dead)	12
PSI-326 (Smart Glue)	15 min	Seawater; 14 wk	5 out of 24 intact	24
PSI-326 (Smart Glue)	15 min	Alcohol dry; seawater; 15 wk	4 out of 12 intact (3 dead)	12
Fastcure epoxy 051135-08107	15 min	Seawater; 4 mo	7 out of 25 intact	25
Fastcure epoxy 051135-08107	15 min	Seawater; 14 wk	8 out of 24 intact	24
Fastcure epoxy 051135-08107	15 min	Alcohol dry; seawater, 14 wk	4 out of 12 intact (1 dead)	12
Stoelting bone cement 51458	20 min	Seawater; 17 wk	0 out of 4 intact	4
Stoelting bone cement 51458	20 min	Seawater; 16 wk	6 out of 12 intact	12
Stoelting bone cement 51458	15 min	Alcohol dry; seawater; 14 wk	9 out of 12 intact (5 dead)	12
Ceramicrete	20 min	Seawater; 20 wk	2 out of 5 intact (1 dead)	5
Ceramicrete	30 min	Seawater; 19 wk	6 out of 9 intact	9
Prism 454	15 min	Seawater, 5 wk	25 out of 25 intact (1 dead)	25

CONCLUSIONS

The most promising adhesives are Fastcure epoxy 051135-08107 (3M Company) and Ceramicrete (developed by Dr. Arun Wagh. Argonne National Lab.). The Ceramicrete powder was mixed with phosphoric acid (diluted to 5% by weight in water) to speed the setting time. Dr. Arun Wagh has recommended another additive (magnesium oxide) to further speed setting. Initial tests of "quick set" Ceramicrete with magnesium oxide additive were disappointing. Stoelting bone cement also proved to be a good adhesive for this application but may be too expensive; additionally, it sets up too fast to use with large numbers of animals at a time. Initial tests of Prism 454 (Henkel Loctite, Rocky Hill, CT) were promising but the cost of this one-part adhesive is high. Equally important for all adhesives are tests for toxicity, which remain to be done. Proper curing of epoxies requires that the ambient temperature be sufficiently high for the thermal energy to support molecular motion so that the chemical reaction of resin and hardener can go to completion. A good rule of thumb is that the reaction should occur at temperatures above the glass transition temperature, T_g. (The glass transition temperature is the temperature at which a polymer changes from a glassy to a rubbery state. Above T_g, portions of the polymer molecules are mobile.) Therefore, application of adhesives in winter poses additional challenges. Resins that are liquid at 0 °C and materials with T_g near 0°C would be useful for cold weather curing.

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EVIDENCE FOR THE INVOLVEMENT OF CYCLIC AMP IN THE METAMORPHOSIS OF BAY SCALLOP, *ARGOPECTEN IRRADIANS* (LAMARCK) LARVAE

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ABSTRACT   The putative involvement of cyclic AMP (cAMP) in the metamorphosis of bay scallop *Argopecten irradians* larvae has been investigated on three integrated aspects. First, we conducted experiments on response of competent larvae to selective inhibitors of phosphodiesterase (PDE), theophylline, and caffeine, which presumably lead to elevated concentration of intracellular cAMP by preventing the degradation of cAMP to 5′-AMP. Second, the endogenous levels of cAMP were determined during larval development. Third, monitoring the variation of cAMP content in larvae when exposed to neuroactive compounds tested (L-DOPA and epinephrine) and to elevated concentrations of potassium ion, was carried out to examine the possible role of cAMP as a second messenger in metamorphic pathway stimulating artificially. Consistent results have been obtained in all the three experiments. The two putative PDE inhibitors that were tested stimulated metamorphosis in *A. irradians* larvae significantly above control level in a dosage-dependent manner. The inductive effects did not vary significantly with exposure time. At the optimum concentration of 1.0 mM, percent metamorphosis increased by 33% and 36.01% when subjected to theophylline and caffeine respectively. The endogenous level of cAMP varied dramatically over larval development. In particular, significant increase in cAMP content from 2129 pmol/(mg protein) for eyed larvae (Day 13 post-fertilization, PF) to 15,195 pmol/(mg protein) for spats (Day 17 PF) occurred during the metamorphic process. This finding indicates that metamorphic pathway involves cAMP in appreciable quantities. Furthermore, the endogenous cAMP content increased significantly in competent larvae exposed to excess potassium ion, epinephrine, or L-DOPA, suggesting cAMP plays an important role in metamorphic signal transduction pathway triggered by the three chemical cues. Evidences presented here show that cAMP becomes involved in the metamorphic pathway of *A. irradians* larvae.

KEY WORDS: cAMP, metamorphosis, *Argopecten irradians*, catecholamines, L-DOPA, PDE inhibitors

INTRODUCTION

Larval metamorphosis is a crucial process in the development of most marine invertebrates. Evidence indicates that this process is triggered by specific endogenous and exogenous chemical cues (Burke 1983, Baloun & Morse 1984, Coon & Bonar 1986, Yool et al. 1986, Bonar et al. 1990, Inestrosa et al. 1993a, Leise & Hadfield 2000, Pires et al. 2000, Zhang et al. 2002a, Zhang et al. 2002b). Recent evidence suggests that neurotransmitters (norepinephrine, dopamine, and 5-hydroxytryptamine) play an important role in regulating metamorphosis of mollusk larvae (Coon & Bonar 1986, Pires et al. 2000, Zhang et al. 2002a). The cAMP/PKA (protein kinase A) pathway is one of the most important signal transduction pathways involved in the neurotransmitter regulation.

Previous studies revealed or inferred that the cAMP, as an important mediator of cellular metabolism and cell-to-cell signaling, was possibly involved in the metamorphosis of certain species of marine invertebrates, such as the polychaete *Phagmatopoma californica* (Jensen & Morse 1990), the barnacle *Balanus amphitrite amphitrite* (Clare et al. 1995), the red abalone *Haliotis rufescens* (Baxter & Morse 1987). It remains unclear as to whether cAMP is involved in the metamorphosis of some marine invertebrate species such as of *Crassostrea gigas* (Coon & Bonar 1987, Bonar et al. 1990, Coon et al. 1990) and *Hydroides elegans* (Holm et al. 1998). The signal transduction pathway involving cAMP, however, is incompletely understood, in the majority of previous studies, because there is no direct proof of variation in endogenous larval cAMP level during the metamorphic process.

It is still unknown whether cAMP is involved in the metamorphosis of the bay scallop *Argopecten irradians* (Lamarck). In this study, we investigated the potential role of cAMP and sought the

direct evidence on the involvement of cAMP in the metamorphosis of *A. irradians* larvae. We designed three experiments to test the putative involvement of cAMP in the metamorphosis of *A. irradians*. The first experiment investigates larval response to phosphodiesterase (PDE) inhibitors. PDE is known to function in stimulating the hydrolysis of cAMP to 5′-AMP. Response of larvae exposed to PDE inhibitors (e.g., theophylline and caffeine), which is assumed to increase endogenous cAMP level, would provide proof revealing function relationship between cAMP and the metamorphic pathway. The second experiment is designed to manifest potential role of cAMP in the natural metamorphic process by monitoring endogenous levels of cAMP over larval development. The third experiment is designed to elucidate the possible relationship between the inductive activities of the commonly adopted exogenous chemical cues and cAMP. Results from the three experiments are expected to provide fuller understanding of the signal transduction pathway that involves cAMP in marine mollusks.

MATERIALS AND METHODS

Collection of Larvae

Larvae of the bay scallop, *Argopecten irradians* (Lamarck), were obtained from Xujia Maidao Hatchery, Institute of Oceanology Chinese Academy of Sciences. Larvae collected with Nitex screen were cultured with the methods as described by Zhang et al. (1986, 1991).

Test of Chemical Cues

All experiments were conducted in 6-well plastic tissue culture plates using 1-μm filtered natural seawater at 23°C, 32 ppt. The selective chemical cues, including the two PDE inhibitors (theophylline and caffeine), L-DOPA, epinephrine (Fluka), KCl, pre-

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pared as 10 stock solutions in distilled water prior to experiments, were kept under 4°C. All chemicals were purchased from Sigma Company unless denoted.

For experiments, the stock solutions of chemicals tested were allowed to be equilibrated to the desired temperature and then were diluted to the appropriate concentration with seawater containing *A. irradians* larvae. Approximately 50–100 larvae in 10 mL of filtered seawater were placed in each well of the plastic tissue culture plates. Seawater in controls was diluted with distilled water to match that in experimental groups. Test solutions of PDE inhibitors were applied at concentrations of 10^{-3}, 10^{-2}, 10^{-1}, 1, and 10 mM in seawater. Exposure time of PDE inhibitors varied from 1 h to 24 h. For the assessment of endogenous cAMP, competent larvae were exposed to L-DOPA or epinephrine at a concentration of 10 μM for 8 h, or to 13.42 mM KCl for 24 h.

On completion of the treatment, larvae were rinsed and replaced in fresh filtered seawater to be ready for other procedures. Larvae were cultured for an additional 72 h before they were fixed with iodine and observed under a dissecting microscope to determine the percentage of larvae that had metamorphosed and the mortality rate. Metamorphosed larvae were verified by the complete formation of dissoconch, the newly grown adult shell. Three replicates were conducted for each experiment with 50–100 larvae per replicate using different batches of larvae.

Analysis of Endogenous cAMP Content

Samples of different developmental stages of *A. irradians* larvae for cAMP assay were taken as follows: D-stage larvae (Day 3 post-fertilization, PF), umbo-stage larvae (Day 7 PF), 10% eyed larvae (Day 10 PF), 100% eyed larvae (Day 12 PF), 100% eyed larvae (Day 13 PF) and spats (Day 17 PF). Larvae following exposure to elevated concentration of K^+, L-DOPA, or epinephrine were also sampled for cAMP assay.

For the measurement of cAMP content ~100–200 larvae were used in each sample. The extraction of cAMP was carried out by homogenizing in 5% trichloroacetic acid and centrifuging at 3,000 rpm for 30 min. The supernatant was washed with saturation ether to remove trichloroacetic acid and then dried on 70–75°C water bath. The residue was redissolved in TE buffer for cAMP assay as described by Gilman (1970). The P-E 240 Elementary Analyzer (Perkin Elmer, USA) was used for protein assay. The amount of larvae was counted prior to the measurement of cAMP content in larvae. The cAMP content is finally expressed as follows, with its

unit of pmol cAMP/(mg protein): Content of cAMP = (cAMP content per larva)/(protein content per larva) Where the protein content per larva was calculated using the following: Protein content per larva = (absolute content of nitrogen × 6.25)/(larval amount).

Data Analysis

Percentage of response of larvae to chemical cues was compared by two-way analysis of variance (ANOVA). All analyses were conducted using Microsoft Excel program.

RESULTS

Influence of Theophylline on Larval Metamorphosis and Mortality

Theophylline exhibited high and consistent inductive activity on the metamorphosis of *A. irradians* larvae. The percentage of metamorphosed larvae increased by over 14% at concentrations of 0.001–10 mM for 1–24 h of exposure compared to controls. Theophylline induced larvae to metamorphose in a concentration dependent manner ($P < 0.1$). Increased concentration of theophylline led to an increase in percentage of larvae that had metamorphosed. At theophylline concentration of 1.0 mM, there is an average maximum increment of 33% over control levels. The mean percentage of larvae metamorphosing increased by 23.15% and 21.97% in response to 0.1 mM and 10 mM theophylline respectively (Table 1). On the other hand, the effect of exposure duration on the metamorphosis of *A. irradians* larvae was not significant ($P > 0.1$). The average metamorphosis increment varied from 19.07% to 26.1% for the exposure duration of 1–24 h at various concentrations of theophylline (Table 1).

Theophylline treatment at concentrations lower than 1 mM for brief periods of time did not cause obvious mortality to the larvae of *A. irradians*. In 11 of 20 cases, larvae in theophylline-treated groups showed higher survival rates than that in the control groups (Table 2). Lethal effect emerged, however, when high concentrations of theophylline or prolonged exposure time were applied. The larval mortality increased by 24.69 ± 3.56% when treated with 10 mM theophylline for 16 h compared with that of the control group. It seemed that prolonged exposure time had more impact on larval survival, as suggested by the increase in larvae mortality, by 17.64 ± 3.56%, 15.48 ± 3.45%, and 39.23 ± 4.36% for 24 h exposure at the concentration of 0.1, 1.0, and 10 mM, respectively. Overall, exposure to theophylline had no significant effect on the

TABLE 1.

Effects of theophylline concentration and exposure time on metamorphosis of *A. irradians*.

Concentration (mM)	Increment of Percent Metamorphosed Larvae Above Controls Level				Average
	1 h	8 h	16 h	24 h	
0.001	22.43 ± 3.45	12.97 ± 2.56	17.50 ± 4.23	20.91 ± 2.56	18.45
0.010	18.74 ± 2.76	17.29 ± 1.29	8.40 ± 1.73	13.84 ± 2.10	14.57
0.100	20.79 ± 2.25	28.64 ± 3.65	23.94 ± 4.72	19.23 ± 2.89	23.15
1.000	21.33 ± 4.67	39.49 ± 6.45	33.37 ± 4.30	37.81 ± 2.57	33.00
10.00	29.54 ± 5.12	32.13 ± 2.64	12.16 ± 1.26	14.06 ± 1.14	21.97
Average	22.57	26.10	19.07	21.17	

Competent larvae were exposed to theophylline as indicated and then allowed for recovery for 72 h. Larvae in control group were kept in filtered seawater that had been diluted to match that in experimental group correspondingly. Three replicates were made with 50–100 larvae per replicate. Metamorphosis was defined as complete formation of dissoconch.
Data are expressed as mean percentage and standard deviation.

TABLE 2.

Effects of theophylline concentration and exposure time on mortality of *A. irradians*.

Concentration (mM)	Increment of Larval Mortality Relative to Controls Level				Average
	1 h	8 h	16 h	24 h	
0.001	2.73 ± 0.45	−2.84 ± 0.23	−2.61 ± 0.78	−8.03 ± 0.89	−2.69
0.010	10.96 ± 2.32	−7.53 ± 1.23	0.26 ± 0.09	0.05 ± 0.02	0.94
0.100	−4.62 ± 0.98	−21.16 ± 2.45	−8.99 ± 2.11	17.64 ± 3.56	−4.28
1.000	−13.65 ± 2.34	−4.00 ± 1.34	−0.91 ± 0.08	15.48 ± 3.45	−0.77
10.00	−4.26 ± 0.89	3.56 ± 0.78	24.69 ± 3.56	39.23 ± 4.36	15.81
Average	−1.77	−6.39	2.49	12.87	

Competent larvae were exposed to theophylline as indicated and then allowed for recovery for 72 h. Larvae in control group were kept in filtered seawater that had been diluted to match that in experimental group correspondingly. Three replicates were made with 50–100 larvae per replicate. Data are expressed as mean percentage and standard deviation.

mortality of *A. irradians* larvae in comparison with larvae in controls ($P > 0.1$).

Influence of Caffeine on Larval Metamorphosis and Mortality

Caffeine like theophylline stimulated the metamorphosis of *A. irradians* larvae remarkably. The metamorphosis increment averaged over 10% for the treatment of caffeine at varying concentrations for 1–24 h. The action of caffeine on the metamorphosis was dose-dependent. Concentration of caffeine had significant effect on the metamorphosis induction ($P < 0.1$). Optimum inducing of metamorphosis was achieved at a concentration of 1.0 mM caffeine, with mean percentage of metamorphosed larvae increasing by 36.01% among the four exposure durations (Table 3). The next effective concentration for caffeine was 10 mM, averaging increment of 26.43% metamorphosis. Caffeine exposure time does not appear to significantly influence the percentage of metamorphosed larvae. Among various durations of exposure time, metamorphosis increased by 19.65–22.02% above the control levels (Table 3). The correlation between treatment duration and efficiency of larval metamorphosis inducing was not significant ($P > 0.1$).

Statistic analysis showed that the caffeine treatment had significant effect on larvae mortality of *A. irradians* ($P < 0.01$). In particular, larvae mortality occurred more than control levels when the exposure time was prolonged to 24 h or when caffeine concentration reached 10 mM. Increment of larvae mortality rose up to 21.29 ± 0.95% when larvae were treated with 10 mM caffeine

for 24 h compared to the control groups. Exposure of *A. irradians* larvae to caffeine below 24 h or 10 mM, however, resulted in the increase in larval survival (Table 4).

Variation of Endogenous Levels of cAMP During Larval Development

The endogenous cAMP level increased over the time-course of the development in *A. irradians* larvae (Fig. 1). The larval cAMP content underwent a gradual increase from 10% eyed larvae to 100% eyed larvae and eventually a sharp climb in the cAMP level following the completion of metamorphosis. The content of cAMP increased by 6.1 times from eyed larvae (100%, Day 13 PF) to spats (Day 17 PF). This result showed that cAMP played an active role in the metamorphic pathway that naturally occurs in *A. irradians*.

Variation of Endogenous cAMP Level Following Excess K$^+$ Treatment

Larvae of *A. irradians* underwent an increase in the cAMP level significantly above the control groups' level when exposed to excess potassium ion (Fig. 2). The content of cAMP in larvae treated with 13.42 mM KCl for 24 h was elevated to 7.8 times higher than that in the control groups, that was, from 2129 pmol/(mg protein) to 18,656 pmol/(mg protein).

Variation of Endogenous cAMP Level Following Treatment of Epinephrine or L-DOPA

The content of cAMP in *A. irradians* larvae increased following the treatment of 10-µM epinephrine or L-DOPA for 8 h (Fig.

TABLE 3.

Effects of caffeine concentration and exposure time on metamorphosis of *A. irradians*.

Concentration (mM)	Increment of Percent Metamorphosed Larvae Above Controls Level				Average
	1 h	8 h	16 h	24 h	
0.001	12.55 ± 1.34	21.52 ± 2.45	16.00 ± 3.13	12.46 ± 2.56	15.63
0.010	7.78 ± 0.99	8.39 ± 1.12	8.40 ± 1.45	24.98 ± 3.56	12.39
0.100	19.10 ± 2.12	12.08 ± 1.56	9.42 ± 1.68	13.70 ± 1.98	13.58
1.000	23.92 ± 2.53	35.73 ± 2.97	46.43 ± 3.76	37.94 ± 3.98	36.01
10.00	34.89 ± 4.13	32.37 ± 2.38	20.00 ± 1.99	18.44 ± 2.01	26.43
Average	19.65	22.02	20.05	21.50	

Competent larvae were exposed to caffeine as indicated and then allowed for recovery for 72 h. Larvae in control group were kept in filtered seawater that had been diluted to match that in experimental group correspondingly. Three replicates were made with 50–100 larvae per replicate. Metamorphosis was defined as complete formation of dissoconch.
Data are expressed as mean percentage and standard deviation.

TABLE 4.

Effects of caffeine concentration and exposure time on mortality of *A. irradians*

| Concentration (mM) | Increment of Larval Mortality Relative to Controls Level | | | | Average |
	1 h	8 h	16 h	24 h	
0.001	−4.50 ± 0.14	−5.60 ± 0.14	−6.43 ± 0.45	5.53 ± 0.97	−2.75
0.010	−8.60 ± 0.87	−9.47 ± 0.87	−6.60 ± 0.76	6.07 ± 0.74	−4.65
0.100	−4.98 ± 0.57	−2.88 ± 0.30	−9.29 ± 0.89	5.10 ± 0.78	−3.01
1.000	−4.07 ± 0.49	−6.59 ± 0.71	−5.73 ± 0.76	9.75 ± 0.89	−1.66
10.00	−3.49 ± 0.37	5.64 ± 0.45	1.07 ± 0.02	21.29 ± 0.95	6.13
Average	−5.13	−3.78	−5.40	9.55	

Competent larvae were exposed to caffeine as indicated and then allowed for recovery for 72 h. Larvae in control group were kept in filtered seawater that had been diluted to match that in experimental group correspondingly. Three replicates were made with 50–100 larvae per replicate. Data are expressed as mean and standard deviation.

3). Larval cAMP content in treated groups increased by 1.5 and 10.7 times than that of the control groups, (i.e., from 4007 pmol/[mg protein] in the control groups to 9882 pmol/[mg protein] and 46,824 pmol/[mg protein]) for epinephrine and L-DOPA treatment groups respectively.

DISCUSSION

It is generally believed that the pathway taking cAMP as second messenger is an important signal-transduction pathway in invertebrate tissues. Involvement of cAMP in larval metamorphosis varies with the species of marine invertebrates and there is no evidence of involvement of cAMP in metamorphosis of Pacific oysters *Crassostrea gigas*. The alpha-1 adrenergic receptor served as the receptor of norepinephrine to regulate metamorphosis of *C. gigas* larvae (i.e., norepinephrine was through intracellular messengers DG [diglyceride] and IP$_3$ [1,4,5-trisphosphoinositide]) not cAMP, to regulate metamorphosis of *C. gigas* larvae (Coon & Bonar 1987, Bonar et al. 1990, Coon et al. 1990). One report revealed that cAMP level in the gastropod *Concholepas concholepas* larvae reduced by 20 times during metamorphosis (Inestrosa et al. 1993b). An earlier report, dealing with the metamorphosis of this species, revealed that the degree of larval internal protein phosphorylation increased during the metamorphosis process

(Campos et al. 1991). Therefore, Inestrosa et al. (1993b) concluded that the phosphorylation of protein following metamorphosis had no relation with PKA but presumably was triggered by other kinds of kinase, including PKC. The investigators, however, did not clarify whether or not cAMP is involved in the metamorphosis of *C. concholepas* larvae.

On the other hand, several studies revealed that cAMP is involved in the process of settlement and metamorphosis in certain invertebrate species (Jensen & Morse 1990, Clare et al. 1995). It has been shown that cAMP is involved in the morphogenetic pathway in the larvae of the red abalone, *Haliotis rufescens* (Baxter & Morse 1987). Cholera toxin has been found to be effective in inducing metamorphosis in *Cassiopea andromeda* larvae, whereas db-cAMP is not effective in initiating the settlement and metamorphosis of the same species (Fitt et al. 1987). Furthermore, endogenous cAMP level in *C. andromeda* larvae did not undergo sharp variation as did in mammal species. Based on earlier observation, the authors concluded that cAMP is involved in the metamorphosis, but not in initiating the settlement and metamorphosis process.

The drug induction method has been used for the research in signal-transduction pathway in the process of marine invertebrate larvae settlement and metamorphosis, i.e., the signal transduction pathway could be inferred from the response of larvae to the specific drug that induces larval settlement and metamorphosis. This method is simple and practical and much progress has been made through this method. Because of the complexity of the biochemical reaction involved in the metamorphic process, there is still limi-
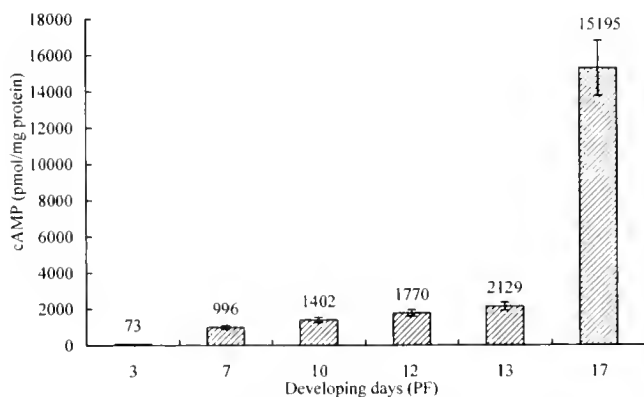
Figure 1. The variation of endogenous level of cAMP in *A. irradians* larvae during consecutive different developing stages as follows: D-stage larvae [Day 3 post-fertilization, (PF)], umbo-stage larvae (Day 7 PR), 10% eyed larvae (Day 17 PF). Data on the top of the bars represents cAMP content. Data are averages of three duplicates with standard deviation indicated as vertical bars.
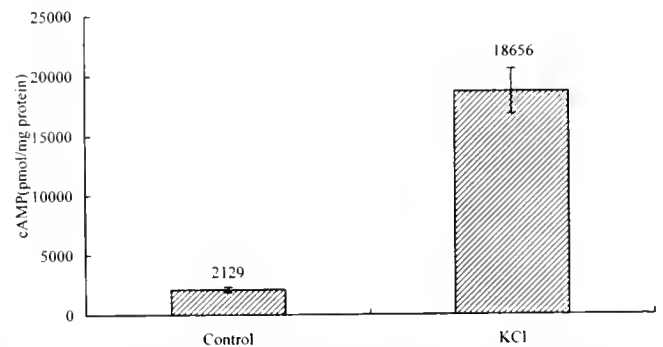
Figure 2. The variation of cAMP content in *A. irradians* larvae exposed to elevated K$^+$. Larvae were treated with 13.42 mM KCl for 24 h. Data on the top of the bars represent cAMP content. Data are averages of three duplicates with standard deviation indicated as vertical bars.
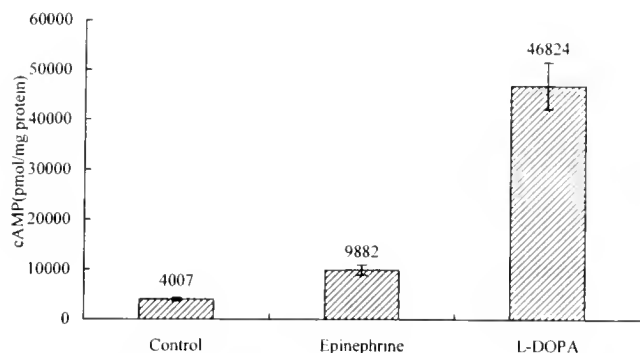
Figure 3. The variation of cAMP content in *A. irradians* larvae exposed to epinephrine and L-DOPA. Larvae were treated with 10 μM of the testing neuroactive drug for 8 h. Data on the top of the bars represent cAMP content. Data are averages of three duplicates with standard deviation indicted as vertical bars.

tation in using this method because of deviation to some extent. Therefore, we combined both drug induction method and direct assay of endogenous level of cAMP in larvae for verification of involvement of cAMP in the process of metamorphosis in *A. irradians*. As for drug induction, theophylline and caffeine, which could affect the intracellular level of cAMP, were used to test the mechanism of *A. irradians* metamorphosis. Results presented here show that both theophylline and caffeine are effective in promoting metamorphosis in *A. irradians* larvae. On the other hand, cAMP level is found to vary different larval developmental stages, especially with significant increase from eyed larvae to spats. Furthermore, the internal cAMP level in larvae increases significantly following exposure to excess K$^+$, epinephrine or L-DOPA that are known as the common inductive agents for settlement and metamorphosis in larval marine invertebrates. All these results suggest that cAMP is involved in the metamorphosis of *A. irradians* larvae. The drastic increase of cAMP, however, occurs after metamorphosis not before. Therefore, we believed that the process of metamorphosis of *A. irradians* larvae was not triggered by cAMP, although cAMP is involved in this process. The triggering process might be through other pathways.

Of particular interest is that mortality of metamorphosing larvae exposed to either theophylline or caffeine in most of the cases was much lower than that in controls. The increase in larval survival was possibly due to the metamorphosis promotion of competent larvae in treated groups, which shortens the time elapsed in the metamorphic process. We have found that the delay of metamorphosis resulted in increasing loss of competent larvae in *A.*

irradians. This finding indicates that theophylline or caffeine is potentially useful for promoting yield of metamorphosed spats in *A. irradians*, which is essential for the efficiency of seed production in commercial hatcheries, and their use as exogenous metamorphosis inducers by hatcheries engaging in seed production of bay scallops in China will result in promising and cost efficient commercialization of bay scallop aquaculture.

In the settlement and metamorphosis model of *C. gigas*, L-DOPA, as the precursor of neurotransmitter, is absorbed by the larvae and transformed into dopamine, which initiates the settlement of *C. gigas* larvae followed by the secretion of neurotransmitters, such as norepinephrine. This process causes metamorphosis in the larvae through the α_{-1} adrenergic receptor (Bonar et al. 1990, Coon et al. 1990). In this study, the intracellular level of cAMP increased significantly following exposure of larvae to L-DOPA and epinephrine, which suggests that β-adrenergic receptor is involved in the metamorphosis of *A. irradians* larvae (i.e., epinephrine regulates the metamorphosis of *A. irradians* through β-adrenergic receptor). It seems that the mechanism of *A. irradians* metamorphosed was obviously different from that of *C. gigas*.

In this study, endogenous level of cAMP increased with exposure of *A. irradians* larvae to excess potassium ion. It is generally believed that K$^+$ induces larval metamorphosis through directly depolarizing excitable cells involved in the larval perception of inductive stimuli (Yool et al. 1986, Baloun & Morse 1984). It, however, remained unknown as to how depolarization causes larval metamorphosis. Based on our results, we propose that as a result of cell membrane depolarization resulting from excess potassium ion exposure, nerve impulse occurs and then increases intracellular level of cAMP through certain pathways, which triggers phosphorylation of PKA, and eventually regulates metamorphosis of larvae of bay scallops.

In summary, this study shows that the second messenger cAMP is involved in the regulation of metamorphosis in *A. irradians* larvae. Since cAMP functions by activating PKA, it means that PKA is possibly involved in the metamorphosis of the bay scallop larvae. However, further proof of PKA involvement in metamorphosis of this species has to be found in future studies.

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DEPURATION CONDITIONS FOR GREAT SCALLOPS (*PECTEN MAXIMUS*)

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ABSTRACT Trials were undertaken to determine appropriate conditions for depurating hand-gathered great scallops (*Pecten maximus*). Scallops were contaminated with *Escherichia coli* to levels consistent with those requiring depuration by relaying in sewage impacted waters for a minimum of 2 weeks. These scallops were then purified for 42–48 h in both laboratory and small-scale commercial depuration systems under varying conditions. Levels of *E. coli* were monitored before and after depuration to assess the effect of temperature, salinity, shellfish-loading arrangements, and the use of artificial seawater on the depuration process. Self-righting trials were used to assess the amount of stress imposed on the scallops caused by transport, handling, and the depuration procedures. Results to date demonstrate that the use of artificial seawater cannot be recommended. During depuration, natural seawater should be maintained at a salinity ≥30‰ and at a temperature ≥10°C. Our results demonstrate that scallops could be depurated in a double layer within trays at a nominal density of 250 scallops m^{-2} with a shellfish-to-water ratio of 1:12 (kg:L).

KEY WORDS: great scallops, depuration conditions, purification

INTRODUCTION

Sewage-contaminated bivalve molluscan shellfish can present a significant health risk if consumed raw or lightly cooked (Rippey 1994, Cliver 1997). To minimize these health risks, most countries operate legislative controls on the harvesting and placing on the market of live bivalve shellfish (Lees 2000). Such controls generally rely on the use of *Escherichia coli* as an indicator of fecal pollution in these shellfish. European Community (EC) Directive 91/492 (Anon 1991) stipulates such controls for the EC and requires classification of shellfish harvested areas depending on the degree of fecal pollution, as judged from monitoring for *E. coli* contamination of bivalve flesh. This classification determines whether bivalve shellfish can be sold direct for consumption or must be treated before sale. There are four classification categories (Table 1). Bivalves from category B areas require short-term self-purification in tanks of clean seawater by a process termed depuration (Richards 1988). All bivalves sold for consumption whether treated or not must comply with an end-product standard of <230 *E. coli* 100 g^{-1}.

There is increasing interest in farming great scallops (*Pecten maximus*) in Europe (Chataigner 1996, Dao et al. 1998) and several studies have examined the environmental requirements for cultivation of this species (e.g., Brynjelsen & Strand 1996, Fleury et al. 1996, Chauvaud et al. 1998, Laing 2000, 2002). However, scallops held in inshore areas have been shown to be as capable of accumulating equal amounts of sewage-derived micro-organism as other commercially cultivated bivalve shellfish (Silk 2000). The availability of pristine (category A) waters for scallop cultivation is limited in the United Kingdom. Most (64%) of the 249 recognized shellfish-harvesting areas in England and Wales are currently classified as category B. About 69% of over 120 Scottish shellfish sites are category B for all or part (seasonal classification) of the year. At least two of the three present scallop farms in Northern Ireland are likely to hold a B classification (Heath & Pyke 2002). The market for scallops is predominantly for a live product. Where bivalves are sold as live product the treatment process most commonly used is depuration, which represents a major control point during the production of bivalve molluscs

world wide (Richards 1998). Depuration has not been applied to scallops landed in the United Kingdom because they are traditionally considered to be fished in offshore locations deemed to be microbiologically secure and so are exempt from classification requirements (Anon 1991). To realize the full aquaculture potential of great scallops in the United Kingdom, there is a need to apply successful treatment processes that will remove microbiological contaminants. Depuration is likely to be the preferred option.

Depuration relies on bivalves continuing filter-feeding activity when placed in tanks of clean seawater and purging themselves of sewage contaminants. To ensure this is achieved, suitable conditions must be met. Criteria for some of these conditions are common to all species, such as adequate water quality, shellfish condition, and system design. However, some conditions, such as temperature, salinity, and loading arrangements, vary depending on the species depurated. These conditions have been carefully determined in the United Kingdom for a variety of bivalve molluscan species, including oysters (*Ostrea edulis, Crassostrea gigas*), mussels (*Mytilus edulis*), cockles (*Cardium edule*), and clams (*Ensis* spp., *Mercenaria mercenaria, Tapes philippinarum, T. decussatus,* and *Spisula solida*). Some preliminary investigations have been conducted to determine the effect of a number of conditions for scallop depuration (Heath & Pyke 2002). However, it was determined that further work would be required to define these and other conditions more closely before regulatory authorities could sanction the use of depuration as a treatment process for scallops.

This study investigated the effect of temperature, salinity, and emersion time before depuration and shellfish-loading arrangements on scallop purification, principally using *E. coli* elimination as a measure of depuration efficiency. The aim of the study was to produce sufficient information that would allow minimum depuration criteria for scallops to be determined.

MATERIALS AND METHODS

Experimental Animals and Environmental Contamination

Market-size scallops were obtained from a commercial cultivation site and were distributed into lantern nets at field sites that were impacted by sewage contamination. The nets were filled with six to seven scallops in each of the 12 compartments and suspended from floating pontoons with the top of the net at least 1 m

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Classification Category	E. coli 100 g^{-1} Flesh	Comment
A	Less than 230	Suitable for consumption. Can be marketed.
B	Less than 4,600	Depuration needed (or relaying in category A area or cooking by an approved method)
C	Less than 46,000	Relaying (minimum of 2 mo) in category A or B area needed (or cooking by an approved method)
Prohibited	Above 46,000	Cannot be taken for placing on the market

from the seawater surface. The nets were deployed for at least 2 weeks to allow microbiological contamination of the scallops. Two field sites were used throughout the study and both had previously been identified as areas where the scallops would reliably accumulate E. coli to a level at which they would require depuration. After contamination, scallops were collected from the field site as required for depuration experiments. They were transported in groups of 60–70 animals in 40-L rectangular plastic bins covered with a dampened hessian sack to maintain a high level of humidity. For all experiments scallops were transported to the laboratory in less than 3 h.

Depuration Tanks

Experiments were conducted in two types of depuration tanks both using UV sterilization. Laboratory scale systems had dimensions of 1050 mm (length) by 500 mm (width) by 450 mm (depth) with a working volume of 200 L. Seawater was recirculated lengthways through the tank at a rate of 400 L h^{-1} and sterilized by irradiation in a 15 W UV sterilizer (type 15/3p; UVAQ Ltd., Sudbury, UK). Temperature was maintained by placing the whole tank in a controlled temperature room. Dissolved oxygen levels were maintained by the use of a spray bar for recirculated water. Shellfish were depurated in plastic mesh baskets (no. 41042; Sommer Alibert [UK] Ltd., Droitwich, UK) raised off the base of the tank to avoid recontamination by voided fecal material.

Standard design small-scale commercial systems (SFIA 1995) had dimensions 1140 mm (length) × 950 mm (width) × 600 mm (depth) with a working volume of 550 L. Seawater was recirculated through the tank at a rate of 900 l h^{-1} and sterilized by irradiation in a 15 W UV sterilizer (type 15/3p; UVAQ Ltd., Sudbury, UK). Scallops were loaded into six mesh baskets (no. 41042; Sommer Alibert [UK] Ltd., Droitwich, UK) stacked three high in two columns. Temperature was maintained by the use of an aquarium heater (Tronic 100 watt; Hagen [UK] Ltd., Castleford, UK) or chiller units (model RA680; Teco Ltd., Ravenna, Italy). Dissolved oxygen levels were maintained by the use of a spray bar for recirculated water. Baskets in the bottom layer were raised off the base of the tank by 50 mm to avoid recontamination by voided fecal material.

Depuration

Natural or artificial seawater was circulated through the depuration system and UV irradiated for at least 24 h before each experiment. Artificial seawater was made using a standard salt mix widely used in the UK for shellfish depuration from a commercial supplier (Seamix; Peacocks Ltd, Glasgow, UK). Contaminated scallops were thoroughly washed and damaged or gaping shellfish discarded. Prior to depuration an initial sample of 20 or 30 scallops was removed and analyzed as duplicate or triplicate samples of ten animals. Scallops were loaded into mesh baskets with cupped shell down generally in a single layer except in loading configuration experiments. For all experiments, except a trial investigating the effect of length of emersion, depuration commenced within 4 h of shellfish collection. After an initial trial using what was believed to be optimal conditions, trial parameters were changed to investigate the effect of artificial seawater, salinity levels, temperature, emersion time before depuration, and loading arrangements. Details of the parameters investigated are discussed further in the relevant part of the results section. A control treatment where the parameter under investigation was not varied was included for each experiment.

All depuration experiments were run for between 42 and 48 h, after which time duplicate samples of 10 scallops for each treatment were removed for E. coli analysis. Levels of dissolved oxygen, temperature, ammonia, and pH were recorded periodically throughout the depuration period.

E. coli Analysis

Scallops were thoroughly washed and scrubbed under running potable water. Dead and open scallops not responding to percussion were discarded. Ten scallops were aseptically opened using a flame-sterilized shucking knife to sever the adductor muscle and meats and intravalvular fluid removed. These were diluted and homogenized as described previously for oysters (Dore & Lees 1995).

Diluted homogenates were assayed for E. coli using a standard most-probable number (MPN) method used for shellfish analysis (Donovan et al.1998). Briefly, this is a five-tube, three-dilution procedure involving inoculation of tubes containing minerals modified glutamate broth (CM607; Oxoid Basingstoke UK) followed by incubation at 37°C for up to 48 h. Tubes displaying acid production were confirmed as containing E. coli by subculture on to Tryptone Bile Glucuronide Agar and incubation at 44°C for 24 h. After incubation, the number of tubes that were positive for β-glucuronidase activity after subculture was recorded. The MPN was then calculated by reference to standard tables (Donovan et al. 1998). The nominal limit of sensitivity for the assay is 20 MPN 100 g^{-1}. All results are expressed as an average for the duplicate samples.

Self-Righting Experiments

Self-righting experiments were performed on some surplus scallops as a simple assessment of pre- and postdepuration stress levels in the animals (Minchin et al. 2000). For these trials, 10–20 scallops were placed upside down (flat shell down) in 25 cm depth of sea water in a 300-L rectangular tank supplied with a continuous flow of aerated unfiltered sea water at ambient temperature and salinity (>30‰). The number of scallops self-righting after 1 h was recorded and the result compared with that for control animals. Repeated observations were made with the same scallops every 3–5 days for up to 15 days or until at least 50% of the scallops in both control and treatment groups righted within 1 h. Mortality of the scallops in the tanks was recorded. After each

experiment the scallops that self-righted were marked with a small spot of permanent ink and all scallops were returned to the normal (cupped side down) position until the next observation in the experiment.

Scallops collected from the cultivation site and delivered directly to the laboratory provided the control for predepuration self-righting trials to ensure that the results from the depuration experiments were not compromised by stress caused by the effects of holding, transporting, and handling of the animals during the contamination phase.

Control scallops for assessing the effect of depuration (in the control depuration treatment) were taken from surplus animals collected from the field site. Comparisons were also made of post-depuration scallops from individual treatments compared with scallops from the control depuration treatment.

RESULTS

Initial Depuration Experiment

An initial experiment was conducted under what was expected be acceptable conditions for scallop depuration based on requirements for oysters. Conditions for the experiment were salinity levels of 36‰, temperature of 15°C ± 1°C, with a scallop to water ratio of approximately 1:50 (1:1 ratio being equivalent to 1 kg to 1 L of seawater). Dissolved oxygen levels were maintained above 90% saturation throughout the experiment. *E. coli* levels of 805 MPN 100 g^{-1}(consistent with a category B classification) were reduced to nondetectable levels indicating that it was possible to purify category B scallops under these conditions. Further investigations varied one parameter at a time.

Artificial Seawater and the Effect of Salinity Concentration

Experiments were conducted to investigate the effect of salinity. Initial trials used fresh tap water to make artificial seawater from standard salt mixes. However these trials produced high mortality rates and poor levels of *E. coli* elimination were observed (Table 2).

These results apparently indicate that decreasing salinity causes an increase in mortality. However the fact that 20% mortality occurred in the control treatment (35‰) compared with no mortalities in the initial trial using natural seawater at a similar salinity described above, indicates that salinity alone was not responsible for the mortalities.

A further trial comparing artificial seawater made up to a final concentration of 30‰ with natural seawater diluted with freshwater to also give a final concentration of 30‰ demonstrated 100% mortality in the tank using artificial seawater compared with

no mortality using diluted natural seawater. *E. coli* levels in shellfish in the natural seawater tank were reduced from 265 MPN 100 g^{-1} to 20 MPN 100 g^{-1} indicating successful depuration.

To determine whether the problem associated with using artificial seawater was because of the salt mix used or the fresh water in which it was diluted, an experiment comparing artificial seawater (30‰) prepared by adding potable water and using freshwater that had been treated by passing through an activated charcoal filter. Scallops depurated in seawater made up in untreated water had a 20% mortality level compared with no mortalities in scallops depurated in filtered water. *E. coli* reductions in the scallops during depuration also differed; initial levels of 2,300 MPN 100 g^{-1} were reduced to <20 MPN 100 g^{-1} in the treated water tank compared with 300 MPN 100 g^{-1} in the untreated water tank. Chlorine and ammonia levels recorded during these experiment were low in both tanks (<0.06 mg mL^{-1} chlorine and <0.02 mg mL^{-1} for ammonia). It therefore appears that there was some unknown constituent in the untreated water salt solution that was causing the mortality in scallops, which was removed by treatment with activated carbon filtration.

Further salinity trials were performed using natural seawater that was diluted in fresh water treated by passing through an activated carbon filter. Other parameters during these experiments were maintained at optimal conditions. Dissolved oxygen levels were maintained above 80% saturation and temperature at 15°C ± 1°C. Scallop to water ratios were maintained at approximately 1:50. Results are shown in Table 3.

A salinity concentration of 28‰ or higher appeared to allow successful elimination of *E. coli* although caution should be used in interpreting some of this data given the relatively low initial *E. coli* levels observed in some of the trials. In all further experiments investigating the effect of other physiologic parameters, full saline natural seawater (range 35 to 38‰) was used.

Temperature Trials

Results from the experiments to investigate the effect of temperature on depuration efficiency are shown in Table 4. Depuration at 10, 16, and 20°C was shown to be effective at reducing *E. coli* to end product levels (<230 *E. coli* MPN 100 g^{-1}) even from levels consistent with a category C classification (>4600 *E. coli* MPN 100 g^{-1}). In contrast a minimal reduction (10%) was observed when depuration was carried out at 7°C.

TABLE 2.

Percentage mortality of scallops during depuration under varying salinity ranges.

Trial Date	Salinity (%)	Mortality (%)
14/2/01	25	100
27/2/01	30	55
14/2/01	35	20
27/2/01	35	20

Artificial seawater was made using standard salt water mixes dissolved in potable standard water.

TABLE 3.

E. coli levels in scallops before and after depuration under varying salinity ranges.

Trial Date	Salinity (%)	E. coli MPN 100 g^{-1}		Percent Reduction
		Predepuration	Post Depuration	
13/3/01	25	465	210	55.2
24/4/01	28	330	<20	>94
13/3/01	30	465	<20	>96
24/4/01	30	330	<20	>94
2/5/01	30	2,300	<20	>99

All values are averages of duplicate samples. Artificial seawater was made using standard salt water mixes dissolved in water treated with an activated charcoal filter.

TABLE 4.

E. coli levels in scallops before and after depuration under varying temperature ranges.

| Trial Date | Temperature (°C) | E. coli MPN 100 g⁻¹ | | |
		Predepuration	Post Depuration	Percent Reduction
31/7/01	10	2200	<20	>99.1
31/7/01	16	2200	<20	>99.1
21/8/01	10	9750	220	95.5
21/8/01	20	9750	<20	>99.8
4/9/01	7	6000	5400	10

All values are averages of duplicate samples.

Emersion Time Before Depuration

One trial was performed to assess the effect of the length of time scallops were emersed before depuration had on the treatment process. Scallops were held out of water at 15°C ± 1°C for a total of 6, 10, and 22 h before being placed in depuration tanks at 14°C ± 1°C for 42 h. Initial *E. coli* levels of 1200 MPN 100 g⁻¹ were reduced to 30, 30, and 145 MPN 100 g⁻¹ for 6, 10, and 20 h emersion treatment respectively. All scallops were successfully reduced from a category B level to end product standard, although it appears that 20 h emersion may have a detrimental effect on the efficiency of depuration compared with a 10-h immersion period.

Loading Arrangements

An initial trial was conducted with 60 scallops loaded in two layers, cup side down, into one basket in a laboratory scale depuration tank under optimal conditions. The scallops moved substantially and several scallops escaped from the basket and on to the base of the tank amongst fecal strands that had settled there. Scallops did not escape from the basket in the control tank where only 20 animals were placed in one basket. After depuration, samples were taken randomly from the top and bottom layers of the treatment basket. Although reductions of *E. coli* were similar between the control and treatment (96% and 98%, respectively), scallops that had escaped the basket and were sitting on the base of the tank were not tested.

Further trials placed mesh nets over the baskets so that the scallops could not escape. A space was left between the scallops and the net so that it did not impinge on the ability of the scallops to open and filter.

A trial was conducted to confirm that scallops could be depurated in two layers. Sixty scallops were loaded into one basket in two layers and 20 scallops into another basket in the same system to act as a control. Initial *E. coli* levels of 3500 MPN 100 g⁻¹ were reduced to 30 and 20 MPN 100 g⁻¹ in the top and bottom layer of the treatment basket, respectively. *E. coli* levels in the control were reduced to 30 MPN 100 g⁻¹.

Three trials were conducted in the commercial scale depuration system fully loaded with scallops on the basis of a double layer of 50–60 scallops in each of six baskets. This gave a scallop to water ratio of about 1:12. Trials were conducted at 15 ± 1°C and salinity of 36‰. Control tanks containing just 20 scallops were also used (scallop to water ratio in excess of 1:50). In all cases *E. coli* levels were reduced to below 230 MPN 100 g⁻¹ (Table 5). Dissolved oxygen decreased in the treatment tanks in all three trials, but

remained above 70% saturation at all times. Total ammonia in the three treatment tanks increased to a level between 2.5 and 5 mg L⁻¹ during the three trials compared with maximum levels 0.5 mg L⁻¹ in the control tank but did not appear to have a detrimental effect.

Self-Righting Experiments

The percentage number of scallops self-righting in the control groups was variable between experiments, from 40–80%, but was generally consistent for each batch of scallops for every repeated observation within experiments. It was often the same (marked) animals that righted on each occasion.

It was shown that holding scallops at the field site and transporting them to and from the site did not apparently impose any stress. In eight righting trials these scallops performed similarly to control scallops delivered directly to the laboratory from the cultivation site. The mean percentage righting responses, for the first observations only, were 64.4% for control scallops and 55.6% for treatment scallops. A paired *t*-test showed that the difference was not significant (t = 1.08, P = 0.314, for 7 df).

The self-righting response of scallops following depuration in the control treatment was similar to that for scallops from the same batch collected from the field site at the same time but not depurated. Mean righting response was 63.8% and 60.8% respectively (paired t = 0.515, P = 0.634, for 4 df).

Scallops from the artificial seawater treatment, which was found to be not suitable for depuration, did not show any self-righting above 10% over the 14 days for which this experiment was continued, by which time there was 40% mortality.

With the salinity experiments, scallops depurated at 25‰ took 20 days to recover to a level of self-righting response of only 28.6%, although there was no mortality in this group. Scallops in the 28‰ treatment showed no difference to the control scallops immediately following depuration at this salinity, with the same number of scallops self-righting in both groups.

At low temperatures, scallops from the 7°C depuration treatment did not show any recovery above 10–20% self-righting over 15 days, by which time there was 70% mortality. In three observations, between 50–70% of the scallops depurated at 10°C self-righted in 1 h, a similar result to those from the control treatment. It was also shown that scallops from an ambient temperature of 18°C that were held at 10°C for 42 h in a simulated depuration experiment showed no sign of stress as measured by these experiments. A similar number of control (untreated) scallops and scallops from this treatment self-righted.

In four separate self-righting trials using scallops from the high density (double layer) depuration experiments, including the three carried out in the commercial scale systems, there were no differences in righting response between high and low stocking densities in the depuration tanks. The mean righting responses, for the first observations only, were 64.8% (control, 20 scallops in tray) and 61.1% (double layer, 50–60 scallops in tray; paired t = 0.502, P = 0.65, for 3 df).

DISCUSSION

The predepuration self-righting experiments showed that the conditions used for holding and handling the experimental animals, including transportation to and from the field site and the laboratory, did not cause stress to the scallops. Most of the other information available on transporting scallops is in respect of mov-

E. coli levels before and after depuration in scallops taken from various positions throughout small-scale commercial depuration systems.

Sample Position	*E. coli* MPN 100 g⁻¹		% Reduction
	Predepuration	Post Depuration	
4/12/01			
Control	9,100	20	99.8
Top basket top layer		30	99.7
Top basket bottom layer		165	98.2
Bottom basket top layer		20	99.2
Bottom basket bottom layer		40	99.6
17/12/01			
Control	925	115	88.1
Left top basket top layer		70	92.4
Left top basket bottom layer		20	97.9
Right top basket top layer		70	92.4
Right basket bottom layer		70	92.4
Left mid. basket top layer		20	97.9
Left mid. basket bottom layer		20	97.9
Right mid. basket top layer		20	97.9
Right mid. basket bottom layer		40	95.7
Left bottom tray		20	97.9
Right bottom tray		20	97.9
26/2/02			
Control	10,000	110	98.9
Left top basket		220	97.8
Left bottom basket		110	98.9
Right top basket		220	97.8
Right bottom basket		220	97.8

Scallops were loaded in plastic mesh trays in double layers at a density of approximately 50–60 scallops per tray.

ing juveniles from a hatchery or collector site to a cultivation site. This has shown that periods of up to 12 h out of water have no observable effect, provided the scallops are maintained in a humid atmosphere (Maguire et al. 1999, Christophersen 2000, Minchin et al. 2000). One of the current depuration experiments showed that the process could be run effectively with scallops that had been immersed for this amount of time. Longer periods of emersion may compromise the ability of the scallops to depurate, and there was some indication of this from the results of this study.

Initial trials had indicated that it was possible to successfully depurate category B level scallops using standard procedures used in the United Kingdom without any detrimental effects on the product quality. Subsequently this study concentrated on finding the minimum acceptable requirements for parameters, such as temperature, salinity, etc., when depurating scallops.

The use of artificial seawater during depuration is common practice for a wide range of species. However, results during this study indicated that the use of artificial seawater is unacceptable under the conditions applied here. This finding does not concur with previous work (SFIA 1996), which demonstrated that it was possible to use artificial seawater in tanks for degritting scallops without any reduction of scallop activity or increased mortality. However, no details of what was used to dilute the artificial salt mix were given for that study. The scallop depuration trials conducted by Heath and Pyke (2001) used natural seawater only. The results obtained here indicated that an unidentified constituent of the tap water used to make the artificial seawater was responsible for the mortalities observed. Artificial seawater has been used on numerous occasions at the CEFAS Weymouth laboratory to undertake depuration trials with other shellfish species without any

effect on shellfish quality, and this result is unique to these scallop trials. Because it was not possible to identify the constituent responsible for the problem, it remains unclear whether this situation is unique to the water used at this laboratory or would be a widespread problem if used in the field. However, until further work is done to investigate the use of artificial seawater during scallop depuration the use of artificial seawater in this role cannot be recommended.

Salinity was found to have a critical effect on efficiency of depuration and levels of 25‰ had a detrimental effect on the rate of *E. coli* clearance. The next lowest concentration of salinity that was investigated was 28‰, and *E. coli* levels were successfully eliminated at this salinity. However only one trial was conducted at this salinity, and the initial level of *E. coli* in this trial was only 330 MPN 100 g⁻¹. It is questionable whether this can be considered a suitable challenge to test this condition. Given this it is recommended that in the absence of further work a minimum salinity concentration of 30‰ for scallop depuration should be maintained during depuration. This is a relatively high salinity compared with minimum concentrations set for other species. This is not surprising given that scallops are an open seawater species that will normally be exposed to full-salinity seawater. It should be noted that the consistent availability of natural seawater at a salinity in excess of 30‰ might present a constraint in some commercial settings. This requirement should be carefully considered by operators at the outset of any plans to depurate scallops.

Temperature was also found to have a significant effect on *E. coli* reduction. Minimal reductions were observed at 7°C, whereas *E. coli* levels were successfully depurated at 10°C. These results do not agree with those from previous studies (Heath & Pyke

2001), which concluded that temperatures as low as 6.6°C could effectively reduce levels of *E. coli*. They are, however, in agreement with the results of McNamara (SFIA 1996), who recommended a temperature range of 10–18°C for scallop degritting based on measurements of shellfish activity. In the absence of any further work, a minimum temperature of 10°C is recommended for use during scallop depuration. No experiments were conducted to define upper temperature limits for depuration. This is because higher temperatures do not usually compromise the depuration process, although they may affect product quality. In the one experiment (Table 4) in these trials in which scallops were depurated at 20°C the scallops depurated effectively, but there was some post depuration mortality. Heath and Pike (2001) recommended an upper temperature limit of 16°C for depuration and McNamara (SFIA 1996) a limit of 18°C for degritting.

Although scallops depurated to below the end product standard in all treatments during the single experiment investigating emersion time, there was some evidence that animals immersed for 22 h reduced *E. coli* levels less successfully compared with those immersed for 10 h. However this single result must be considered inconclusive, although it does concur with previous work (SFIA 1996). That study concluded that scallop activity was reduced during degritting in scallops that were immersed for 24 h before processing. A general conclusion from various studies on the effect of emersion time on viability of great scallops is that they should not be kept out of water for longer than 12 h (Maguire et al. 1999, Christophersen 2000, Minchin et al. 2000).

It was possible to depurate successfully in a double layer with nominal capacity of 250 scallops m^{-2}. Scallops loaded at this density showed a considerable tendency to move and, if left unconfined, escaped from the basket and deposit themselves on the base of the tank. This is considered unacceptable during depuration as much of the fecal material excreted by the scallops during the depuration cycle will settle on the base of the tank. Movement of the scallops in this area will resuspend this material, which may be reingested and recontaminate the scallops with microbiological organisms present in the sediment. During this trial, plastic mesh was placed over the baskets to prevent the scallops escaping. It is critical for the depuration process that scallops should be contained within the basket. Any procedure for doing this must not interfere with the ability of the scallops to open and filter. The lowest scallop to shellfish water ratio investigated in this trial was 1:12 because this was the maximum that could be achieved using the double layer arrangement. This is a higher ratio than may be found in some of the high-intensity systems that may be used to depurate other species. These may have shellfish-to-water ratios of as low as 1:3 when fully loaded. However it is considered unlikely that high-density depuration of scallops is likely to be required in the near future. Given this and results from density trials conducted elsewhere (Heath & Pyke 2001), it is recommended that scallop to water ratios should not fall below 1:12.

In general, results from the post depuration self-righting (stress) experiments gave good agreement with the results from the depuration experiments. That is, scallops from conditions that supported effective depuration showed no difference in stress to control scallops, whereas scallops from conditions in which they did not depurate showed high levels of stress, sometimes accompanied by subsequent high mortality. Also, the lower temperature and salinity limits, below which the scallops will not depurate effectively, are similar to the lower limits for optimum growth performance of great scallops (Laing 2000, 2002).

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CIRCADIAN METABOLIC RATE AND SHORT-TERM RESPONSE OF JUVENILE GREEN ABALONE (*HALIOTIS FULGENS* PHILIPPI) TO THREE ANESTHETICS

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ABSTRACT Time–course experiments were performed on juvenile green abalone (*Haliotis fulgens*) to assess the degree of stress caused by the anesthetics magnesium sulfate (MS), benzocaine (BZ), and phenoxyethanol (PE). Metabolic rate (VO_2) of abalone was reduced by 65, 35, and 18% during short-term (10 or 20 min) exposure to MS, BZ, and PE, respectively. Abalones significantly increased their VO_2 above control values (1.5-fold) after removal of PE from metabolic chambers, whereas those treated with MS or BZ recovered their VO_2 to preanesthesia values. Visual criteria of recovery generally coincided with those of metabolic measurements (i.e., 80% of abalone regained "normal" activity after 35 min postanesthesia), yet metabolic measurements showed that "fast" recovering abalone treated with PE maintained high VO_2 values during 3-h postanesthesia. Abalone treated and nontreated with anesthetics exhibited a circadian metabolic rhythm, with 20–35% higher rates observed during dark than light hours. Despite the short-term metabolic alterations with MS, BZ, and PE, the present study suggests that all three anesthetics may be safely used in abalone. However, detailed evaluations are still needed to assess the effect of anesthesia on other physiological variables. The results obtained in this study highlight the importance of physiological evaluations when different chemical substances are used in aquatic invertebrates.

KEY WORDS: abalone, anesthetics, circadian rhythm, metabolism, *Haliotis fulgens*

INTRODUCTION

Current culture methods for abalone involve various steps in which organisms need to be dislodged from their rearing substratum for the purpose of size grading, adjusting densities, tagging, and/or transferring from indoor to outdoor culture facilities (Hahn 1979, Juefeng & Shiuan 1996). Because of the natural ability of abalone to strongly adhere on most surfaces, forced removal not only results in excess mucus production with the consequent energy losses (Peck et al. 1987, Davies & Williams 1995, McBride et al. 2001), but it can also result in injuries in the soft tissues that may eventually result in death (White et al. 1996). It is thought that the lack of clotting mechanisms in abalone facilitate prolonged bleeding and/or the appearance of bacterial infections in wounded tissues (Armstrong et al. 1971), thus increasing the probabilities of death (Juefeng & Shiuan 1996).

Farms have produced several solutions to remove abalone from the substrate without producing injuries. All of them are aimed to relax the soft tissues or decrease the degree of awareness in abalone and include thermal shock and desiccation (Hahn 1989) in addition to the use of anesthetic substances, such as CO_2, urethane, chloral hydrate, barbitol, diethyl carbonate, benzocaine, ethyl alcohol, propylene phenoxytol, potassium chloride, procaine hydrochloride, MS-22, pentobarbital, magnesium sulfate, and phenoxyethanol (Hahn 1989, Juefeng & Shiuan 1996, White et al. 1996, Aquilina & Roberts 2000). The last three substances have been reported as effective and nonlethal anesthetics for abalone because organisms usually recover within the first few hours of application (Hahn 1989, White et al. 1996, Aquilina & Roberts 2000).

Excess magnesium sulfate interfere with neuromuscular transmission signals in mammals because magnesium ions block the release of the neurotransmitter acetylcholine from motor nerve endings, by competitively binding to *N*-methyl-d-aspartate (NMDA), a glutamic acid receptor (Iwatsu et al. 2002). The overall effect of this blockade is a sedative effect of the neuromuscular system, followed by muscle paralysis, respiratory depression, coma, and death (Swain & Kaplan–Machlis, 1999). It is thought that phenoxyethanol also binds competitively to NMDA receptors (Mushoff et al. 1999) and causes depression of the central nervous system and hypoxia when delivered in excess (American Veterinary Medical Association 2001). The mechanism of action of local anesthetics, such as benzocaine (BZ), is a blockade of the voltage-activated sodium channel at the neuronal cell membrane, which prevents the generation and conduction of the nerve impulse (Catterall & Mackie 1996). Excess BZ in mammals may result in prolonged sedation, cardiac arrhythmias, respiratory depression, tremors, and death (Catterall & Mackie 1996).

The effectiveness of anesthetics in marine molluscs has been largely evaluated on the basis of visual observations, such as the degree of gaping response after tactile stimuli in bivalves (Culloty & Mulcahy 1992, Heasman et al. 1995, Mills et al. 1997), degree of muscle relaxation, and coloration in squids (García–Franco 1992), and degree of adhesion, muscle relaxation, and mortality in abalone (Hahn 1989, White et al. 1996, Aquilina & Roberts 2000). In most cases, short-, medium-, or long-term effects of anesthetics have not been studied in detail, even though the magnitude of stress during and shortly after the application of anesthetics is well documented with visual observations. Therefore, detailed understanding of the effect of anesthetics on abalone is still needed at the physiologic level, especially because they represent a potential tool for research and management.

Several physiological parameters have been reported to increase at night in abalone, including motor (Donovan & Carefoot 1998), feeding activities (Barkai & Griffiths 1987), and metabolic rate (Uki & Kikuchi 1975). The latter is known as a highly sen-

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sitive parameter in molluscs because it readily changes in response to stress factors, such as temperature (Newell 1973, Paul & Paul 1998), pH (Harris et al. 1999), nitrite (Harris et al. 1997) and ammonia concentrations (Harris et al. 1998), and starvation (Garcia-Esquivel et al. 2002). Respiration is controlled by the central nervous system, and therefore it is not surprising that metabolic rate of abalone is affected by substances such as magnesium sulfate (Edwards et al. 2000). In the present study, time–course measurements of metabolic rate were performed in juvenile green abalone (*Haliotis fulgens*) in the presence and absence of anesthetics to assess the magnitude and duration of metabolic stress produced by magnesium sulfate, phenoxyethanol and benzocaine.

METHODS

Experimental Conditions

One and a half year-old juvenile abalone (*Haliotis fulgens*) with shell lengths ranging from 25 to 35 mm, originally obtained from BC Abalone farm in Erendira B.C., Mexico, and maintained at the laboratory facilities at the University of Baja California, were used for the different experiments. Abalone was kept in a shallow water tray (180 × 90 × 20 cm, length × width × height) under flow-through (ca. 300 mL min^{-1}), aerated seawater conditions. Seawater temperature was maintained at 23 ± 1°C with a digitally controlled heater (CLEPCO, 1000 watts) located in a reservoir. Inert food was offered at night on a regimen of 12 h per day, with a diet (Table 1) made in the laboratory as recommended by Viana et al. (1996). Light intensity was kept at ca. 2 × 10^{-5} μE/s/cm^2 with several layers of a plastic mesh (70% shed) placed around the system. A photoperiod of 12:12 (Light: Dark) was maintained throughout the study.

Experimental Design

Experiments were of two types: (1) Time series to identify circadian changes in the metabolism of *H. fulgens*, and (2) Time series to assess the short-term (3 h) and medium-term (2 days) effects of magnesium sulfate (MS), phenoxyethanol (PE), and BZ on the metabolic rate.

Circadian Changes

Twenty-four abalone were randomly selected from the maintenance tray and distributed among eight respiration chambers (three organisms per chamber) with a volume of 1.8 L each. Four additional chambers were also used as controls (without organisms). Chambers were maintained with open flow and without food during 24 h for the abalone to acclimate to the system. Feces and any remaining particles were siphoned out from each chamber before beginning the first measurement of oxygen consumption. Respiration was measured using closed-cell respirometry. Incubations of ca. 1- to 1.5-h duration were conducted every hour during a total period of 48 h. At the end of this period, the live weight and total length of the experimental abalone was recorded.

Anesthetics

Two experiments were carried out with using the anesthetics magnesium sulfate (MS, Sigma M-7506) at a final concentration of 4% w/v (Hahn 1989); phenoxyethanol (PE, Sigma P-1126) at 0.1% v/v (Edwards et al. 2000) and benzocaine (BZ, Sigma E-1501) at 0.01% v/v (Hahn 1989). The latter was dissolved in 95% ethanol (10% w/v) before use. All final solutions were prepared in 5-μm filtered seawater just before application.

Ingredients (g/100 g diet)	Balanced Diet
Fish meal*	30.00
Corn starch	14.66
Kelp meal[a]	10.00
Corn flour[b]	10.00
Gelatin (50 blooms)	10.00
Soybean meal[c]	8.00
Cellulose[d]	5.00
Modified starch[e]	5.00
Mineral mixture[f]	4.00
Vitamin mixture[g]	1.50
Fish silage[h]	1.40
Stay-C[i]	0.20
Choline chloride	0.10
Sodium benzoate	0.10
BHT[j]	0.04
Composition (%)	30.8 ± 0.7
Protein	12.6 ± 0.1
Ash	6.3 ± 0.1
Nitrogen-free extract	50.3 ± 0.4

* 64% protein.
[a] Made from *Macrocystis pyrifera*.
[b] Corn flour (Maseca).
[c] 39% protein, 21% lipid.
[d] α-cellulose (Alphacel).
[e] Modified corn starch (Clearjel®).
[f] ICN vitamin diet fortification.
[g] ICN salt mixture #5 Brigges.
[h] Acid fish silage from tuna viscera.
[i] Ascorbyl polyphosphate (kindly donated by Roche).
[j] Butylated hydroxy toluene.

In the first experiment, a step-wise approach was used for quantifying the short-term response of juvenile abalone exposed to anesthetics. Metabolic rate was measured before, during, and after the application of anesthetics. Each anesthetic was evaluated on different days using four replicate chambers per anesthetic (three organisms per chamber), three control chambers (abalone without anesthetic), and three reagent controls (seawater with anesthetics, but no abalone). Experimental organisms were transferred to the chambers 24 h before the treatment, as described in the previous section. At the end of this period, chambers were cleaned and respiration rate of abalone was measured during 1–1.5 h. The water was completely renewed (100% oxygen saturation) and anesthetics were added directly into the incubation chambers, while recordings of oxygen consumption continued. Abalones were in contact with anesthetics for a fixed period of 10 min (BZ and PE) or 20 min (MS). These treatment periods were based on preliminary visual observations of the organism's response to these anesthetics. Chambers were flushed with fresh seawater (ca. 6 volume changes) after the exposure period to eliminate anesthetics. It was assumed that anesthetics got rid off the chambers during flushing, as the reagent control and experimental chambers regained a constant oxygen baseline afterwards. Incubations continued every 1 or 1.5 h during the following 3 h to measure the metabolic response postanesthesia on the same organisms.

The second experiment consisted of a time series of 48 h with simultaneous measurement of VO_2 in abalone treated with all three anesthetics (MS, PE, BZ) to assess the duration of metabolic stress. Experimental abalone were removed from the maintenance tray with the help of a spatula and transferred to plastic buckets containing 1 L of seawater. When all organisms had adhered to the walls, all three anesthetics (MS, PE, BZ) were added separately into the buckets and the anesthetized organisms were transferred to metabolic chambers (3 abalone per chamber), where respiration measurements began 3h later. Incubations lasted between 1.5 and 2 h, with a measurement frequency of ca. 8h, and a total elapsed time of 47 h. At the end of each trial, oxygen consumption rate was measured in the same experimental chambers without abalone, to correct for the oxygen consumed by sources other than abalone (electrodes, microorganisms). A total of three replicates per treatment (anesthetics) and two control replicates (abalone without anesthetics) were used for this trial.

Measurements

Metabolic Rate

Oxygen consumption by *H. fulgens* was recorded every 30 sec with two computer-controlled polarographic oxygen sensors (Strathkelvin Instruments Ltd., Ireland). Each oxygen meter had six channels, such that 12 chambers could be monitored simultaneously. Aerated seawater was used for calibration to 100%, and sodium sulfite was used for 0% calibration. A magnetic stir bar (10-mm diameter × 8-mm length) was used for mixing the water in each incubation chamber under a perforated acrylic sieve (4-mm mesh) to prevent a direct contact between the stir bar and organisms. After incubations all abalone from the chambers were blot dried with a piece of cloth, measured with digital calipers (MAX-CAL, ± 0.03 mm) on their longest dimension, and weighed in a portable scale (AND SV-200, ± 0.01g). Oxygen consumption rate (metabolic rate, VO_2) was estimated from the corrected slope of the oxygen evolution curve (abalone minus non-abalone chambers), after transforming the %O_2 saturation to μmol of dissolved O_2 in seawater, from known values of oxygen solubility (Green

and Carritt, 1967). The following equation was used for calculating metabolic rate:

$$VO_{2e} = (Cs * m * 60)/(100\% * Wwt) \qquad (1)$$

where VO_{2e} = metabolic rate of the experimental organism (μL O_2 /g/h)

Cs = total amount of O_2 in the incubation chamber at 100% saturation (μL O_2).

m = slope of the O_2 evolution curve (%O_2/min)

60 = factor used to transform from minutes to hours

Wwt = live weight (g) of organisms in the incubation chamber.

Visual Assessment of Anesthesia and Recovery

Direct observations of abalone behavior during and after application of each anesthetic were conducted on 36 organisms distributed in 12 buckets (three organisms per bucket). The time taken from the application of anesthetics to the moment an abalone fell off the walls of the bucket was considered as the period needed for induction to anesthesia. Similarly, the time taken for an anesthetized abalone to regain an upright position (ventral side firmly attached to the container's walls) was considered a visual criterion for recovery postanesthesia (White et al. 1996). Mortality was evaluated on anesthetized organisms after 2 or 4 weeks postanesthesia.

Statistics

In all cases, time-dependent changes of metabolic rate were statistically tested using an analysis of variance (ANOVA) with repeated measures. When significant differences were found, least squares pre-planed comparisons of means were used to identify specific differences. These tests were carried out using a general linear model procedure (GLM) included in the statistical package SAS, version 6.08 (SAS, 1998).

RESULTS

Short-Term Effect of Anesthetics

Juvenile abalone treated with all three anesthetics exhibited time-dependent differences in their metabolic rate (VO_2), as this was significantly reduced (Table 2) by 65% (MS), 35% (BZ), and 18% (PE) of initial values during the exposure period (Fig. 1a–c).

TABLE 2.

Results of repeated analysis of variance for comparison of short-term (5 h) and long-term (48 h) changes of metabolic rate of juvenile abalone, *Haliotis fulgens*, after exposure to three anesthetics (Anest).

Source of Variation	DF	Mean Squares			F		
		MS	PE	BZ	MS	PE	BZ
Short term							
Replicates	3	28.0	60.9	46.9	0.9 NS	3.8*	1.0 NS
Between Subjects (Anest)	1	390.5	1137.8	30.7	12.8**	70.3**	0.7 NS
Within Subjects (time)	3	824.5	431.3	234.5	27.0**	26.6**	5.1**
Time × Anest	3	842.0	439.6	378.3	27.6**	27.1**	8.2**
Error	17	30.5	16.2	46.2			
Long term							
Replicates	2		51.8			3.1 NS	
Between Subjects (Anest)	3		141.2			8.4**	
Within Subjects (time)	6		115.22			68.8**	
Time × Anesth	18		46.9			2.8**	
Error	47		16.7				

Short-term trials were conducted independently for each anesthetic and its control whereas the long-term trial was conducted simultaneously for the anesthetics magnesium sulfate (MS), phenoxyethanol (PE), benzocaine (BZ) and a control without anesthetics (C).

* $P < 0.05$; **$P < 0.01$; NS = not significant at $P > 0.05$.
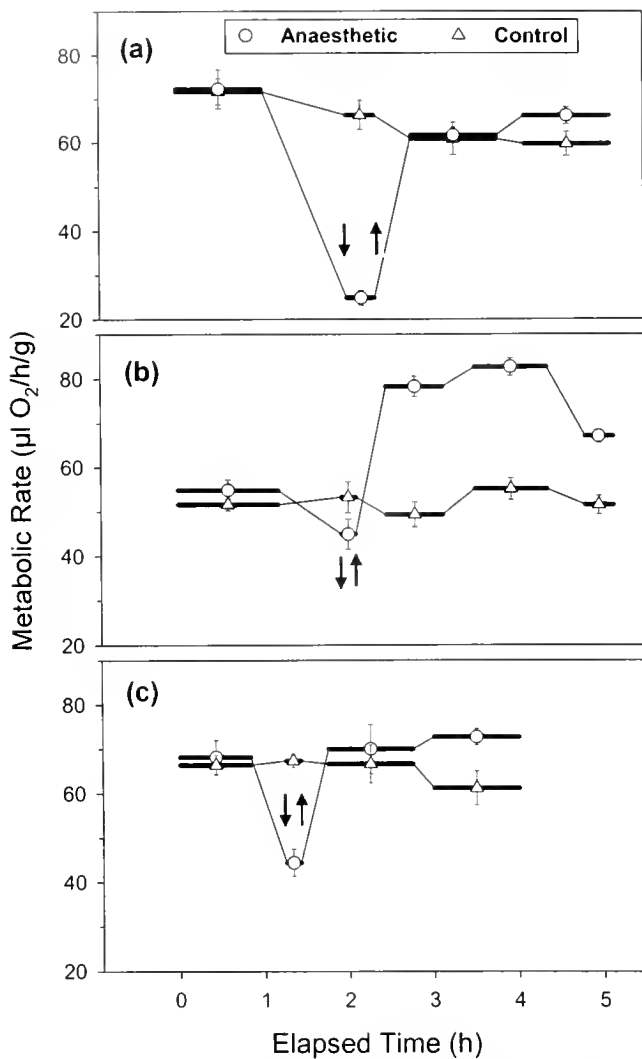
Figure 1. Short-term changes of metabolic rate in juvenile green abalone (*Haliotis fulgens*) before, during, and after exposure to magnesium sulfate (a), pheoxyethanol (b), and benzocaine (c). Arrows indicate the moment when anesthetics were added ↓ and removed ↑ from the respiration chambers.

Abalone from the MS and BZ treatments re-established their VO_2 after flushing away the anesthetic from the respiration chambers, and remained similar to control values thereafter ($P > 0.05$). In contrast, abalone treated with PE significantly increased their VO_2 by a factor of 1.5 above control values (Table 2) and remained high for the next 2 h posttreatment, with a trend to decrease thereafter (Fig. 1b). Oxygen consumption of reagent control chambers (anesthetics, without abalone) also increased in the presence of these chemicals. It accounted for 10% (MS), 13% (PE), and 54.3% (BZ) of the total O_2 consumed by experimental abalone during the exposure period, yet no significant O_2 consumption was observed in the reagent chambers ($P > 0.05$) after anesthetics were flushed away (data not shown).

Diel Changes of Metabolic Rate

A circadian rhythm was observed in juvenile abalone in the absence of anesthetics (Fig. 2a), with significantly higher meta-

bolic rate observed during dark than light hours ($P < 0.01$). Metabolic rate (VO_2) decreased and remained relatively constant (42–52 µL O_2/h/g Wwt) during the period of 10:00 to 16:00 h (light conditions), and significantly increased ($P < 0.01$) by ca. 20% during the period of 22:00 to 4:00 h (dark conditions). The transition period (light switched on or off, at 8:00 and 20:00 h, respectively) was characterized by rapid changes of metabolic rate, such that abalone exhibited most of the time a dark- or a light-adapted metabolic rate (Fig. 2a). The circadian VO_2 pattern was maintained throughout the 48-h measuring period, even though the absolute values showed a trend to decrease in the second day of the trial ($P < 0.05$). Abalone exposed to anesthetics exhibited significant anesthetic and time effects (Table 2), and the same circadian rhythm identified above. VO_2 values obtained at 8:00 or 20:00 h (Fig. 2b) also corresponded to the transition period. Higher values (65 to 70 µLO_2/h/g Wwt) were observed during dark hours and lower values during daylight hours (Fig. 2b). Time vs anesthetic interaction was also significant (Table 2). PE-treated abalone exhibited significantly higher VO_2 values than control organisms ($P < 0.05$) during the first measurement (3 h postanesthesia), yet these differences were not statistically significant thereafter ($P > 0.05$).

Visual Criteria

Based on motor activity, it was observed that SM acted slowly and asynchronously on juvenile abalone. These organisms showed a complete relaxation of the mantle and became narcotized some-
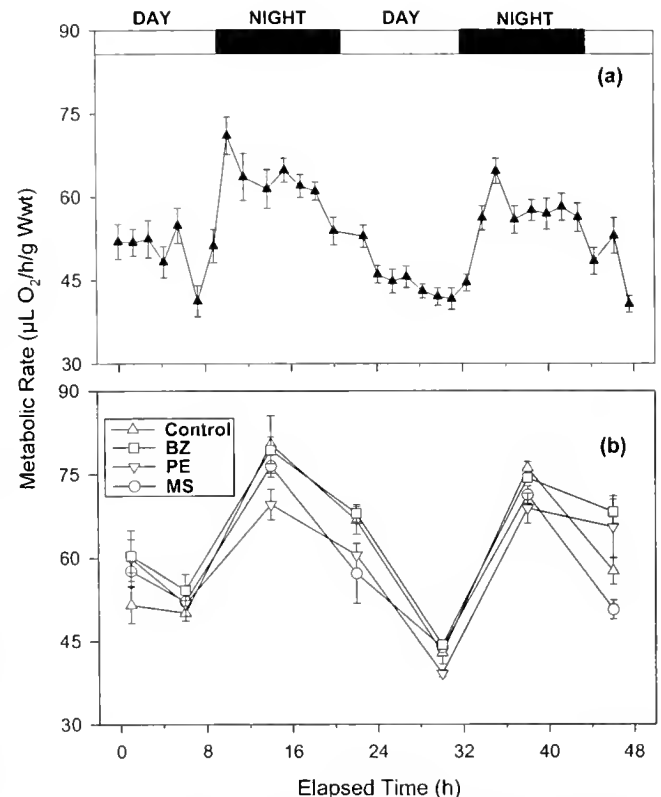
Figure 2. Two-day recordings of metabolic rate exhibited by juvenile green abalone (*Haliotis fulgens*) without anesthetic treatment (a) and after a 10-min exposure (b) to the anesthetics magnesium sulfate (MS), phenoxyethanol (PE), and benzocaine (BZ). A control treatment (abalone, no anesthetics) is also shown. Time 0 = 10:00 AM.

TABLE 3.

Visual assessment of minimal (min) and maximal (max) exposure period (minutes) necessary to anesthetize juvenile abalone, *Haliotis fulgens*, by magnesium sulfate (MS), phenoxyethanol (PE) and benzocaine (BZ).

ID	MS		PE		BZ	
	Min	Max	Min	Max	Min	Max
1	2.4	19.0	2.3	4.2	4.5	4.5
2	3.2	5.1	1.1	4.2	3.7	6.5
3	2.3	25.0	1.9	2.7	2.7	4.4
4	2.7	18.6	2.3	5.7	2.6	4.2
5	4.0	20.0	2.3	4.2	3.0	9.6
6	2.1	23.0	2.1	5.6	2.5	4.4
7	2.4	34.0	2.8	3.0	2.4	9.2
8	4.0	19.0	3.2	4.3	4.5	4.8
9	1.5	23.0	2.6	3.2	2.9	2.9
10	14.0	23.0	2.4	3.1	6.5	7.1
11	12.0	26.0	3.0	4.7	2.4	3.1
12	2.4	18.0	2.6	3.3	3.7	6.1
Mean + SE	4.4 + 1.2	21.1 + 1.9	2.4 + 0.2	4.0 + 0.3	3.4 + 0.3	5.6 + 0.6

Data based on evaluations of 12 independent chambers per each anesthetic (15 abalone/chamber).

time between 2 and 21 min after exposure. In contrast, organisms exposed to PE and BZ became generally narcotized within the first 2 to 5 min (Table 3) and were characterized by their rigidity. Recovery postanesthesia varied among anesthetics. About 80% of abalone regained their normal upright position after 18 min (PE) 25 min (BZ) and 35 min (MS) post-anesthesia, and nearly 100% had been recovered after 1 h in all treatments (Fig. 3).

DISCUSSION

Short-Term Effect of Anesthetics

The induction/recovery periods visually determined for *H. fulgens* in this study were similar to those reported for other molluscan species, including the abalone *H. gigantea* and *H. midae* (Hahn 1989, White et al. 1996), the scallop *Pecten fumatus* (Heasman et al. 1995) and the pearl oysters *Pinctata albina* and *P. margaritifera* (Norton et al. 1996). The heterogeneous anesthetizing effects of MS contrasted with the homogeneous, rapid action of PE and BZ assessed visually. Similar observations have been previously reported for *H. midae* (Hahn 1989, White et al. 1996) and may be related to the degree of access of these anesthetics to the site of action. It is known that topical anesthetics like BZ readily and locally interact with any nerve cell receptor (American Veterinary Medical Association 2001), whereas MS affects the smooth muscle or the central nervous system of vertebrates (Swain & Kaplan-Machlis 1999) by blocking the release of the neurotransmitter acetylcholine. The relaxation symptoms of abalone tissues observed in this and other studies (White et al. 1996) are consistent with the symptoms described for the neuromuscular system of humans (i.e., Swain & Kaplan-Machlis 1999) and other vertebrates (American Veterinary Medical Association 2001).

To our knowledge, this is the first study that documents in detail a time-course response of metabolism in marine invertebrates exposed to anesthetics, including the observation of rapid depression of the respiratory system and concomitant recovery following the elimination of anesthetics from the chambers (Fig. 1). White et al. (1996) recorded an inhibitory response of the tarsal muscle of *H. midae* when exposed to MS, PE, and procaine, but

their study was more focused at demonstrating the effectiveness of these substances as anesthetics, rather than documenting time-dependent physiologic effects on abalone. The short-term (i.e., <3 h postanesthesia) metabolic response of abalone was only partially coincidental with visual criteria of recovery. In this regard, the VO_2 exhibited by *H. fulgens* immediately after MS and BZ were flushed away from the incubation chambers were similar to the controls, whereas organisms treated with PE maintained a high VO_2 even after 3 h postanesthesia (Fig. 1b). Conversely, visual observations suggested that organisms exposed to PE and BZ recovered faster and more uniformly than those exposed to MS (Fig. 1). Although no other physiological variables were measured in this study, it has been reported that the trout *Oncorhynchus mykiss* experienced an increase in blood pressure after being exposed to PE (Fredricks et al. 1993). Therefore, the actual physiologic state of abalone (this study) was more likely reflected in the metabolic response curve, as this variable is highly sensitive to exogenous
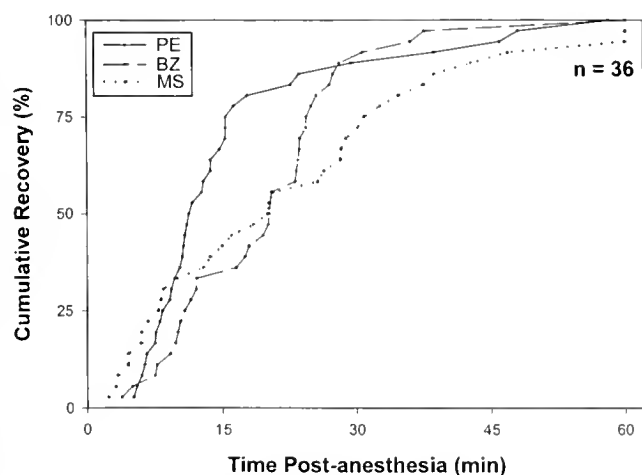
Figure 3. Cumulative percent recovery from anesthesia of juvenile abalone, *Haliotis fulgens*), based on visual criteria. Magnesium sulfate (MS), phenoxyethanol (PE), and benzocaine (BZ).

and endogenous perturbations. The reason for the post-anesthesia increase of VO_2 in the presence of PE is not known and need further and detailed studies in abalone.

Diel Changes

The consistent circadian rhythm exhibited by control and anesthetic-treated *H. fulgens* suggests that these chemicals did not significantly affect the functioning of the central nervous system after a few hours of application. Furthermore, the observed rhythm is consistent with previous reports of night-accelerated metabolism in the Japanese abalone *H. discus hannai* (Uki & Kikuchi 1975). Other physiologic variables, such as food intake (Barkai & Griffiths 1987) and motor activity (Donovan & Carefoot 1998), typically increased at night in abalone. Circadian physiologic rhythms have also been observed in other molluscan species. For example, Watanabe (1983) reported that the repair and growth of shell in the bivalves *P. martensii* and *Helisoma duryi* was highest under continuous darkness. Taken together, these results suggest that the endogenous clock of these species may be cued by light. It has been demonstrated that changes in light intensity/photoperiod are among the major environmental cues responsible for the activation of clock genes in organisms ranging from fruit flies to mammals (Schibler & Lavery 1999). In addition, it has been shown that the ocular circadian rhythm of the marine snail *Bulla gouldiana* is a complex process regulated at the level of transcription, translation, and phosphorylation and involves the presence of a cyclin-dependent protein kinase, whose activity coincides with a circadian clock (Krucher et al. 1997). Earlier reports suggest that the hypothesis of a light-controlled circadian rhythm may still be controversial haliotids. In this regard, Jan et al. (1981) found that *Haliotis diversicolor supertexta* exhibited a circadian metabolic rhythm when exposed to a natural diurnal cycle, but not under continuous light. Accordingly, Peck et al. (1987) did not find significant differences in the metabolic rate of *H. tuberculata* between day and night (12:12 L:D photoperiod), and suggested that such behavior resulted from an excess in the available food. The contradictory results found among haliotids may either suggest that there are species-specific differences in the response to environmental cues and/or there are subtle methodological differences that may explain the observed results. In this regard, it was shown that the gastropod *A. californica* exhibited a circadian feeding rhythm under conditions of 12:12 L:D photoperiod, with shorter feeding response associated with light hours. Such a pattern continued when organisms were shifted to a 0:24 (L:D) photoperiod (Kohn 1983), but the whole experimental period lasted only three days, and therefore no conclusive results could be drawn as to the role of light on cueing the observed rhythm. Therefore, detailed and long-term experiments are needed to test whether the circadian rhythm observed in this and other studies can be generalized in haliotids. Despite these controversies, the results of the present study may have implications for growth/production protocols in *H. fulgens* because the total energy drain (i.e., respiration) of *H. fulgens* is highest at night. The trend of decreasing metabolic rate observed during the second day of measurement (Fig. 2a) was likely due to a higher amount of food remaining in the abalone's gut (i.e., SDA component of VO_2) in the first day of measurement. It has been shown that a complete gut evacuation can take between 18 h to 7 days in abalone (Wee et al. 1992, Maguire et al. 1993, Britz et al. 1996, Mai et al. 1998).

Overall, the results of this study highlight the importance of physiologic evaluations when different chemical substances are used in aquatic invertebrates. The combined visual and metabolic evaluations confirmed that all three anesthetics might be potentially used for handling abalone, since all of them effectively induced anesthesia, rapid post-anesthesia recovery and no mortality. Nevertheless, careful evaluations are still needed to assess the long-term effects of anesthesia on other physiologic variables such as growth, food intake and activity of abalone. In this regard, Edwards et al. (2000) found that the abalone *H. laevigata* and *H. rubra* exhibited a significantly lower growth rate than control organisms after 6 weeks of exposure to PE and BZ.

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LABORATORY HYBRIDIZATION OF THE MUSSELS, *MYTILUS TROSSULUS* AND *M. GALLOPROVINCIALIS*: LARVAL GROWTH, SURVIVAL AND EARLY DEVELOPMENT

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ABSTRACT Experiments were performed to determine whether hybrid larvae of *Mytilus trossulus* (Baltic mussel) and *Mytilus galloprovincialis* (Mediterranean mussel) could be produced in a shellfish hatchery environment and whether early development, survival, or growth differences existed between the two species and their reciprocal hybrids at full and reduced salinity. Hybrids of these two species are uncommon in Puget Sound, Washington and on the northern west coast of North America. Broodstock were screened morphologically and positively identified at two nuclear DNA loci using polymerase chain reaction and restriction fragment length polymorphism techniques. Hybrid larvae were produced in both reciprocal combinations, and were successfully reared through metamorphosis. There was no apparent hybrid vigor because hybrids did not grow consistently larger (or survive better) than the parental crosses, nor did one reciprocal cross grow consistently larger than the other. Both reciprocal hybrid crosses and the parental cross, *M. trossulus*, grew faster than the other parental cross, *M. galloprovincialis*, at low salinity (20 ppt). These results concur with the two species' physiologic and ecological characteristics. *Mytilus trossulus* grows well in areas of low and variable salinity (much of Puget Sound) and *M. galloprovincialis* grows well in areas of stable, full salinity, and recruits poorly in Puget Sound. Hybrids showed generally lower fertilization rates and slower early development than parental crosses, although they were sufficient to produce larval cultures and postlarvae. The successful fertilization, growth, and survival of these hybrids suggests that some factor other than genetic incompatibility is likely responsible for the rarity of these hybrids in Puget Sound. One such factor could be the limited overlap of the spawning periods of the two species in this region. A differential species growth-response to salinity was observed in this study.

KEY WORDS: hybrid, mussel, *Mytilus trossulus*, *galloprovincialis*

INTRODUCTION

Two species of marine mussels found in Puget Sound, Washington are *Mytilus trossulus* (Baltic mussel) and *M. galloprovincialis* (Mediterranean mussel). The two species occur both sympatrically and allopatrically in Puget Sound. Both species possess distinct ecological and physiologic characteristics (Johnson 1978, Kautsky 1987, Brooks 1991, Margus 1991, Sarver & Loudenslager 1991, Sarver & Foltz 1993, Geller et al. 1994, Hoffman & Somero 1995, 1996). *Mytilus trossulus* thrives in areas of widely fluctuating salinity, as is the character of much of Puget Sound, whereas *M. galloprovincialis* occurs in bays with more stable, high salinity (Brooks 1991).

These two species hybridize naturally in isolated populations in Puget Sound (Brooks 1991, Suchanek et al. 1996). Their hybrids also occur in other bays in Washington, Oregon, and California. Although these two species often co-occur throughout their distribution within Puget Sound, the overall frequency of hybrids has remained very low (Brooks 1991, Suchanek et al. 1996). One reason this laboratory attempt at hybridizing these two species was performed was to help elucidate what sort of barrier to hybridization may be responsible for this lack of hybrids in the wild. Barriers to hybridization can be rooted in genetic incompatibility or physiologic and ecological differences, such as disease, salinity tolerance, or the timing of spawning events.

Both mussel species are commercially important within the United States and throughout the world. Their physiologic and ecological differences pose challenges to those who culture them. *Mytilus trossulus* suffers high mortalities before the end of its second year of life because of high summer water temperatures and the disease hemic neoplasia (Brooks 1991). Up to 75% of a *M. trossulus* mussel crop in Penn Cove, Puget Sound often dies before

it is old enough to be harvested (Brooks 1991). *Mytilus galloprovincialis* typically grows to a larger size than *M. trossulus* and is resistant to hemic neoplasia (Brooks 1991). *Mytilus galloprovincialis* has been observed suffering significant mortalities when salinities have dropped to 20 parts per thousand (ppt), and 100% mortality below 10 ppt (Kautsky 1987, Margus 1991). Low salinity conditions have repeatedly coincided with substantial mortalities of *M. galloprovincialis* in Holmes Harbor, Puget Sound, Washington (Kurt Johnson, Taylor Resources, personal communication), eventually leading to the closure of the mussel farm there. The financial implications of the aforementioned mortalities are severe enough to warrant examining biologic alternatives that might ameliorate lost farm revenues and even closures as a result of salinity- and disease-related crop loss. One such alternative worth examining is hybridization of the two mussel species. *Mytilus trossulus* and *M. galloprovincialis* possess characteristics that if expressed in a hybrid (variable salinity tolerance of *M. trossulus* and the disease resistance of *M. galloprovincialis*) might result in increased mussel production for industry. *Mytilus galloprovincialis* × *M. edulis* hybrids carry some of *M. galloprovincialis*' disease resistance to a trematode parasite (Coustau 1991). Sturgeon hybrids have been found to be more resistant to thermal and salinity shock than either parental species (Chikhachev 1979). Both interspecific and intraspecific hybrid vigor have been documented in bivalve mollusks (Loosanoff 1954, Hedgecock et al. 1996, Bayne et al. 1999) and it could occur in hybrids of these two *Mytilus* species. Hybrid vigor is defined here as an increase in growth or survival of hybrid crosses over pure-species crosses.

This investigation was performed to determine whether hybrid larvae of two locally occurring species of marine mussels (*M. trossulus* and *M. galloprovincialis*) could be produced in a shellfish hatchery environment, and whether survival and growth differences existed between the two species and their reciprocal hybrids at full and reduced salinity. This was the necessary first phase of evaluating the culture potential of hybrid mussels.

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METHODS

Broodstock

Broodstock mussels were collected from areas known to have essentially monospecific populations. After preliminary Broodstock selection was made based on morphology, molecular methods were used to positively identify all of the Broodstock in this study. Penn Cove was chosen for collection of *M. trossulus* based on Brooks 1991, 1996 and 1997. Only mussels several years old that fit the typical morphology for its species were collected. Hybrids often have a shell morphology intermediate to that of the parent species (Lubet 1984). Large mussels (1.5 inches or longer) of either species are easier to tell apart than very young ones, so only larger mussels were collected.

These two species have different morphologies (Brooks 1991, McDonald & Koehn 1991). The valves of *Mytilus trossulus* are typically narrow and long. The ventral shell margin is usually concave or straight. The anterior end (the umbo) is gradually bent; sometimes referred to as "beaked." The periostracum of *M. trossulus* is typically thin and rubs off near the umbo. In sagittal section, the ventral shell margin is straight. *M. galloprovincialis* has a very broad valve. The ventral margin is often convex. The umbo appears sharply hooked. *M. galloprovincialis'* periostracum is typically thick and black. In sagittal section, the ventral shell margin is rolled inward at the joining of the two valves. Mussels that fit these criteria were chosen for Broodstock. Those that appeared intermediate to these morphotypes were not collected to avoid hybrid Broodstock.

Molecular Identification

Broodstock mussels were identified using two different types of nuclear DNA markers (Heath et al. 1995, Rawson et al. 1996). These diagnostic molecular markers enabled positive discrimination between the three members and hybrids of the Mytilus complex. Both of these nDNA markers were based on the polymerase chain reaction and one used restriction fragment length polymorphism analysis. The first marker is based on the Glu gene, which encodes the mussel polyphenolic adhesive protein (Rawson et al. 1996). That protein is key in mussel attachment to the substrate. The Glu-5' marker enables differentiation between all three species in the Mytilus complex; *Mytilus trossulus*, *M. galloprovincialis*, and *M. edulis*.

In this study, tissue samples were digested using CTAB isolation buffer (IB) and proteinase k (10 mg/mL). CTAB was used to remove mucopolysaccharides in the bivalve tissue that could co-extract with the DNA and negatively affect later polymerase chain reaction (PCR). The CTAB IB (2% w/v CTAB, 1.4 M NaCl, 0.2% w/v 2-mercaptoethanol, 20 mM EDTA, 100 mM Tris/HCl, pH 7.5) was preheated at 50°C. Mantle edge tissue was chopped up with a razor blade and put into 1.5-mL polypropylene centrifuge tubes with 10 μL of proteinase k and an equal volume of CTAB IB. The mixture was incubated at 55°C for 3 h in a Rossi agitating incubator, vortexed for 10 s each, and then held at 50°C in a water bath overnight. In the morning, DNA was extracted from the tissue digestion with an equal volume of 24:1 chloroform:isoamyl alcohol mixture. The mixture was centrifuged at 11,500 g in a microfuge for 10 min. It was necessary to repeat the extraction two or three times to get a clear supernatant. Two volumes of 100% ethanol were added and the mixture was held in a −20°C freezer over night to allow precipitation of the DNA. The next day, the

extraction was centrifuged for 30 min at 11,500 g. The alcohol was removed and the pellet was rinsed with 0.5 mL of 70% ethanol. The pellets were dried in a centrifugal evaporator and then dissolved in 100 μL of TE (10 mM Tris/HCl, 1 mM EDTA, pH 7.6). A "Gene Quant" spectrophotometer (Pharmacia) was used to quantify DNA stock solutions. The stock solutions were diluted to make a 100 ng/μL working solution for use in PCR and were stored in refrigerator at 4°C. The stock solutions were frozen at −20°C for long term storage.

The sequences of the primers used for Glu-5' (Rawson et al. 1996) in this study were: 5'-GTAGGAACAAAGCATGAACCA-3' (forward) and 5'-GGGGGGATAAGTTTTCTTAGG-3' (reverse) The PCR recipe of Rawson et al. (1996) and their thermal cycler protocol were adapted. The end concentrations of chemicals in the PCR were 0.8× TBE buffer (20× TBE buffer solution: 121 g/L Tris base, 61.7 g/L boric acid, 7.44 g/L Na2EDTA*2H2O), 0.32 dNTPs, 1.5 mM MgCl$_2$, 4 μM forward primer, 4 μM reverse primer, 4 ng/μL of DNA template, and 0.04 U/μL of Taq DNA polymerase. The total reaction volume was 12.5 μL, and samples were amplified in a Techne thermal cycler using a hot-start protocol. The thermal cycler protocol used for this marker was one cycle of 94° for 3 min and then 24 cycles of 94° for 20 sec, 53° for 20 sec, and 72° for 45 sec. After PCR, the products were size-fractionated on 3% agarose TBE gels and stained with SYBR green (Molecular Probes) for approximately 1 h. They were visualized using a Molecular Dynamics 575 FluorImager. The banding pattern observed for Glu-5' in *M. galloprovincialis* was one band of 300 base pairs (bp) and one 500 bp band or just one 300 bp band. One 240 bp band was observed for *M. trossulus*.

The second DNA species marker used was also PCR-based but was followed by restriction fragment length polymorphism analysis (Heath et al. 1995). This codominant marker was based on internal transcribed spacer (ITS) regions between the 18S and 28S nuclear rDNA coding regions. Heath et al. (1995) showed that it worked very well in distinguishing *M. trossulus* from *M. galloprovincialis* or *Mytilus edulis*. This marker cannot distinguish between *M. galloprovincialis* and *M. edulis*, but because *M. edulis* is not yet known to occur in any of the Broodstock collection sites, or anywhere else in Puget Sound. It was reasonable to use this marker in conjunction with the Glu-5' marker and morphologic screening. The sequences of the primers used (Heath et al. 1995) in this study for the ITS marker were 5'-GTTTCCGTAGGT-GAACCTG-3' (forward) and 5'-CTCGTCTGATCTGAGGTCG-3' (reverse). The Heath et al. (1995) PCR recipe and thermal cycler protocol were both optimized for the facility where the work was performed. The end concentrations of chemicals in the PCR were 1× buffer, 0.8 mM dNTPs, 1.5 mM MgCl$_2$, 0.3 μM forward primer, 0.3 μM reverse primer, 0.5 ng/μL of DNA template, and 0.05 U/μL of Taq DNA polymerase. The thermal cycler protocol used was 94° for 3 min and then 30 cycles of 94° for 20 sec, 50° for 20 sec, and 72° for 45 sec. The total reaction volume was 16μl and a Techne thermal cycler was used. The PCR was hot-started. After PCR, 1 μL of each product was electrophoresed on a 1.5% agarose gel to check for amplification success. The products were then cut with *Hha*I restriction endonuclease overnight. Conditions for one digestion reaction was 0.04 μL *Hha*I enzyme, 1.0 μL 10× NEB #4 buffer (50 mM potassium acetate, 20 mM Tris acetate, 10 mM magnesium acetate, 1 mM DTT, pH 7.9 @ 25°C), 0.1 μL of 100× bovine serum albumin, and 3.5 μL of sterile double distilled water, and 5 μL of template DNA in TE (100 ng/μL). The frag-

ments were separated on a 3% agarose gel, stained with SYBR green for approximately 1 h and visualized using a FluorImager.

Heath et al. reported that in *M. edulis* and *M. galloprovincialis*, the 1250-bp PCR product was cut into two 450-bp fragments and two 180-bp fragments. In *Mytilus trossulus*, the 1250-bp product was cut into two 280-bp fragments, two 180-bp fragments, and a few fragments smaller than 100 bp. In this study, the PCR product was closer to 1050 bp long in both species. The two species markers, Glu-5′ and ITS both worked well at distinguishing *M. trossulus* from *M. galloprovincialis* and from hybrids. All Broodstock individuals were positively identified using the Glu-5′ marker. All but three males and two females were identified at the ITS locus. Those few were most likely not identifiable because of sample degradation. Both banding patterns were seen when equal portions of DNA from each species were mixed together and amplified (with either marker). This shows that one species' DNA was not preferentially amplified over the other's. Hybrid mussels would show the banding patterns of both of their parents (Rawson et al. 1994). The use of two loci increased the power of detection of hybrids.

Larval Rearing

Mussels were spawned and reared in 12-L plastic bags at the Taylor Shellfish Hatchery on Dabob Bay, Washington. It was necessary to condition *M. trossulus* Broodstock for a few weeks before spawning was attempted. *M. trossulus* normally spawn in March through May in Puget Sound (Johnson 1978). The mussels were held in tanks at ambient Dabob Bay temperature (10 to 12°C) and were fed large amounts of algae to encourage the necessary development of the gonad. Brenko and Calabrese (1969) found that food was the primary controlling factor for gonad development in Baltic mussels, and that rise in water temperature was the triggering factor for spawning in the natural environment. It was not necessary to condition the fully ripe *M. galloprovincialis* Broodstock, as they were already in spawning condition. They could not be held in water overnight because they spawned out in the holding tank before morning when this was attempted. *M. galloprovincialis* are typically in spawning condition December through March in Puget Sound. Up to 100 mussels of each type were used in spawning attempts for the first experiment to ensure that enough mussels actually spawned to make the crosses. Only approximately one fourth of those mussels that were induced, spawned enough gametes to produce a culture. Approximately 300 mussels of each species were induced in the spawning attempts that led to the second experiment. The mussels were induced to spawn by agitation, followed by heat shock (Loosanoff & Davis 1963). The mussels were taken from ambient 11° or 14°C water, shaken in buckets for approximately two minutes, and then placed into spawning trays with flowing seawater. The water temperature in the trays was changed from as high as 24° to as low as 11°C repeatedly. Dense, live algal food was also added occasionally for periods of about 20 min to encourage spawning.

Once a mussel began spawning, it was immediately removed from the tray, its sex was identified, the mussel's interior and exterior was rinsed with seawater, it was placed in a separate clean dish, and was allowed to spawn further. After that, its interior and exterior and its dish were rinsed a second time. Then it was allowed to spawn the gametes that would be used in the crosses. When all of the individuals had spawned, the mussels were removed from the dishes and the gametes were screened. All screens

were cleaned and soaked with hot fresh water between batches of gametes. Spawning mussels, each in its own dish, were kept separated in different areas on different tables by both sex and by species. Every batch of eggs and sperm were carefully examined under the microscope for contamination by other gametes. A batch of eggs or sperm was only used in a cross if it was observed to have zero signs of development in it, and any contaminated gametes were discarded. Fertilization was confirmed in each culture under the microscope. After fertilization, the embryos were screened and rinsed to remove excess sperm. All screens were cleaned and soaked with hot fresh water between batches of embryos. Two samples of one ml each were taken after fertilization for early development analyses and later growth measurements.

Sixty-four different mussels were used in all, to produce the 32 pair matings used in the experiment. Eight individuals of each sex were used to establish each cross (16 parents for each cross). Each replicate represented one single-pair mating. No replicates shared either a sire or dam. The four crosses made were *M. trossulus* × *trossulus (TT)*, *galloprovincialis* sperm × *trossulus* egg *(GT)*, *trossulus* sperm × *galloprovincialis* egg *(TG)*, and *galloprovincialis* × *galloprovincialis (GG)*. The low-salinity treatment was 20 ppt and the high salinity was 30 ppt. The embryos were then placed in 32 separate 12-L culture bags, with four replicate bags per cross by salinity treatment. (See Fig. 1 for a graphical description of the experimental design.) The culture bags were hung in a water bath with a thermostat-controlled immersion heater and circulating pumps. Culture temperatures were maintained at 18°C. The cultures were covered with shade cloth to prevent algal growth.

The larval density and the algal density of each culture were both standardized (regularly made equal between cultures). Larval density was equalized twice per week (at each water change) to prevent density-dependent growth or survival. This was performed by counting the larvae in each culture, and then decreasing the water volume in all bags until they had the same larval density as the culture with the highest survival (10 larvae/ml initially, decreasing to 3 larvae/mL by day 14). The algal density was equalized once per day, by counting the algae in each culture, and then feeding a different amount to each culture to maintain the desired algal density (20,000 cells/mL initially, gradually increased to 80,000 cells/mL at pediveliger).

Larvae were initially fed 20,000 cells/mL of naked flagellates
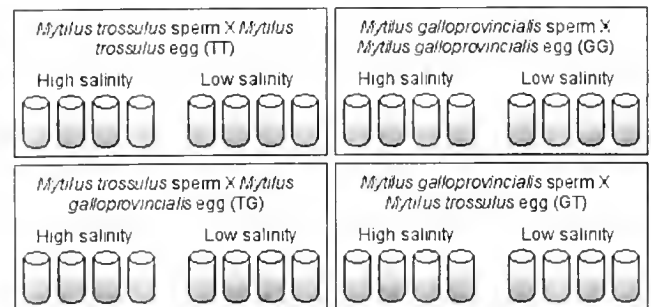
Figure 1. Two-factor experimental design used in examining survival and growth in larvae of *M. trossulus*, *M. galloprovincialis*, and their reciprocal hybrids at high and low salinities (32 ppt and 20 ppt, respectively). The 32 larval cultures were produced by 32 separate-pair matings. Eight cultures were used in each of four crosses, and four cultures were used for each salinity level within each cross. For example, the *M. trossulus* sperm by *M. galloprovincialis* egg cross included four cultures at high salinity and four at low salinity.

(Isochrysis sp. Tahitian isolate). Algal cell concentration was determined using a Coulter Counter model ZBI. On the day following fertilization, the cultures were fed a mixture of flagellates and diatoms (*Tahitian Isochrysis, Chaetoceros calcitrans, Thalassiosira pseudonana* [University of Washington 3H clone], and *Skeletonema* [species unidentified]). A mixture of two algal species supported faster growth than one alone, according to Bayne (1965), when he fed *Isochrysis galbana* and *Monochrysis lutheri* together. The amount of food given increased incrementally to a maximum of 80,000 cells/mL at the pediveliger stage. The density of larvae and algae was kept equal between cultures to reduce the possible influence on larval growth rate due to crowding. Sampling for survival was done twice per week at each water change. Each culture was condensed to 100 mL and one count was taken. The variability between counts was kept below 5% (tested beforehand) by condensing the culture and using a paddle stirrer. Cultures were resuspended in one liter between counting and bag refilling. Bags were cleaned with bleach, sodium thiosulfate, and rinsed with hot water at each water change. Bag water volumes were then adjusted to equalize larval density and larvae were resuspended in their bags. Two samples of 1 mL each were taken after fertilization for early development analyses and later growth measurements. Estimates of the proportions of larvae at each developmental stage present in the cultures were made from those samples as well. A total of 200 larvae were counted from each sample, and the number of larvae at each developmental stage was noted. Fifty larvae were chosen randomly and the distance from umbo to lip (shell length) of each was measured in microns using a compound light microscope and ocular micrometer.

RESULTS

Early Development

Early development was measured as the proportion of embryos that had developed to the blastula stage or beyond, at 12 h post-fertilization. This proportion was arcsine transformed to conform to the normality and homogeneity of variance assumptions of the analysis of covariance. It was also adjusted for egg density by using egg density as a covariate. The regressions for the covariate were significant for developmental success (proportion of blastulas; $P < 0.0001$). The slopes of the lines for the different crosses were equal for developmental success (NS cross by egg density interaction). Cross ($P = 0.045$) was a significant factor. The mean development of the TT cross was significantly higher than that of the TG cross' mean ($P = 0.007$, Fig. 2). No other differences were significant.

Growth

At day 3, the mean length of the GG cross was significantly higher than those GT and TT crosses ($P = 0.026$ and < 0.001, respectively). The TG cross's mean length was significantly higher than the TT cross's mean length also ($P = 0.001$, Fig. 3). These results are similar to an earlier experiment performed with the same crosses (Matson 2000). The low salinity treatment was applied at day 3. No significant differences existed in growth between crosses or salinities from day 3 to 7.

Salinity was a highly significant factor affecting growth between day 3 and 14 ($P < 0.001$, Fig. 4, Table 1). Cross was not a significant factor from day 3 to 14 ($P = 0.256$). The three crosses with a *M. trossulus* component (TT, GT, and TG) had higher mean
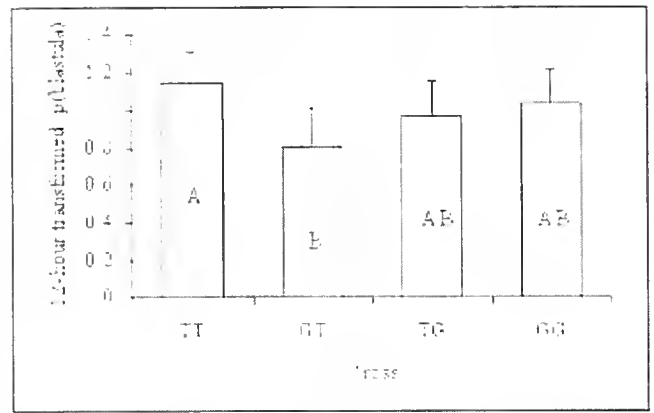
Figure 2. Early development of hybrid and pure species *Mytilus* larvae. Larvae of the TT cross developed significantly faster than those of the GT hybrid cross ($P = 0.007$). TT, *M. trossulus*; GT, *M. galloprovincialis* sperm × *M. trossulus* egg; TG, *M. trossulus* sperm × *M. galloprovincialis* egg; GG, *M. galloprovincialis*. Bars represent the mean transformed proportion of the larvae that were at or beyond the blastula stage after 12 h at 16°C at a salinity of 30 ppt.

growth in the low salinity treatment than in the high salinity treatment, while the GG cross did not. It had a slightly higher mean growth in the high salinity treatment than in the low salinity treatment.

Survival

Cross was a significant factor ($P = 0.011$) affecting day 3 survival. The TT cross's mean survival was significantly higher than those of the GT, TG, and GG cross's mean survival ($P = 0.042, 0.026$, and 0.020 respectively, Fig. 5). No significant differences existed in survival between crosses or salinities from day 3 to 7, or from day 3 to 14, though there was an interesting pattern in the means. Each hybrid cross survived most like its sire (TG cross survived better at low salinity, GT cross survived better at high salinity).
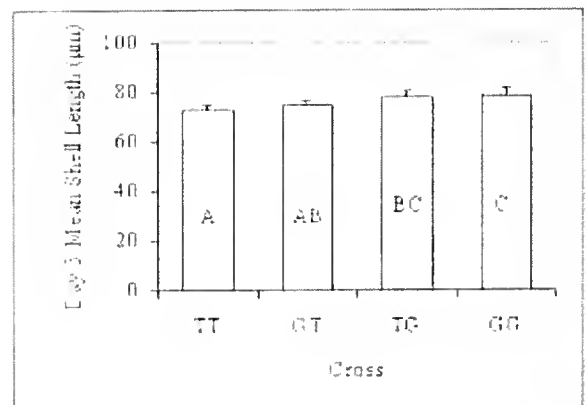
Figure 3. Shell length of hybrid and pure species *Mytilus* larvae at day 3. At day 3, the mean length of the GG cross was significantly greater than those GT and TT crosses ($P = 0.026$ and <0.001, respectively). TT, *M. trossulus*; GT, *M. galloprovincialis* sperm × *M. trossulus* egg; TG, *M. trossulus* sperm × *M. galloprovincialis* egg; GG, *M. galloprovincialis*. Cultures were maintained at 18°C in 30 ppt seawater.
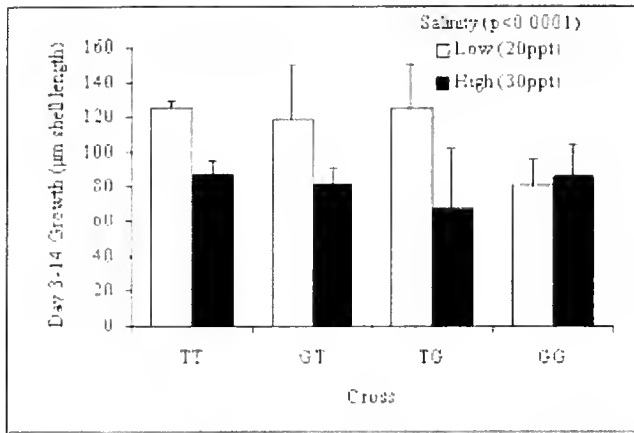
Figure 4. Change in shell length of hybrid and pure species *Mytilus* larvae from day 3 to day 14 at two salinities (20 ppt and 30 ppt). Salinity was a significant factor affecting growth of the larvae ($P <$ 0.001). Larvae of the TT, GT, and TG crosses grew more than at low salinity than at high salinity. Cultures were maintained at 18°C. TT, *M. trossulus*; GT, *M. galloprovincialis* sperm × *M. trossulus* egg; TG, *M. trossulus* sperm × *M. galloprovincialis* egg; GG, *M. galloprovincialis*.

Figure 5. Survival of hybrid and pure species *Mytilus* larvae at day 3. The mean survial of the TT cross was significantly higher than the mean survival of the GT, TG, and GG crosses (P = 0.042, 0.025, and 0.020, respectively). TT, *M. trossulus*; GT, *M. galloprovincialis* sperm × *M. trossulus* egg; TG, *M. trossulus* sperm × *M. galloprovincialis* egg; GG, *M. galloprovincialis*. Cultures were maintained at 18°C in 30 ppt seawater.

DISCUSSION

Barriers to Hybridization

Hybrid larvae were produced in both species–egg combinations and larvae were successfully reared through settlement. The successful fertilization, growth, and survival of these hybrids suggest that some factor other than genetic incompatibility is responsible for the rarity of these hybrids in Puget Sound. One such factor could be the limited overlap of the two species' spawning periods in Puget Sound. This would be an example of a partial temporal barrier to hybridization. Both *M. trossulus* and *M. galloprovincialis*, have one peak or mass-spawning time per year and one or more periods when a much smaller proportion of each species spawns (trickle-spawning). Mass spawning of *M. galloprovincialis* occurs in the late-winter through early spring in Totten Inlet (Dr. Jonathan Davis, personal communication). Brooks (1991) found ripe *M.*

galloprovincialis during November and December of 1988 though 1990 in Puget Sound. *M. trossulus* that were examined at the same time had gonads that were still in the resting stage with little gamete formation. *M. trossulus* typically mass-spawn in March or April in Holmes Harbor in Puget Sound (Johnson 1978). These observations support *M. galloprovincialis* being primarily a winter-spawner and *M. trossulus* being a primarily spring-spawner in Puget Sound, and thus also support the theory of a partial temporal barrier to hybridization. The comparatively low abundance of *M. galloprovincialis* in the region (Suchanek et al. 1996) may interact with or exacerbate the effects of a temporal barrier. There would likely be fewer opportunities for hybrids to be formed if the two species spawning times are different and if one of the species was present in much lower numbers than the other. *Mytilus galloprovincialis*, in this case, occurs in Puget Sound at much lower abundance than *M. trossulus* (Suchanek et al. 1996, Brooks 1991), probably due to *M. galloprovincialis*' preference for high, stable

TABLE 1.

Two-factor analysis of variable table for growth (change in shell length from day 3 to 14) of the four crosses of *Mytilus* pure-species and hybrid larvae at high and low salinities.

	Dependent Variable: LENGTH Difference in Microns Day 3 to 14							
	Type III Sum of Squares	df	Mean Square	*F*	Sig.	Eta Squared	Noncent. Parameter	Observed Power
Source corrected model	14626.461	7	2089.494	4.539	0.003	0.58	31.771	0.965
Intercept	284579.457	1	284579.5	618.1	0	0.964	618.145	1
Cross	1993.008	3	664.336	1.443	0.256	0.158	4.329	0.33
Salinity	8030.93	1	8030.93	17.44	0	0.431	17.444	0.979
Cross* salinity	3901.449	3	1300.483	2.825	0.061	0.269	8.474	0.6
Error	10588.661	23	460.377					
Total	315391.818	31						
Corrected total	25215.122	30						

TT, *M. trossulus*; GT, *M. galloprovincialis* sperm × *M. trossulus* egg; TG, *M. trossulus* sperm × *M. galloprovincialis* egg; GG, *M. galloprovincialis*. Salinity was a significant factor affecting growth of the larvae ($P <$ 0.001).
Computed using alpha = 0.05
R squared = 0.580 (Adjusted R squared = 0.452)

salinity bays and *M. trossulus'* preference for low temperature, variable salinity bays (Margus 1991, Sarver & Foltz 1993, Hilbish 1994, Geller 1994, Hoffman & Somero 1995, 1996).

There are many examples of barriers to hybridization among mollusk and echinoderm species, and some of these are temporal in nature. A temporal barrier to hybridization is believed responsible for the lack of natural hybridization of the sea stars *Leptasterias polaris* and *Asterias vulgaris* (Hamel & Mercier 1994). The two sympatric species were shown to hybridize readily in the laboratory, although they do not in the wild, due to their distinct breeding seasons. Another example of a temporal barrier to hybridization is found in Montastraea corals. Szmant (1997) found that the coral, *Montastraea faveolata* consistently spawned 1 to 1.5 h before *M. franksi* and *M. annularis* in experiments. These species have very specific spawning periods that are both seasonal and closely related to lunar cycles. Szmant discussed this as a potential temporal barrier to fertilization and hybridization in these species because there was no inherent prezygotic barrier to cross-fertilization among three species he studied.

Other examples of barriers to hybridization include genetic, ecological, geographic, and physiologic barriers. In the hard clam, *Mercenaria mercenaria* × *M. campechiensis* hybrids were shown to have an excessive susceptibility to gonadal neoplasia, relative to either parental species (Bert et al. 1993). This cellular disease acts as a barrier to hybridization by decreasing fitness of the hybrids relative to the parental species. Genetic barriers to hybridization have been shown in oyster species. Allen et al. (1993) attempted hybridization between *Crassostrea gigas* and *C. virginica*, as well as between *C. rivularis* and *C. virginica*. They found that larvae survived only 8 to 10 d and grew little; therefore, the hybrids were considered genetically inviable. Allen and Gaffney (1993) found that *C. gigas* and *C. rivularis* yielded viable hybrids when crossed in the laboratory. *Crassostrea gigas* and *C. sikamea* have also been hybridized, although they were only successful in one direction (Dr. Anja Robinson, pers. comm.).

An unusual physiologic barrier to hybridization is seen in the interaction of the urchins, *A. vulgaris* and *S. droebachiensis* gametes. Although shown to be physiologically able to hybridize with *L. polaris* (which has a different spawning period), the eggs of *A. vulgaris* were observed to disable heterospecific sperm from *S. droebachiensis* (believed to have an overlapping spawning period) within 12 sec (Hamel & Mercier 1994). It is thought that a very diffusible substance involved in the phenomenon is secreted only from mature eggs that appears to disable sperm in the direct vicinity of an egg.

Salinity, Growth, and Survival

A differential species response to salinity was observed in this study. From day three to 14, the TT, GT, and TG crosses all grew much faster at low salinity, and the GG cross grew slightly faster at high salinity (Fig. 3). These differences agree with the two species' physiologic and ecological characteristics (Kautsky 1987, Margus 1991, Hoffman & Somero 1995, 1996, Sarver & Foltz 1993, Geller et al. 1994, Johnson 1978, Brooks 1991), and are likely to be inherited genetic differences. *Mytilus trossulus* has been shown to be a low, variable salinity mussel and *M. galloprovincialis* has been shown to be a high, constant salinity mussel (Brenko et al. 1977, His 1989, Margus 1991, Sarver & Foltz 1993, Hilbish 1994, Hoffman & Somero

1996). These data suggest that the hybrids inherited the ability to grow well in low salinity water from their *M. trossulus* parent.

Significant cross-dependent differences in survival existed only during the first week, where the TT cross survived better than the GT, TG, and GG crosses (Fig. 5). There were no significant between-cross differences in larval survival after the first week. These data are in agreement with a previous experiment by Matson (2000). Hybrid larvae of both reciprocal crosses survived through settlement and as juveniles. No significant survival differences existed between crosses or salinities from day 3 to 7, or from day 3 to 14, though there was an interesting pattern in the means by day 14. Each hybrid cross survived most like its sire (TG and TT crosses survived better at low salinity, GT and GG crosses survived better at high salinity). It may be worth examining this more closely, perhaps with greater replication (more than 4x per cross at each salinity, more than $n = 32$ total) to see if salinity tolerance/preference may be paternally inherited.

Hybrid Vigor

Although significant cross-dependent differences were found in early growth, survival, and early development, most of them seem to have been due to factors other than the phenomenon of hybrid vigor. Hybrid vigor was defined here as an increase in fitness of the hybrids over either of the parental crosses, exhibited in either growth or survival. Hybrids were not consistently larger than parental crosses, nor did one reciprocal consistently grow faster than the other. These findings concur with a previous between-cross experiment (Matson 2000). The hybrid cross using *M. galloprovincialis* eggs generally grew larger than its reciprocal, which used *M. trossulus* eggs, during the first week. This may have been because of maternally dependent conditioning effects, or species-specific temperature effects that were also maternally dependent. Early cross-dependent larval growth was probably also influenced by maternal effects (Lannan et al. 1980). These maternal effects may have been the result of species- or population-dependent differences in egg nutrition (Bayne 1978), or differences in egg condition (Lannan et al. 1980), reflected by the different peak spawning times of each species in Puget Sound (Johnson 1978, Brooks 1991). When spawned, the *M. galloprovincialis* mussels were at the end of their spawning season, and the *M. trossulus* mussels were almost at their peak. This observation concurs with previous seasonal examinations of these two species gonadal condition (Johnson 1978, Brooks 1991).

These results are similar to those of Beaumont et al. 1993 (in terms of lack of hybrid vigor), who hybridized *M. galloprovincialis* with *M. edulis*. Beaumont et al. (1993) found that after initially higher mortality, veliger larvae of both reciprocal hybrid crosses grew as fast (Trial Three) or significantly faster than (Trial One) *M. galloprovincialis* larvae in one trial, but not the other. Hybrid crosses didn't grow consistently faster than parental crosses. Lubet (1984), who created hybrid *M. galloprovincialis* x *M. edulis* mussels and examined their juveniles and adults in the field, concluded that those two species are closely related, and exhibit minimal barriers to hybridization as well as minimal fitness differences between hybrids and parentals.

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RIBOSOMAL RNA CHARACTERIZATION OF NON-TRANSCRIBED SPACER AND TWO INTERNAL TRANSCRIBED SPACERS WITH 5.8S RIBOSOMAL RNA OF *PERKINSUS* SP. FOUND IN UNDULATED SURF CLAMS (*PAPHIA UNDULATA*) FROM THAILAND

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ABSTRACT The genetic divergence of *Perkinsus* sp. found in the undulated surf clam (*Paphia undulata*) from the Gulf of Thailand and other known *Perkinsus* species was examined using the non-transcribed spacer and two internal transcribed spacers with 5.8 S rRNA gene. The sequences of non-transcribed spacer (NTS) and internal transcribed spacer region (ITS) that includes the 5.8S rRNA gene flanked by ITS1 and ITS2 (ITS1-5.8S-ITS2) were cloned and sequenced. The sequences were compared with those of *Perkinsus olseni* from Australia, *P. atlanticus* from Korea, *P. marinus* and *P. andrewsi* from the United States and *P. qugwadi* from Canada. The lengths of the obtained nucleotide sequences of NTS, ITS-1 5.8S rRNA and ITS-2 were 1,167, 183, 159, and 371 bp, respectively. The nucleotide sequences of NTS and ITS-5.8S rRNA of Thai *Perkinsus* and *P. olseni* showed 98.69% and 99.85% identity, respectively. When compared with *P. atlanticus* identities were 96.27 and 99.71%, in *P. marinus* 75.38 and 94.88% and in *P. andrewsi* 46.55 and 86.23%. The nucleotide sequences of ITS-5.8S rRNA between Thai *Perkinsus* and *P. chesapeaki* showed an identity of 87.05%. This is the first report of the occurrence of *Perkinsus* sp in the Gulf of Thailand.

KEY WORDS: *Perkinsus* sp., *Paphia undulata*, nucleotide sequence, non-transcribed spacer, internal transcribed spacer1, internal transcribed spacer 2

INTRODUCTION

The pathogenic protozoans, *Perkinsus* spp. causes Perkinsosis disease in marine bivalves (Andrews 1988). According to Perkins (1976) and Levine (1978), they were classified as an apicomplexan. However, recent molecular phylogenetic analyses by Siddall et al. (2001) and Reece et al. (1997) have placed these parasites within the Dinoflagellata. Traditionally, diagnosis of *Perkinsus* infection depends on the fluid thioglycollate medium (FTM) assay for identification and Choi's 2 M NaOH digestion technique on FTM cultivated tissues for quantification (Choi et al. 1989, Almeida et al. 1999). However, the FTM assay does not discriminate different *Perkinsus* species and has a potential to introduce misleading positive results between *Perkinsus* and other dinoflagellate species (Almeida et al. 1999). A more precise application for detection, identification, and numeration of these parasites is based on molecular characterization. The internal transcribed spacers (ITS), 5.8 S regions of the ribosomal RNA (rRNA) and non-transcribed spacer gene (NTS) can be used to discriminate among the *Perkinsus* species because these regions are largely non-coding with high evolutionary rate, and have been used to identify *Perkinsus* species isolated from different hosts and geographical regions (Kotob et al. 1999, Robledo et al. 1999, Robledo et al. 2000). These NTS and ITS regions have also been used to distinguish between strains and species of other protozoa (Cai et al. 1992, Goggin 1994, Cunningham 1997). We have identified *Perkinsus* in the undulated surf clam, *Paphia undulata*, a major commercial species from the Gulf of Thailand using FTM assay. In this study, we have characterized the Thai *Perkinsus* ribosomal RNA, the nucleotide sequences of ITS-5.8S rRNA, and NTS and com-

pared the sequences with rRNA sequences that have been reported for other known *Perkinsus* species.

MATERIALS AND METHODS

Isolation of Prezoosporangia

Live specimens of the undulated surf clam (*Paphia undulata*) were obtained from food markets in Chonburi Province, Thailand. The infected gills of clams were cultured in fluid thioglycollate medium supplemented with streptomycin (500 µg/ml) and penicillin G potassium (500 unit/ml) at 27°C in the dark for 3 days. The tissue was then digested by trypsin (0.25% in sterilized seawater) at room temperature for 3–4 h, and the obtained prezoosporangia were then isolated by filtration through a silk net. The resulting pellets were finally washed 3 times using sterilized seawater and at each washing the pellets were centrifuged at ×490g for 8 min.

DNA Isolation

Genomic DNA was extracted from prezoosporangia using a DNA trap kit, according to details provided by the manufacturer (Tissue Protocols for DNA isolation, DNA TEC., Thailand).

PCR Amplification

The complete region of ITS1-5.8S-ITS2 and NTS genes were amplified from genomic DNA using a forward primer of the small subunit (SSU) 5'AGGAAGGAGAAGTCGTAACAAGG 3' (Hamaguchi et al. 1998) and a reverse primer of the large subunit (LSU) 5'ACCCGCTGAATTTAAGCATA 3' (Goggin 1994). The NTS region was amplified by using a forward primer 5' AAGTCCTTAGGGTGCTGCTGGCT 3' and reverse primer 5' CTACTGGCAGGATCAACCAGGT 3' (Park et al. 2002). The polymerase

chain reactions were performed in a final volume of 20 μL reaction mixture containing 2 μL of 10× reaction buffer (50 mM KCL; 10 mM Tris-HCL, pH 8.3; 1.5 mM MgCl$_2$). 2 μL of 1 mM deoxyribonucleoside-5'-triphosphate (dNTPs)(Promega Corp., Madison, WI), 1 μl of 5 μM upstream primer, and 1 μL of 5 μM downstream primer, 0.8 μL of 5 units Taq DNA polymerase (Promega Corp.) and 4 μL of 5 ng/μl of DNA template. The final volume was adjusted with sterilized distilled water to 20 μL. The mixture was amplified in a PCR Thermal Cycler (GeneAmp®PCR system9700, Albertville, MN) for 35 cycles with initial denaturation at 94°C for 3 min. The process was followed by 35 cycles of denaturation at 94°C for 30 sec and annealing at 55°C for 30 sec. The primer extension was performed at 72°C for 1 min followed by a final extension at 72°C for 5 min. The PCR products were analyzed utilizing 1% agarose gel electrophoresis and visualized for DNA bands under UV light and photographed using digital photography (Delidow et al. 1993, Hamaguchi et al. 1998, Carnegie et al. 2000).

DNA Cloning and Sequencing

The PCR products of the ITS1-5.8S rRNA- ITS2 and NTS fragments were excised from the agarose gel and purified by a cleaning reagent consisting of exonuclease 1 (Exo 1) and shrimp alkaline phosphatase (SAP). The NTS and ITS-5.8S fragments were cloned into pGEM®-T Easy vector (Promega Corp.) and isolated. At least one clone of each NTS and ITS-5.8S fragment was sequenced following standard procedures in an automatic DNA sequencer, ABI PRISM model 377 (Dicker et al. 1993, Hamaguchi et al. 1998). The nucleotide sequences of NTS were analyzed for nucleotide similarities with *P. olseni* (GenBank accession number AF466527), *P. atlanticus* (AF438150), *P. marinus* (AF497479), *P. andrewsi* (AF102171). The nucleotide sequences of ITS1-5.8S-ITS2 were analyzed for nucleotide similarities with *P. marinus* (AF497479), *P. andrewsi* (AF102171), *P. atlanticus* (AF473840), *P. olseni* (U07701), *P. qugwadi* (AF151528), and *P. chesapeaki* (AF091541) by BLAST and CLUSTALW programs provided by GenBank and the European Bioinformatics Institute.

RESULTS

From this study, the sequences of the NTS, ITS-1, ITS-2, and 5.8S rRNA fragments PCR amplified from prezoosporangias of Thai *Perkinsus* found in *P. undulata* were 1167,183, 371, and 159 bp in length, respectively. These sequences were submitted to GenBank and given an accession number (AF522321). The nucle-

otide sequences of NTS from Thai *Perkinsus* were compared with the completed sequences of *P. olseni* isolate P 01 (Murrell et al. unpublished data), *P. atlanticus* (Park et al. 2002), *P. marinus* isolate TXsc (Robledo et al. 1999), and *P. andrewsi* (Coss et al. 2001). The sequence similarity between the NTS region of Thai *Perkinsus* and *P. olseni, P. atlanticus, P. marinus,* and *P. andrewsi* were 98.69%, 96.27%, 75.38%, and 46.55%, respectively (Table 1).

To determine the ITS with 5.8S rRNA sequence similarities of the Thai *Perkinsus*, we compared the complete sequences of this species with completed sequences of *P. marinus* isolate TXsc (Robledo et al. 1999), *P. andrewsi* (Coss et al. 2001), *P. atlanticus* (Park et al., in press), *P. olseni* (Goggin 1994), *P. qugwadi* (Hervio et al., unpubl. data), and *P. chesapeaki* (Kotob et al. 1999). The ITS-5.8S rRNA sequences of Thai *Perkinsus* was 94.88% similar to *P. marinus* isolate TXsc, 88.34% similar to *P. andrewsi*, 99.71% similar to *P. atlanticus*, 99.85% similar to *P. olseni*, 68.02% similar to *P. qugwadi*, and 87.05% similar to *P. chesapeaki* (Table 1). The sequence of 5.8S rRNA of Thai *Perkinsus* showed 100% similarity to *P. olseni, P. atlanticus,* and *P. marinus*.

DISCUSSION

Several species of *Perkinsus* have been reported from different locations in the world including Australia (Goggin 1994), China (Liang et al. 2001), Japan (Blackbourn et al. 1998, Hamaguchi et al. 1998, Choi et al. 2002), Korea (Park & Choi 2001), New Zealand (Goggin 1994), Portugal (Azevedo 1989) and USA (Mackin et al. 1950). There has been no report of *Perkinsus* sp. in any species of shellfish in Thailand and no species of shellfish in Thailand has been reported to exhibit symptoms of Perkinsosis diseases. However, additional research in this area may reveal otherwise.

In our study, we targeted and analyzed the NTS, ITS-1, ITS-2, and 5.8S rRNA genes for species-specificity of Thai *Perkinsus* found in *P. undulata*. The results showed that the NTS region of Thai *Perkinsus* is slightly different from that of *P. olseni* (1.31%) and *P. atlanticus* (3.73%) but highly different to *P. marinus* (24.62%) and *P. andrewsi* (53.45%). As proposed by Coss et al. (2001), this implies that the NTS region of *P. andrewsi* is dramatically different in both length and sequence from those of *P. marinus* and *P. atlanticus*. Robledo et al. (1999) concluded that the NTS region can accumulate a high degree of sequence variability between closely related species. The sequence of 5.8S rRNA of Thai *Perkinsus* showed 100% similarity to *P. olseni, P. atlanticus,*

TABLE I.

The length and sequence similarity (%) of non-transcribed spacer, internal transcribed spacer1, 5.8S ribosomal RNA and internal transcribed spacer2 of Thai and other *Perkinsus* species.

Organism	Length (bp)	NTS (% similarity)	Length (bp)	ITS-1 (% similarity)	Length (bp)	5.8S rRNA (% similarity)	Length (bp)	ITS-2 (% similarity)	Accession No.
Thai *Perkinsus*	1,167	100	183	100	159	100	371	100	AF522321
P. marinus	1,158	75.38	197	85.27	161	100	372	93.27	AF497479
P. andrewsi	1,551	46.55	185	79.45	159	98.74	368	82.60	AF102171
P. atlanticus	—	—	183	99.45	159	100	371	99.73	AF473840
P. atlanticus	1,146	96.27	—	—	—	—	—	—	AF438150
P. olseni	—	—	183	99.45	159	100	371	100	U07701
P. olseni	1,153	98.69	—	—	—	—	—	—	AF466527
P. qugwadi	—	—	204	47.05	158	93.63	363	63.08	AF102171
P. chesapeaki	—	—	188	87.76	159	96.22	379	82.45	AF091541

and *P. marinus*. As reported by Goggin (1994), the 5.8S rRNA sequence regions from *P. olseni*, *P. atlanticus*, *P. marinus*, and unidentified *Perkinsus* (from *A. trapezia* and *C. pacificus*) were all identical. However, the 5.8S rRNA sequence of Thai *Perkinsus* differs at 2 positions when compared with *P. andrewsi* and 10 positions when compared with *P. qugwadi*. Coss et al. (2001) reported that 5.8S rRNA of *P. andrewsi* differed from 5 isolates of *Perkinsus* spp. reported by Goggin (1994) in 2 positions but differed from *P. qugwadi* in 14 positions. Murrell et al. (2002) recently updated the phylogenetic position of the genus *Perkinsus* and considers *P. olseni* and *P. atlanticus* to be synonyms.

Our results show high levels of sequence homology in ITS-5.8S rRNA region among Thai *Perkinsus*, *P. olseni*, and *P. atlanticus*. The nucleotide sequences of ITS-5.8S rRNA in Thai *Perkinsus* were highly similar to *P. olseni* (99.85%) and *P. atlanticus* (99.71%). In this region, Thai *Perkinsus* differs from *P. olseni* at 1 position in ITS-1 but differs from *P. atlanticus* at 2 positions in both ITS-1 and ITS-2. Goggin (1994) found that *P. olseni* from Australia and *P. atlanticus* from Portugal had an identical sequence for ITS1 but differed in ITS-2 at 3 positions by substitution of one nucleotide and he suggested that these two species belong to a single species. From our study the Thai *Perkinsus* is most closely related to *P. olseni* and *P. atlanticus*. At the same time, the Thai *Perkinsus* showed genetic divergence at the ITS-5.8S rRNA region from *P. marinus*, *P. andrewsi*, and *P. qugwadi*. The nucleotide sequence of Thai *Perkinsus* ITS-5.8S rRNA showed 94.88% homology to *P. marinus*, 86.23% homology to *P. andrewsi*, and 68.02% homology to *P. qugwadi*. At this fragment, the sequences of Thai *Perkinsus* versus those of *P. marinus* and Thai *Perkinsus* versus *P. andrewsi* were more different in ITS-1 (14.73% and 20.55%) than ITS-2 (6.73% and 17.40%). Goggin (1994) also reported that the sequences of ITS-1 and ITS-2 of *P. marinus* from

American oysters differed significantly from *P. olseni*, *P. atlanticus*, and an unidentified *Perkinsus* from *Anadara trapezia* and *Chama pacificus*. Furthermore, he found that the variation among 4 isolates of *Perkinsus* and *P. marinus* was greater in the ITS-1 (23%) than the ITS-2 (7-8%) region. Goggin (1994) concluded that 12% differences of nucleotide deletions were most common in the ITS-1. Our study shows that the sequences of ITS-1 and ITS-2 in Thai *Perkinsus* and *P. qugwadi* were substantially different. Coss et al. (2001) also found genetic divergence from ITS-1 and ITS-2 regions, between *P. andrewsi* and *P. qugwadi* and suggested that *P. qugwadi* is not closely related to the other *Perkinsus* species.

In conclusion, molecular evidence of the ribosomal RNA from *Perkinsus* found in *Paphia undulata* from the Gulf of Thailand shows that it is distinctly different from *P. marinus*, *P. andrewsi*, and *P. qugwadi*. Although homology of NTS, ITS-1, 5.8S rRNA, and ITS-2 sequence in Thai *Perkinsus* with *P. olseni* and *P. atlanticus* are high, the level of homology required to discriminate between species of *Perkinsus* have not been determined (Goggin 1994). Therefore, we do not specify a species-specific name for *Perkinsus* sp. found in *Paphia undulata* from the Gulf of Thailand at this point in time.

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A STUDY OF GONADAL DEVELOPMENT IN *RUDITAPES DECUSSATUS* (L.) (MOLLUSCA, BIVALVIA), USING IMAGE ANALYSIS TECHNIQUES: INFLUENCE OF FOOD RATION AND ENERGY BALANCE

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ABSTRACT   This study evaluated the influence of food availability on sexual maturation in *Ruditapes decussatus* (L.) in conditions of positive (daily rations of 0.10, 0.24, 0.42, and 0.96%), zero (0.05% ration), and negative energy balance (0.025% ration). The percentages correspond to the organic weight of the phytoplankton supplied as a proportion of the live weight of the clams. The gonadal occupation index (GOI) and the percentage of ripe oocytes in the gonad, calculated using image analysis techniques, were taken as indicators of the degree of sexual maturity. Gonadal development in *R. decussatus* occurred under all food rations and energy balance conditions, even when the organic weight of the clams decreased during the period of sexual development. All conditions registered a gradual increase in GOI and the percentage of ripe oocytes throughout the experimental period. Maximum values for GOI varied between 30% and 40% in females and between 55% and 75% in males, according to the amount of food available. Similarly, mature sexual cells were observed under all experimental conditions, with maximum percentages in females of between 30% and 40%. The extent of gonadal development is directly related to the amount of food available, which in turn has a direct bearing on the rate of gonadal development, with smaller rations leading to a lower rate of increase in the gonadal occupation index and the percentage of ripe oocytes.

KEY WORDS: food availability, gonadal development, image analysis, *Ruditapes decussatus*

INTRODUCTION

The majority of studies of the reproductive cycle of *R. decussatus* in its natural habitat (Pérez-Camacho 1980, Beninger 1982, Shaffee & Daouidi 1993, Villalba et al. 1993), are based either on indirect indicators of gonadal development (condition index, gonadosomatic index, flesh weight), the discharge of gametes, smear techniques (Berthou et al. 1980), or histologic studies of the gonad that describe the various stages of gametogenesis (Holland & Chew 1974).

A more objective determination of the degree of maturity is provided by methods that measure the area occupied by sexual cells and the frequency distribution of oocyte sizes. As a result, it has been possible to produce more accurate inter- and intraspecies comparative analyses of several bivalve species (Navarro et al. 1989, Xie & Burnell 1994, Laruelle et al. 1994, Rodríguez-Moscoso & Arnaíz 1998). However, the data on bivalve reproductive histology provided by image analysis are more accurate and precise than that obtained by the traditional stereological method in which an ocular graticule is used (Lowe et al. 1982).

Temperature is one of the main factors influencing the gametogenic cycle in bivalves (Sastry 1975, Mann 1979). It would appear to define both the starting point and the rate of gonadal development, whereas diet appears to have a direct effect on the duration of gametogenesis (Lubet 1980–1981). The above-mentioned studies, however, tend to support the involvement of several environmental parameters on sexual activity in bivalves. The reproductive phenomenon is studied in the natural habitat, making it difficult to separate the particular effect of one factor from those of the others. In fact, there are very few studies of the individual influence of each environmental variable on the reproductive process under controlled conditions (Sastry 1966, Gimazane 1972, Bayne et al. 1975, 1978, Pipe 1985). The use of image-analysis techniques to determine the effects of a single environmental variable, in this case food availability, on gonadal development in *R. decussatus* is the main aim of this study.

MATERIALS AND METHODS

Breeding Stock

The experiments were performed in two years running, using clams of two sizes. In the first experiment specimens of *R. decussatus* with a length of 20.8 ± 0.15 mm (mean plus standard deviation) and a live weight of 1.60 ± 0.31 g were used. In the second experiment, average clam length was 36 ± 0.19 mm and live weight 9.97 ± 1.53 g.

Experimental Design and Conditions

The experiments were performed in a flow-through system containing seawater filtered through a μm cartridge and maintained at a constant temperature (18°C) and salinity (33‰). As a consequence of the large number of individuals in each experiments (400 and 420) and long duration of the surveys (46 and 70 days), clams were maintained within large groups, in plastic tanks of 12 l. In this way, food concentration is more stable and equal for all clams at each experimental conditions are closer to natural ones. Food consisting in different rations of the microalga Isochrysis galbana was added to the circulating water on a continuous basis by means of a variable flow peristaltic pump. The different rations were obtained by maintaining food concentration constant and varying both the flow of water into the tanks and the number of clams per tank. Through-flow in the vessels was reduced after each sampling, to adjust it to the number of clams remaining.

Experiment 1

The following daily food rations, with percentages corresponding to the organic weight (ask free dray weight) of food supplied as a proportion of the live weight of the clams, were assayed in this experiment: 0.24% (A1), 0.48% (A2), and 0.96% (A3).

The initial number of specimens was 140 for ration, and the number of clams for tank 140, 70, and 35 for the rations A1, A2, and A3 respectively. The experimental period lasted 46 days, with samples being taken on days 12, 26, 35, and 46. On each occasion 10 specimens from each diet were used to determine soft tissue dry

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weight, with a further 10 specimens used for histologic studies. Where necessary, the number of specimens per sample was increased to obtain a minimum of four specimens of each sex.

Experiment 2

The rations used in this experiment were 0.025% (B1), 0.05% (B2), and 0.10% (B3). The initial number of specimens was 200 for ration B1, and 100 for rations B2 and B3, and the number of clams for tank 200, 100, and 50, for the rations B1, B2, and B3, respectively. The experimental period lasted 70 days and samples were taken on days 25, 41, and 70, with 10 specimens being used to determine soft tissue dry weight and a further 10 specimens used for histologic studies. Where necessary, the number of specimens per sample was increased to obtain a minimum of four specimens of each sex.

Soft Tissue Growth: Total, Somatic and Gonadal

The anatomic features of the gonad in this species make it difficult to separate from the rest of the organism, so indirect methods are usually used to determine the changes that take place (Pérez Camacho 1979). In our case, total clam flesh growth (FG) corresponds to the difference between initial and final dry weight (DW). DW was obtained by freeze-drying the total amount of soft tissue.

When there was an increase in weight during the experimental period, gonadal growth (GG) was calculated from the difference between the DW of the initial sample (when gonadal development was nil, or very little) and that of the final sample (when the gonad was well developed). To discount any growth of the organism during the experimental period, initial DW was calculated for a standard clam of the same length as the mean length of the final sample, using the length–DW equation of the initial sample. Somatic growth (SG) was taken as the difference between the increase in total DW and gonadal growth (FG–GG).

Histology and Image Analysis

A conventional histology protocol was followed. The soft tissues were fixed with Bouin's fixative, sealed in paraffin, and 4-μm slices were taken. Harris' hematoxylin and eosin stain was used (Bancroft and Stevens 1996). For each specimen, nine fields of vision of the gonad were chosen at random, corresponding to three different depths in the body of the clam. MicroImage software (Olympus) was used to process and analyses the images obtained.

Females

Because sexual maturation in venerids is characterized by an increase in size of the gonadal follicles and their progressive occupation by ripe gametes, which then separate from the follicle walls, it was decided to focus on the area of the gonad occupied by oocytes. The area of each of the oocytes visualized was obtained automatically (MicroImage software). On average, measurements of more than 500 oocytes were obtained for each specimen.

The gonadal occupation index was defined as follows:

GOI: (area occupied by gametes/area of the field analyzed) × 100.

Gametogenic development in females is also characterized by a considerable increase in oocyte size, and maximum diameters were therefore measured. Oocytes were considered to be in the final

stage of vitellogenesis, or ripe, when their maximum diameter exceeded 50 μm (Vilela 1950).

Males

Colorimetrics was used to analyze images of the male clams, with each different part of the soft tissue being color-coded. This division of soft tissue corresponded to gametes (deep purple stain), muscle tissue and reserves (deep and pale pink stain), and empty zones (white). The area occupied by each color in the image being studied was measured, and the previously mentioned expression (GOI) was calculated, the area occupied by gametes corresponding to that occupied by spermatozoids, spermatids, spermatocytes and spermatogonia. Each specimen was assigned a mean value for GOI and a percentage of ripe oocytes present in the gonad, obtained from the nine images analyzed in each case.

Statistical Methods

Comparisons between the different rations for flesh dry weight, conditioning index, gonadal occupation index and oocyte diameter were established by analysis of variance (ANOVA) for a significance level of 95%, and by analysis of covariance (ANCOVA) to compare slopes of the regression lines of those equations having the greatest determination coefficient. Cochran's test was used to guarantee the homogeneity of the variances. When there was a direct relationship between the mean and the standard deviation, logarithmic transformation was used to homogenize the variances. Parameters expressed as percentages were modified, prior analysis using angular transformation (arcsine$\sqrt{\%}$). Multiple comparisons between experimental conditions were performed with the multiple rank test using the least significant difference (LSD) method. All the statistical analyses were performed with Statgraphics plus 3.0 software, according to the methods described by Snedecor and Cochran (1980) and Zar (1974).

RESULTS

Total, Somatic, and Gonadal Growth

The clams in experiment 1 were fed daily rations of 0.24, 0.42, and 0.96%. All three diets produced a positive energy balance, leading to a considerable increase in flesh dry weight (DW) that was directly proportional to the amount of food available (Fig. 1a). The total increase in DW, expressed as a percentage of initial DW, was 35.8% for ration A1, 48.9% for A2, and 80.4% for A3. The differences between the increases in DW recorded for each of these diets were statistically significant (ANOVA, $P < 0.001$; multiple rank test (LSD), $P < 0.05$).

In experiment 2, diet B3 (0.10%) produced a positive energy balance leading to an increase of 18.6% in DW over the initial value. For diet B2 (0.05%) DW stayed approximately constant during the experimental period, indicating a zero energy balance, as corresponds to a maintenance diet. Diet B1 (0.025%) led to a negative energy balance and a loss of 20% DW by the end of the experimental period (Fig. 1b). The differences between the variations in DW of clams fed with these diets were statistically significant (ANOVA, $P < 0.05$; multiple rank test (LSD), $P < 0.05$).

Most of the energy acquired by the clams in positive energy balance conditions in our experiments was expended on gonadal development. Accordingly, as can be seen in Figures 1a and 1b, gonadal growth accounts for 90% of the total increase in DW for the highest diets (experiment 1), and 98% for diet B3 in experi-

a

b

□ GG ▣ SG □ FG
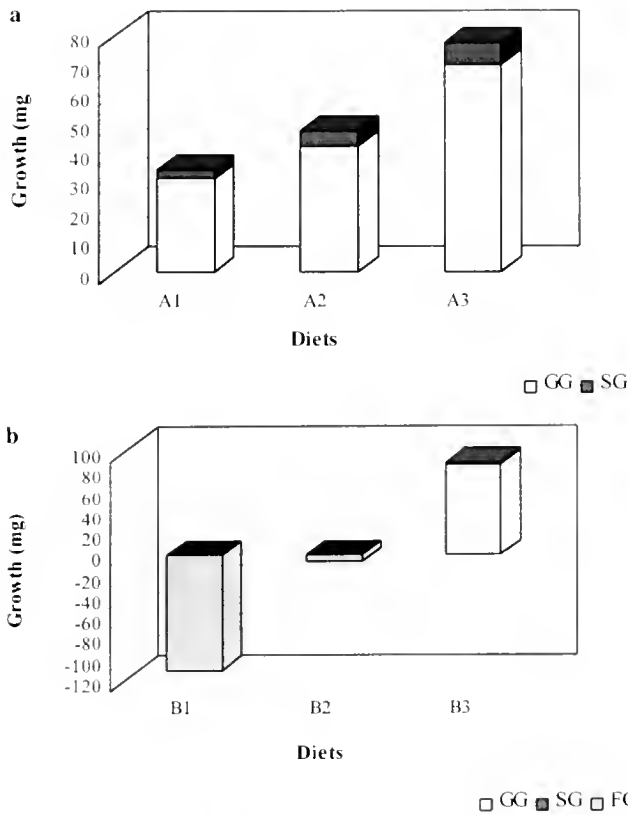
Figure 1. Total flesh growth (FG), somatic growth (SG), and gonadal growth (GG) during the experimental period. (a) Experiment 1 (46 days): diets A1 (0.24%), A2 (0.48%), and A3 (0.96%). (b) Experiment 2 (70 days): diets B1 (0.025%), B2 (0.50%), and B3 (0.10%).

ment 2. Gonadal development in diets B1 and B2 occurred at the expense of previously stored reserves, and cannot therefore be quantified by the same method.

GOI

GOI increased throughout the experimental period in both males and females for all diets. Although there was clear evidence of gonadal development in all cases, there were obvious differences, attributable to the different rations. Statistical comparisons were based only on data from samples taken up to days 26 (experiment 1) and 41 (experiment 2). Partial spawning observed in the experimental tanks after these dates would have affected the interpretation of the data corresponding to later samples.

Experiment 1

Females

The two highest rations in experiment 1 (A3 and A2) both produced a rapid increase in the GOI to approximately 35% by day 12, after which it remained constant (Fig. 2a). The rate of increase for ration A1 was slower, and although maximum GOI was similar to those for diets A2 and A3 (Fig. 2a) this did not occur until day 35.

GOI was related to time by means of a potential equation (Table 1). A comparison of the slopes of these equations after applying logarithmic transformation reveals statistically significant differences between the lowest ration (A1) and the two high-

a

Females

○ A3   × A2   △ A1

b

Males

○ A3   × A2   △ A1
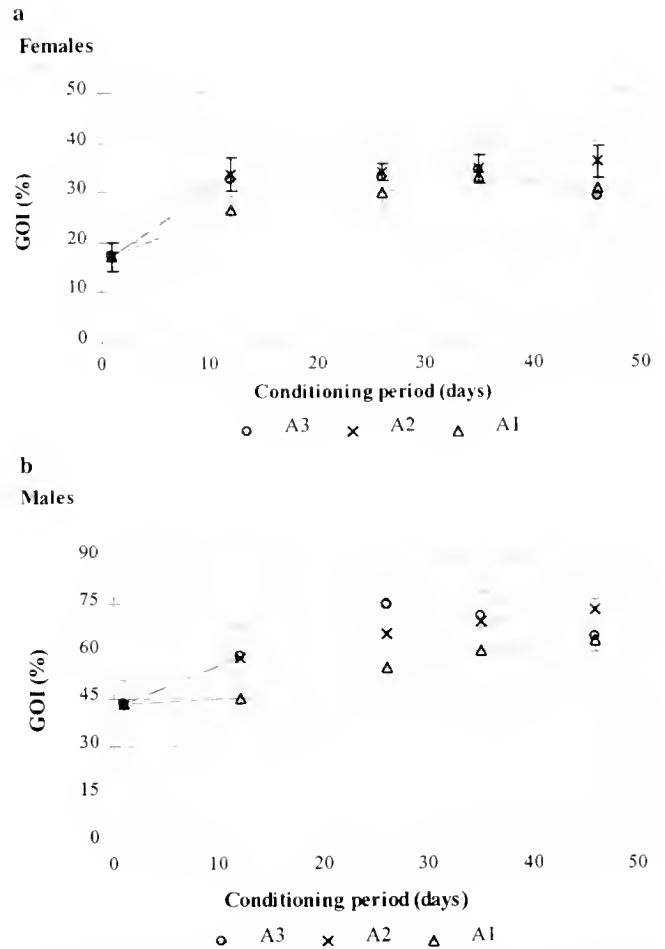
Figure 2. Gonadal occupation index (GOI) during experiment 1 with diets A1 (0.24%), A2 (0.48%), and A3 (0.96%). (a) Females. (b) Males. Average data (± SD).

est (A2 and A3). No significant differences were observed between the latter two rations ($P > 0.05$). The amount of food available did not lead to any significant difference in the maximum GOI for any of these diets (ANOVA, $P > 0.05$).

Males

The GOI was much higher in males than in females. Maximum values of between 60 and 75% were obtained, according to the amount of food available. This factor, together with the energy balance, has a more noticeable effect on variations in the male GOI: there is a constant increase throughout the experimental period, with the highest diets showing the greatest rate of increase (Fig. 2b). There was a marked decrease in the GOI of clams fed on ration A3 after day 26, once maximum GOI (75%) had been reached. This coincided with the partial spawning observed in the experimental tanks.

The best fit between GOI and time (Fig. 2b) is given by a linear equation ($y = a + bx$). Comparison of pairs of regression lines (Table 1) shows significant differences between the slopes of these equations ($P < 0.05$). The ANOVA performed between the maximum values of the GOI for each ration shows significant differences ($P < 0.05$) between the lowest diet (A1) and the two highest (A2 and A3). No statistically significant differences were observed between the latter two rations.

TABLE 1.

Parameters of the regression lines between the gonadal occupation index (%, y) and time (days, x).

	Diets	a	b	r^2	P	n	Comparison of Slopes	P
Females	A1 (1)	17.18	0.57	0.74	0.0040	12	A1–A2	0.0100
	A2 (1)	18.65	0.65	0.75	0.0001	13	A1–A3	NS
	A3 (1)	18.51	0.59	0.69	0.0005	13	A2–A3	0.0200
Males	A1 (2)	33.61	6.64	0.63	0.0036	11	A1–A2	0.0040
	A2 (2)	33.10	11.15	0.70	0.0007	12	A1–A3	0.0020
	A3 (2)	27.87	15.55	0.88	0.0000	11	A2–A3	0.1040
Females	B1 (3)	6.45	18.63	0.85	0.0001	10	B1–B2	0.0010
	B2 (3)	6.78	26.26	0.91	0.0000	13	B1–B3	0.0001
	B3 (3)	5.69	31.59	0.97	0.0000	9	B2–B3	0.0500
Males	B1 (1)	8.41	14.32	0.78	0.0001	13	B1–B2	NS
	B2 (1)	9.38	13.25	0.80	0.0004	10	B1–B3	NS
	B3 (1)	13.24	13.35	0.66	0.0001	16	B2–B3	NS

(1) Potential model ($y = a.x^b$); (2) Linear model ($y = a + bx$); (3) Logarithmic model ($y = a + b\ln x$). NS, not significant; n, number of observations.

Experiment 2

Females

Maximum GOI (39.81%) was reached after 41 days (Fig. 3a) for those clams fed on a ration that produced a positive energy balance (B3). Clams fed on ration B2, the maintenance ration, did not reach the same GOI until the end of the experimental period (day 71). Diet B1, with a clearly negative energy balance, gave both a slower rate of increase in the GOI and a lower maximum value (35.5%). The differences in maximum GOI values between rations were not, however, statistically significant (ANOVA, $P > 0.05$).

Although maximum gonadal occupation is similar for all the diets in this experiment, the rate of gonadal development is determined by the amount of food available, and a comparison of the GOI time regression slopes (Table 2) shows statistically significant differences (ANCOVA, $P < 0.05$).

Males

Variations in the GOI of males in experiment 2 were similar for all rations, with maximum values of around 60% (Fig. 3b). Comparisons between the slopes of pairs of regression lines (Table 2) show no significant differences between any of them (ANOVA, $P > 0.05$), and neither were there any significant differences between the maximum values obtained for each ration (ANOVA, $P > 0.05$).

Percentage of Ripe Oocytes

Experiment 1

The percentage of ripe oocytes (i.e., with diameters of over 50 μm) in the clams in experiment 1 increased rapidly during the first two weeks of the experimental period to approximately 25%. This rate of increase then diminished, and by day 26 average values of 26.3, 32.3, and 34.8% were recorded for rations A1, A2, and A3, respectively. After this date partial spawning was observed in the tanks containing clams fed on the two highest rations (A2 and A3), this being reflected in a decrease in the percentage of ripe oocytes, followed by a subsequent recovery (Fig. 4a).

Although the maximum percentage of ripe oocytes is similar for all three rations at close to 40% (ANOVA, $P > 0.05$), the rate of increase of this percentage is directly related to the amount of food available, and the increase of the slopes of the regression lines between the percentage of ripe oocytes and time coincides with an increase in food (Table 2). The corresponding ANCOVA shows significant differences between the slopes of rations A1 and A3, at a 95% confidence level. No statistically significant differences
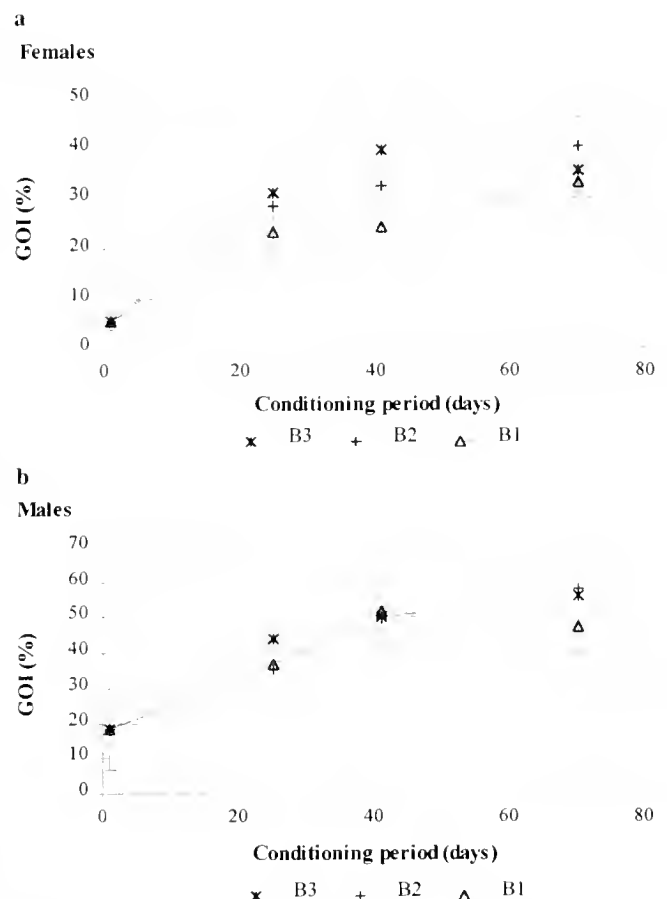
Figure 3. Evolution of the gonadal occupation index (GOI) during experiment 2 with diets B1 (0.025%), B2 (0.05%), and B3 (0.10%). (a) Females. (b) Males. Average data (±D).

TABLE 2.

Parameters of the regression lines between the proportion of ripe oocytes (%, y) and time (days, x).

Diets	a	b	r^2	P	n
A1	9.92	10.29	0.71	0.0003	13
A2	10.39	12.96	0.70	0.0004	13
A3	8.72	13.6	0.76	0.0000	15
B1	−7.41	7.47	0.73	0.0320	10
B2	−11.66	12.87	0.87	0.0000	13
B3	−11.33	12.74	0.92	0.0001	9

Linear model (y = a + bx). *P*, probability level; *n*, number of observations.

were observed between the slopes of rations A2 and A3, or A1 and A2.

Experiment 2

If the amount of available food is lower, as in experiment 2, where daily rations of 0.025, 0.050, and 0.10% were used (rations B1, B2, and B3, respectively), the percentage of ripe oocytes in the gonad increases at a lower rate than for the higher diets in experiment 1 (daily rations of between 0.26% and 0.96%). Under these conditions, after 25 days the average percentages of oocytes with a diameter greater than 50 μm were 12.8, 14.6, and 23.5%, for rations B1, B2, and B3, respectively, and 70 days were needed to reach values similar to those of the third sample (day 25) in ex-
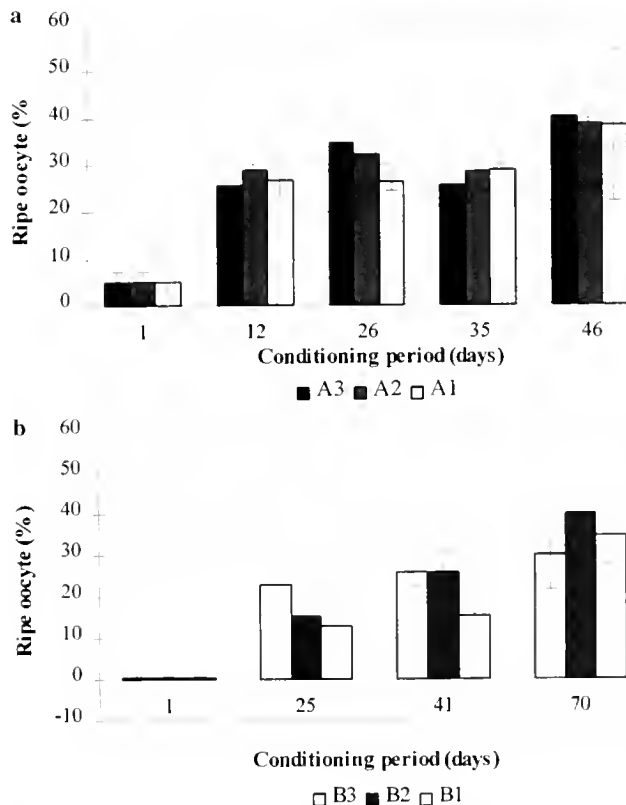
Figure 4. Percentage of ripe oocytes during the experimental period. (a) Experiment 1: diets A1 (0.24%), A2 (0.48%), and A3 (0.96%). (b) Experiment 2: B1 (0.025%), B2 (0.05%), and B3 (0.10%). Average data (±SD).

periment 1 (Fig. 4b). The lower percentages recorded at this point for clams fed with ration B3 coincide with partial spawning observed in the experimental tanks.

As in experiment 1, the increase in the percentage of ripe oocytes was directly related to the amount of food available, although the differences between maximum percentages of ripe oocytes for each ration were not statistically significant (ANOVA, $P > 0.05$). Accordingly, comparison of the slopes of the regression lines between the percentage of ripe oocytes and time (Table 2) shows statistically significant differences (ANCOVA, $P < 0.05$) between the lowest ration (B1: 0.025%) and the two highest (B2: 0.05%, B3: 0.10%). No statistically significant differences were observed between the latter two rations.

DISCUSSION

Image analysis has been used by several authors to compare the reproductive cycles of different species, using methods based on the frequency of different sizes of oocyte or the proportion of gonadal tissue occupied by oocytes. For example, Laruelle et al. (1994) and Xie and Burnell (1994) detected differences in the extent and intensity of reproductive activity in species, such as *R. decussatus* and *Ruditapes philippinarum* (Adams and Reeve). The parameters used in the present study, i.e., the percentage of ripe oocytes and the gonadal occupation index, would also seem to be good indicators of the degree of gonadal maturity in *R. decussatus*, although they give no indication of the total amount of gonadal tissue.

Navarro et al. (1989) associated interannual differences in the reproductive cycle of *Cerastoderma edule* (L.) with fluctuations in the nutrient storage cycle caused by variations in food availability. The amount of food available in the environment is a determining factor of the amount of energy incorporated by the animal, and must therefore affect processes such as somatic and reproductive growth, as our experiments clearly show.

Accordingly, when the daily amount of available food (expressed as a percentage of clam live weight) is equal or greater than 0.10%, as in rations B3, A1, A2, and A3, a positive energy balance ensues. In these situations, there is a corresponding increase in the amount of clam soft tissue, which under the temperature conditions prevailing in our experiments, corresponds principally to an increase in reproductive tissue.

Ration B2 produces a zero energy balance, in which energy acquisition and expenditure by the organism were equal. When there is a negative balance, as in the case of ration B1, the energy obtained from food is insufficient to meet the energy demands of the organism, resulting in a considerable loss of body weight. Gonadal development took place in both situations, possibly as a result of the high temperature at which the experiments were performed, but in this case at the expense of previously stored reserves.

Our results show that the amount of available food influences both the extent of gonadal development and the rate of gonadal maturation, with the higher rations producing a faster rate. Similarly, Buchanan et al. (1998) detected differences in the gametogenic development and the conditioning index of *Crassostrea virgínica* (Gmelin), which he associated with nutritional and temperature differences between laboratory conditions and the natural medium that produce a faster rate of gonadal development in specimens conditioned at a higher temperature and optimal nutritional conditions.

There are noticeable differences in the GOI of males and females, with maximum values ranging from 55–75% for the former and 35 and 40% for the latter (Figs. 2 and 3). Spontaneous release of gametes can occur when these values are reached. A similar phenomenon is observed regarding the proportion of ripe oocytes, with spawning taking place when percentages reach between 30 and 40% (Fig. 4).

These are not total spawnings because the variations in DW, GOI, and the percentage of ripe oocytes are only moderate, and in the case of the last-mentioned parameter they are followed by a rapid recovery. In this respect our results coincide with the period of continued spawning described by Laruelle et al. (1994) and Rodríguez-Moscoso (2000) for *R. decussatus*, characterized by partial but continued release of gametes once a certain level of gonadal occupation has been reached. This reproductive strategy regulates the continued and progressive process of follicular occupation, which does not appear to be compensated by an adequate degree of reabsorption of gametes in this venerid. The spawning period starts earlier under favorable nutritional conditions, since the first partial spawnings correspond to the diets with the greatest abundance of food. These partial discharges of gametes may, on the other hand, be responsible for the reduced synchronization between specimens, and for the high degree of variation in the data from the final stages of the experiment. Toba et al. (1993) also describe a greater synchronization between specimens in the early stages of gonadal maturation in *R. philippinarum* in Tokyo Bay, which decreases considerably in the later stages of maturity.

Bayne (1975), however, in contrast with the findings of our study, discovered a certain increase in the rate of gametogenic development in *Mytilus edulis* (L.) under conditions of nutritional stress during the initial stages of gametogenesis, although in this species this process is completed by the reabsorption of gametes. In a later study on the effects of thermal and nutritional stress on the eggs of *M. edulis*, Bayne et al. (1978) establish a relationship between decreases in the volumetric fraction of gametes and spawning periods when temperatures are high and food abundant. When food is scarce, these decreases correspond to reabsorption processes or a low level of gametogenesis. In our case, and has already been mentioned, decreases in GOI for the higher diets are associated with spontaneous spawnings, but we have seen no significant decreases associated with nutritional deficiency in either zero or negative energy balance situations.

Based on the relationship between gonadal development and the accumulation and use of nutrients, species can be classified as being either conservative or opportunist (Bayne, 1976). In the former category, gametogenesis takes place at the expense of previously accumulated reserves (Zandee et al. 1980, Bayne et al. 1982). In the latter, gametogenesis occurs when there is an abundance of food in the environment, and sexual maturation parallels the accumulation of nutrients.

Our results show that the behavior of *R. decussatus* varies according to the amount of food available. When there is an abundance of food it adopts an opportunist behavior, developing the gonad at the expense of ingested food, but when food is scarce it behaves like a conservative species, with gametogenesis taking place at the expense of accumulated reserves.

ACKNOWLEDGMENTS

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ABSORPTION OF BIOCHEMICAL COMPONENTS AND FEEDING BEHAVIOR WITH NATURAL AND CARBOHYDRATE-RICH DIETS IN *RUDITAPES DECUSSATUS* AND *VENERUPIS PULLASTRA* CLAMS

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Abstract The feeding behavior and the efficiency of the absorption of biochemical components in the diet of specimens of two species of clams, *Ruditapes decussatus* and *Venerupis pullastra* fed on natural and carbohydrate-rich diets were studied. Both the natural diet, which consisted of the microalga *Isochrysis aff. galbana*, clone T-ISO and ashed sediment, and the carbohydrate-rich diet, which consisted of microalgae and corn starch as organic ingredients, and ashed sediment as the inorganic component, were assayed at a concentration of total particulate matter close to 1 mg TPM L^{-1}, and a concentration of particulate organic matter of approximately 0.6 mg POM L^{-1}, which are similar conditions to those found in the Galician Rías. The feeding behavior of both species for each diet is described with reference to the clearance and ingestion rates, whereas the absorption of the biochemical components of the two diets was determined by biochemical analysis of the diet and the resulting feces. Both ingestion and absorption rates were higher for *V. pullastra* when the clams were fed on a natural diet. Enriching the diet with carbohydrates led to a notable increase in the ingestion and absorption rates in both species, although this increase was greater in *R. decussatus* than in *V. pullastra*, and in consequence the energy absorbed from the carbohydrate-rich diet was greater in the case of *R. decussatus*. The energy absorbed by *R. decussatus* fed on this latter diet was three times greater than that absorbed on the natural diet, allowing it to maintain similar rates of protein absorption for both diets. However, in the case of *V. pullastra*, the amount of total energy absorbed that derived from proteins is 50% lower in the carbohydrate-rich diet than in the natural diet. The energy absorbed from carbohydrates in the carbohydrate-rich diet was greater for *R. decussatus* than for *V. pullastra*. The contribution of lipids to the total energy absorbed was found to be almost double in *R. decussatus* fed on the carbohydrate-rich diet, in comparison with the natural diet, although in *V. pullastra* this contribution was lower. Thus, the effect of diet on the feeding behavior of both species, i.e., the increase in the ingestion rate and the corresponding increase in the absorption rate, allows *R. decussatus* to compensate for the nutritional deficiencies of the carbohydrate-rich diet, whereas in the case of *V. pullastra* it does not appear to be sufficient for the clams to maintain the same protein absorption rate as on the natural diet. These results are discussed in relation to the possible existence of major differences in the metabolism of the two species of clams, differences which would be connected to the habitats in which they live.

Keywords: absorption, biochemical components, clams, diets, feeding behavior, *Ruditapes*

INTRODUCTION

Differences in the characteristics of the habitat occupied by a given species, particularly food availability and quality, give rise to functional adjustments in individual members of the species to allow them to maintain adequate levels of energy acquisition. These adjustments can take place at different levels, e.g., filtration activity, production of pseudo-feces, ingestion rate, digestive capacity, transfer of food to the digestive gland, and enzyme production. The efficiency with which the food is absorbed after ingestion, i.e., absorption efficiency, is one of the most decisive parameters in establishing the amount of energy available to a specimen for growth and reproduction.

Although the absorption processes of bivalves, in terms of total organic matter, have been the subject of extensive study (Thompson & Bayne 1972, Widdows 1978, Griffiths & King 1979, Navarro & Winter 1982, Bayne & Newell 1983; Bayne et al. 1989, Beiras et al. 1993, Navarro & Thompson 1996, Pérez–Camacho et al. 1997, amongst others), there are few references in the bibliography on the efficiency with which each individual biochemical component in the diet is absorbed (Langdon 1989, Bayne et al. 1993; Kreeger & Langdon 1994, Ibarrola et al. 1996, 1998), it having been observed that the quality of the diet affects the effi-

ciency with which its different components are absorbed, this being closely related to the digestive processes.

Studies of the absorption efficiencies of specific elements of the diet, such as carbon or nitrogen, are to be found in greater number (Hawkins and Bayne 1985, Cranford 1995, Iglesias et al. 1996, Urrutia et al. 1996), and from these it is possible to predict efficiencies for proteins in relation to carbohydrates and lipids. Another approach to establishing the nature of the mechanisms by which different components of the diet are used is based on the oxygen consumption : nitrogen excretion (O:N) ratio, which is an indirect indicator of the relative use of protein (Kreeger & Langdon 1993).

As a result of the work of our group in recent years on the two species of clams included in the present study, *Ruditapes decussatus* and *Venerupis pullastra*, we have established the existence of major differences between these two species in terms of both nutritional requirements and physiological parameters, as a result of the different ecological niche they each occupy (Labarta et al. 1997). The purpose of the present work has been to study the absorption of the biochemical components of the diet and the feeding behavior of the two species of clam when fed on a natural diet and on a carbohydrate-rich diet.

MATERIAL AND METHODS

Acclimatization

Specimens of the clams, *R. decussatus* and *V. pullastra*, of approximately 40 mm in length were collected in the surrounding

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area and transferred to the Centro Oceanográfico de A Coruña, where they were acclimatized to laboratory conditions over a minimum of 7 days. Throughout the whole of the acclimatization process, clams were kept in an open-flow system with a flow rate of approximately 2 L ind^{-1} h^{-1} of seawater filtered to 1 μm and enriched with the microalga *Isochrysis aff. galbana*, clone T-ISO. The organic weight of microalgal cells was calculated by filtration of a volume of the algal cultures through Whatman GF/C glass fibre filters that had previously been ashed and then rinsed with a 0.5-M ammonium formate solution. Filters were dried to constant weight at 100°C and ashed at 450°C in a mufle furnace. The concentrations of the microalgal cultures were determined using a Multisizer Coulter Counter. The daily food ration during the acclimatized period, approximately 3%, expressed as a percentage of organic matter in the diet in relation to total flesh dry weight, was supplied at a concentration of approximately 0.5 mg MO L^{-1}, these being similar conditions as those applying during the experimental period. Water temperature was maintained at 19 ± 1°C.

Experimental Conditions

Similar-sized specimens (*n* = 10) of each species were chosen from the stock of acclimatized clams and placed in individual vessels connected to an open-flow system by multichannel peristaltic pumps. Each vessel was fitted with an inlet-tube at the base and an outlet-tube near the surface, the latter being covered with a nylon mesh to prevent loss of feces. Each pump was also connected to two vessels containing no clams to obtain samples of the diet supplied. The flow-rate was 2 L ind^{-1} h^{-1} and the temperature was maintained at 19 ± 1°C in a controlled environment.

Experimental Diets

The natural diet was designed so as to reproduce the annual average values of total particulate matter (TPM; mgL^{-1}), hence particulate organic matter (POM; mgL^{-1}), and percent organic matter observed in the Galician Rias. The diet comprises two particulate components: *Isochrysis aff. galbana*, clone T-ISO, cells, and sediments from underneath the bottom that had been ashed and freeze-dried.

The carbohydrate-rich diet consisted of a mixture of microalgae and corn flour starch (commercial corn starch MAIZENA from Bestfoods España, S.A.) as its organic components and ashed sediment as the inorganic component. The stability of the diet over a 24-h period, in both quantitative and qualitative terms, was monitored from samples obtained from the outlet tubes of the clam-free control vessels. The daily ration of corn flour starch and sediment was resuspended in seawater, using an electrical stirrer and sieved at 60 μm before adding to the system. Size of the corn starch

particles used ranged from 4 to 30 μm, being the mean particle size 15 μm.

Both diets (Table 1) were assayed at a concentration of total particulate matter of approximately 1 mg TPM L^{-1}, and a concentration of particulate organic matter of around 0.6 mg POM L^{-1}, these being similar to the conditions prevailing in the Galician Rías (Babarro et al. 2000). The concentration of organic matter in the carbohydrate-rich diet was increased to 0.77 mg POM L^{-1}, so that when expressed in units of energy (Table 1) this concentration would be equivalent to that assayed in the natural diet, given the lower energy content of corn flour starch in comparison with microalgae. Both experiments were conducted in summer, being the water temperature for both experiments around 19°C.

Samples (2 L) were taken daily from the outlet-tubes of the clam-free vessels directly on to Whatman GF/C fiberglass filters that had previously been washed, ashed, and weighed. After filtration, these filters were rinsed with a 0.5 M ammonium formate solution. Samples were taken in triplicate over a 24-h period to determine both particulate matter, whether total (after oven-drying to constant weight at 100°C) or organic (after ashing in a muffle furnace to constant weight at 450°C) and biochemical components. The filters used for biochemical analysis were freeze-dried and stored at –30°C until the analyses were performed.

Physiological Parameters

The physiological rates were established from the total amount of feces produced over a specific period of time by means of the biodeposition method (Iglesias et al. 1998). The clams were maintained on the experimental diet for 24 h, after which they were cleansed of feces and the period of accumulation of total feces commenced, these being collected after 24 h. The total feces produced were collected on Whatman GF/C filters that had been treated as described above. A proportion of the feces were used to establish their inorganic content and thus determine ingestion rates and absorption efficiency. The remainder were collected on filters, which were freeze-dried, weighed to obtain the total ingestion rate, and then stored at –30°C until biochemical analyses were performed.

The sum of the weight of the feces distributed among the different filters (total egestion rate), together with their inorganic content (inorganic and organic egestion rate) and the inorganic content of the diet allows us to calculate the clearance rate, which when multiplied by the concentration of organic matter in the diet gives us the organic ingestion rate. Absorption efficiency was obtained from the organic content of the feces (*e*) and the diet (*f*), according to the formula established by Conover (1966):

$$AE = (f - e)/((1 - e)*f).$$

TABLE 1.

Characteristics of the diets used: natural diet: *Isochrysis galbana*, clone T-ISO, and ashed sediment; carbohydrate-rich diet: *I. galbana*, clone T-ISO, corn flour starch, and ashed sediment.

Diet	TPM	POM	Energy	POM/TPM	Protein	Carbohydrate	Lipids
	mg L^{-1}		J L^{-1}		% total POM		
Natural	0.77 ± 0.07	0.56 ± 0.07	14.1	0.73	40.5 ± 6.5	17.1 ± 2.5	42.4 ± 4.8
Carbohydrate-rich	0.95 ± 0.10	0.77 ± 0.06	15.1	0.82	7.0 ± 0.1	79.7 ± 0.2	13.3 ± 0.3

TPM, total particulate matter; POM, particulate organic matter. Average values ± standard deviations are shown (n = 6).

Clearance rates were standardized for both species for a specimen of 1 g flesh dry weight using the expression:

$$CR_s = (1/W_e)^b CR_e$$

where CR_s is the standardized clearance rate, W_e is the flesh dry weight of each specimen, and CR_e is the observed clearance rate of the same specimen. The exponent applied, b, was 0.68, which relates clearance rate to the size of the specimen, expressed in terms of weight, for clams (Delgado 2002).

Absorption of Biochemical Components

The biochemical composition of the diet and the feces produced was ascertained by analyzing the contents of the filters of food and feces, according to the following methodology. Proteins were calculated using the method described by Lowry et al. (1951) after alkaline hydrolysis with NaOH 0.5N/30°C. Carbohydrates were quantified as glucose by the phenol-sulphur method (Strickland & Parsons 1968). Lipids were extracted according to a modified Bligh and Dyer (1959) method (Fernández-Reiriz et al. 1989). Total lipids were determined by the Marsh and Weinstein method (1966), with tripalmitine used as a standard. Based on the results of the biochemical analyses of the contents of the food and feces filters, ingestion rates for the different biochemical components were calculated from the product of the organic ingestion rate and the proportion of each biochemical component in the diet. The absorption efficiencies of the various components (AE_{comp}: AE_P, AE_C, and AE_L) were obtained by applying the following formula (Ibarrola et al. 1998):

$$AE_{comp} = (comp_D - comp_F (1-AE))/ comp_D$$

in which $comp_F$ (P_F, C_F and L_F) and $comp_D$ (P_D, C_D and L_D) are the contents of each component in the feces (F) and the diet (D), respectively. The absorption rates of the different biochemical components were obtained from the product of the ingestion rate of the biochemical component in question and its absorption efficiency. Component absorption rates were transformed to energetic units using the following energy equivalents: 18.0 Kj (g protein)$^{-1}$, 17.2 Kj (g carbohydrate)$^{-1}$, and 35.2 Kj (g lipid)$^{-1}$ (Beukema & de Bruin 1979).

Statistical Analysis

The differences observed in the different physiological parameters between the experimental diets used and between the two species studied in this experiment were submitted to statistical analysis of variance (ANOVA, $P < 0.05$; Zar 1984). Angular transformation (arc sin $\sqrt{(AE/100)}$) was used to transform the results for absorption efficiency in order to guarantee standardisation of the data. The Bartlett test was used to check homogeneity of the variances. In the case of non-homogenous variances, logarithmic or reciprocal transformation was used to transform the data, after which their homogeneity was once again checked.

RESULTS

Characteristics of the Diets

Table 1 shows the characteristics of the diets used. The main components of the organic fraction in the natural diets were proteins and lipids, each accounting for approximately 40%, whereas the proportion of carbohydrates is much lower at 17.1%. In the carbohydrate-rich diet, however, the relative percentages of pro-

teins and lipids are much lower, with values of 7.0 and 13.3%, respectively, the main component being carbohydrates, which account for 79.7%.

Both diets were assayed at concentrations similar to those observed in their natural environment (Navarro et al. 1991, Babarro et al. 2000). The ratio of the concentration of organic matter to total particulate matter was 0.73 for the natural diet and 0.82 for the carbohydrate-rich diet. Food concentrations, expressed as energy equivalents, were similar for both diets, being 14.1 and 15.1 J L^{-1} for the natural and carbohydrate-rich diets, respectively.

Physiological Parameters

Average clearance rates (CR), organic ingestion rates (IR_o), organic absorption efficiencies (AE_o) and organic absorption rates (AR_o) together with their standard deviations for a specimen of 1 g flesh dry weight for each species of clam and for both diets are shown in Table 2. Organic ingestion rates of natural diet were significantly higher in *V. pullastra* than in *R. decussatus* ($P < 0.05$, ANOVA test). When clams were fed on the carbohydrate-rich diet organic ingestion rates were significantly higher than those registered for the natural diet. This increase in the ingestion rate was much more noticeable in *R. decussatus* than in *V. pullastra*, thus leading to higher rates in *R. decussatus*

The absorption efficiencies of total organic material were similar for both species fed on the natural diet (ANOVA; $P > 0.05$), with a value of close to 70%. However, when the clams were fed on the carbohydrate-rich diet, absorption efficiencies decreases in both species at around 37%. Thus, the increase in the proportion of carbohydrates in the diet leads to an increase in the ingestion rate, and this in turn supposes a decrease in the efficiency with which the ingested food is absorbed. The relation between the ingestion rate and the absorption efficiency is given by a model that fits the equation $AE = a*IR^b$, in which a = 6.37 ($\pm$0.575) and b = -0.404 ($\pm$0.095) (r = -0.9493, $R^2 = 90.13\%$, $P = 0.0507$).

The organic absorption rate (AR_o) behaves in a similar manner to the IR_o in natural-diet fed clams: the AR_o was significantly higher (ANOVA, $P < 0.05$) in *V. pullastra* than in *R. decussatus*. When the carbohydrate diet was used, organic absorption rate was three times greater than that for the natural diet in the case of *R. decussatus*, but only 50% higher in comparison with the natural diet in the case of *V. pullastra*.

Absorption of Biochemical Components

The difference in biochemical composition between the two diets determines the ingestion rates of each biochemical component of the diet. In *V. pullastra*, although the total ingestion rate of the carbohydrate-rich diet is three times greater than that of the natural diet, the quantity of protein ingested in the former is only half that in the latter (Table 2). The value of lipids ingested is similar in both diets in this species, whereas the quantity of carbohydrates ingested is much greater in the carbohydrate-rich diet. In the case of *R. decussatus*, however, the protein ingestion rate is the same for both diets whereas lipid ingestion doubles with the carbohydrate-rich diet, in which the quantity of carbohydrates ingested increases considerably.

Although total organic absorption efficiency is the same for both species when fed on the same diet, the efficiency with which proteins are absorbed by *V. pullastra* on the carbohydrate-rich diet is less than that of *R. decussatus*, and this, together with the smaller amount of proteins ingested by *V. pullastra*, as described above,

TABLE 2.

Absorption of biochemical components in *Ruditapes decussatus* and *Venerupis pullastra* clams fed on different diets; natural diet: *Isochrysis galbana*, clone T-ISO, and ashed sediment; carbohydrate-rich diet: *I. galbana*, clone T-ISO, corn flour starch, and ashed sediment.

Diet	CR	IR_O	IR_P	IR_C	IR_L	AE_O	AE_P	AE_C	AE_L	AR_O	AR_P	AR_C	AR_L
Ruditapes decussatus													
Natural	0.30 ± 0.10[a]	169.1 ± 57.3[a]	68.5 ± 23.2[a]	28.8 ± 9.8[a]	71.7 ± 24.3[a]	67.0 ± 5.7[a]	78.5 ± 1.7[a]	67.2 ± 3.8[a]	56.0 ± 3.0[a]	113.3 ± 38.4[a]	53.8 ± 1.1[a]	19.4 ± 1.1[a]	40.2 ± 2.1[a]
Carbohydrate-rich	1.28 ± 0.38[b]	991 ± 311.5[b]	69.4 ± 21.8[a]	789.9 ± 248.3[b]	131.8 ± 41.4[b]	37.6 ± 10.8[b]	77.1 ± 5.5[a]	30.9 ± 2.6[b]	56.9 ± 14.3[a]	372.8 ± 17.2[b]	53.5 ± 3.8[a]	244.4 ± 20.8[b]	75.0 ± 18.8[b]
Venerupis pullastra													
Natural	0.46 ± 0.12[c] (1.53)	253.9 ± 71.9[c] (1.50)	102.9 ± 29.1[b] (1.50)	43.3 ± 12.3[c] (1.50)	107.7 ± 30.5[c] (1.50)	71.2 ± 6.4[a] (1.06)	82.1 ± 2.9[a] (1.05)	68.7 ± 5.5[a] (1.02)	61.7 ± 3.8[b] (1.10)	180.6 ± 51.2[c] (1.59)	84.5 ± 3.0[b] (1.57)	29.8 ± 2.4[c] (1.54)	66.4 ± 4.1[b,c] (1.65)
Carbohydrate-rich	0.95 ± 0.24[d] (0.74)	731.3 ± 196.2[d] (0.74)	51.2 ± 13.7[c] (0.74)	582.9 ± 156.4[d] (0.74)	97.3 ± 26.1[c] (0.74)	36.8 ± 8.0[b] (0.98)	68.9 ± 4.7[b] (0.89)	30.6 ± 2.2[b] (0.99)	56.9 ± 10.8[a,b] (1.00)	268.8 ± 72.1[d] (0.72)	35.3 ± 2.4[c] (0.66)	178.2 ± 12.9[d] (0.73)	55.4 ± 10.5[c] (0.74)

CR, standardized clearance rates, expressed in L h^{-1} for a specimen of 1 g flesh dry weight. IR, ingestion rates expressed in μg h^{-1} of total organic matter (IR_O), proteins (IR_P), carbohydrates (IR_C), and lipids (IR_L) h^{-1}. AE, absorption efficiencies expressed as a percentage. AR, absorption rates, expressed in μg of biochemical component h^{-1}.

Average values ± standard deviations are shown.

The same superindex in each column indicates that the differences are not significant (ANOVA, $p < 0.05$). An index of comparison between the two species was calculated on the basis of the quotient *V. pullastra/R. decussatus* for each of the physiological rates, and this is shown in brackets.

gives us a protein absorption rate which is 60% lower than that observed for the natural diet. Lipid absorption efficiencies are similar between the two species, and between diets for each species, at around 56%, thus producing the same difference in lipid absorption rates as have previously been described for ingestion rates, i.e., *R. decussatus* doubles its lipid absorption rate, whereas that of *V. pullastra* remains the same when the diet changes. Carbohydrate absorption efficiencies decrease for both species with the carbohydrate-rich diet, but because carbohydrate ingestion rates are very high on this diet, the absorption rates for this component are nevertheless much higher than those observed for the natural diet.

Whereas the highest absorption rate was given by proteins in both species when fed on the natural diet, the main component absorbed were carbohydrates when clams were fed on the carbohydrate-rich diet, due to the fact that they comprise the highest quantity of the organic matter ingested. In *R. decussatus*, protein absorption rates maintain similar values (53.5 $\mu g\ ind^{-1}\ h^{-1}$) to those described for the natural diet (53.8 $\mu g\ ind^{-1}\ h^{-1}$), in spite of the noticeable decrease of the protein content of the diet. *V. pullastra*, however, is unable to maintain the same level of protein absorption as with the natural diet (84.5 $\mu g\ ind^{-1}\ h^{-1}$), dropping to 35.3 $\mu g\ ind^{-1}\ h^{-1}$, a lower rate than any of those obtained for *R. decussatus* on either of the diets. The increase in total ingestion for both species fed on the carbohydrate-rich diet leads to a considerable increase in the carbohydrate absorption rate when compared to that obtained with a natural diet, although this increase is much greater in *R. decussatus*, with an AR_C of 244.4 as opposed to 178.2 μg of carbohydrates absorbed in *V. pullastra*. With regard to lipid absorption, this is maintained at a similar level to the natural diet (AR_L, 66.4 $\mu g\ ind^{-1}\ h^{-1}$) by *V. pullastra* when fed on a carbohydrate-rich diet (AR_L, 55.4 $\mu g\ ind^{-1}\ h^{-1}$; ANOVA, $P = 0.0838$) whereas in the case of *R. decussatus* it doubles (75.0 $\mu g\ ind^{-1}\ h^{-1}$) its value with respect to the figure obtained for the natural diet (40.2 $\mu g\ ind^{-1}\ h^{-1}$).

Absorption Levels Expressed in Units of Energy

Figure 1a and b shows the absorption rates of the biochemical components of the diet expressed in their energy equivalents. In both species the greatest amount of energy absorbed comes from lipids, when they are fed on a natural diet (54% of total energy absorbed), but from carbohydrates when they are fed on a carbohydrate-rich diet. On a natural diet, the energy absorbed by *V. pullastra* is 70% higher than that absorbed by *R. decussatus*, but on a carbohydrate-rich diet *R. decussatus* absorbs 40% more energy than *V. pullastra*. When fed on a carbohydrate-rich diet, *R. decussatus* absorbs three times the energy than it does when fed on a natural diet and is thus able to maintain protein absorption, in energy terms, at similar levels for both types of diet. However, *V. pullastra* reduces the contribution of energy supplied from proteins to the total of absorbed energy by 50% when fed on a carbohydrate-rich diet in comparison with a natural diet. The energy absorbed from carbohydrates when both species were fed on a carbohydrate-rich diet was greater in *R. decussatus* than in *V. pullastra*. In the case of lipids, the amount of energy deriving from this component in *R. decussatus* when fed on a carbohydrate-rich diet is almost double that obtained on a natural diet, whereas in *V. pullastra* this figure decreases.
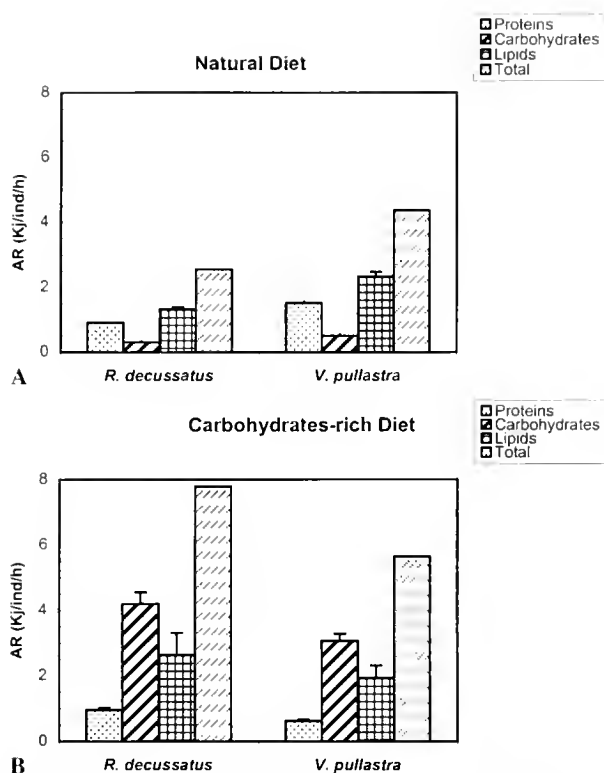
Figure 1. Absorption rates (AR) of biochemical components, expressed in energy equivalents, in two clam species, *Ruditapes decussatus* and *Venerupis pullastra* when fed on a natural diet (a) and a carbohydrate-rich diet (b).

DISCUSSION

Physiological Parameters

The most important difference observed between the feeding physiology of the two species of clam when fed on a natural diet are caused by the ingestion rates. According to the results of our study, organic ingestion rates in *V. pullastra* are 50% higher than in *R. decussatus* (Table 2; *V. pullastra*/*R. decussatus* index, Vp/Rd = 1.50), which when taken together with the slightly higher food absorption efficiency in *V. pullastra* gives a total organic absorption rate for this species that is almost 60% higher than that of *R. decussatus* (Vp/Rd = 1.59). This difference in energy absorbed is in consonance with the findings of other authors (Pérez–Camacho 1980, Beiras et al. 1993, Albentosa et al. 1996, Laing et al. 1987, Laing & Child 1996), who in their studies note that both growth and food consumption rates in *R. decussatus* are lower than those observed in other venerids such as *V. pullastra* or *Ruditapes philippinarum*.

When the clams are fed on a carbohydrate-rich diet, important differences can also be observed in the feeding behavior of the two species, although of an opposite nature to those described for the natural diet. In these circumstances, the ingestion rate for *R. decussatus* when fed on a carbohydrate-rich diet is higher than that observed for *V. pullastra*, giving us in this case an index of Vp/Rd = 0.74. An increase in ingestion is observed in both species when fed on the carbohydrate-rich diet, this increase being of the order of 6 and 3 times greater in *R. decussatus* and *V. pullastra*, respectively. It is therefore true to say that the effect of the diet is the same in both species, i.e., an increase in ingestion when compared

with the natural diet, although quantitatively much greater in the case of *R. decussatus*. If we bear in mind that the food concentration, expressed as total particulate matter, is only 1.4 times higher in the carbohydrate-rich diet than in the natural diet, these quantitative differences would not account for the increase in ingestion observed. Furthermore, when expressed in terms of energy, the food content of both diets was similar (Table 1). Navarro et al. (2000) describe a feeding behavior similar to the one observed in our study, in *Argopecten purpuratus*. These authors describe an increase in ingestion of up to 6 times, just as is the case with *R. decussatus* in our study, when the microalgal diet is supplemented with carbohydrates obtained from potato starch. They also note a similar behavior when the diet is supplemented with lipids, but this time the ingestion rate increases by a factor of 8 in comparison with that obtained on a pure microalgal diet. They suggest the existence of chemical receptors on the gills or labial palps that are capable of detecting specific nutritional components of the diet and which would stimulate an increase in the clearance rate and hence the ingestion rate. The corn flour starch used in the present study consists of particles of a much greater density than the microalgal cells, or if expressed in terms of unit volume, the organic content of corn flour starch particles are some 4 times greater than that of microalgae cells (unpublished data). If we consider that bivalves are continuous filter-feeders, i.e., their digestive system is continuously occupied by food, then we can assume that the digestive capacity of both species, expressed in terms of the amount of organic matter that can be contained inside the digestive tract, must be much greater when the clams are fed on a carbohydrate-rich diet than when fed on a natural diet, because of the above-mentioned difference in particle density between the two diets. Given the great similarity of food concentration at which both diets were assayed (0.6–0.8 mg POM L^{-1}), the total occupation for an equal volume of the digestive system would be obtained by the existence of higher clearance rates for the carbohydrate-rich diet, which would account for the differences found between the ingestion rates for the two diets.

Total organic matter absorption efficiency is reduced by half in both species when they are fed on a carbohydrate-rich diet, owing to the considerable increase in the ingestion rate. The relation between food ingestion rate and absorption efficiency has been much studied in bivalves (Foster-Smith 1975, Navarro & Winter 1982, Bayne & Newell 1983, Beiras et al. 1993, Albentosa et al. 1996, Ibarrola et al. 1998) with a similar behavior being described in all instances, i.e., a decrease in absorption efficiency as the ingestion rate increases, principally because of the reduced transit time through the digestive tract and hence the reduced length of time during which food is exposed to the digestive enzymes. Navarro et al. (2000) also describe a decrease in absorption efficiency when the microalgal diet is supplemented with either carbohydrates or lipids, although to a lesser extent than that observed in the present study.

Total organic matter absorption efficiency within each diet was the same for both species, so the differences observed between species in absorption rates (Vp/Rd = 1.59 for the natural diet and Vp/Rd = 0.72 for the carbohydrate-rich diet) reflect the differences observed in the ingestion rate. Although there is a considerable decrease in the efficiency with which ingested food is absorbed when the clams are fed on a carbohydrate-rich diet, the total organic matter absorption rates are higher, even more so in the case of *R. decussatus* (Vp/Rd = 0.72). The absorption of total organic matter was three times higher in the carbohydrate-rich diet than in

the natural diet for *R. decussatus*, whereas in the case of *V. pullastra* this increase was only 1.5 times greater.

Absorption of Biochemical Components

There are few references in the literature to the process of absorption of the various biochemical components of the diet in bivalves (Kreeger & Langdon 1994, Ibarrola et al 1996, 1998), particularly when the biochemical composition of the diets differs as much as it does in the present study. Ibarrola et al. (1998), in studies of specimens of *Cerastoderma edule* fed on diets consisting of microalgae and sediment in varying proportions (some of which are comparable with the natural diet assayed in our study), show that the most efficiently absorbed biochemical component in high quality diets (i.e., diets with the highest proportion of organic matter) are carbohydrates, whereas in low quality diets lipids are the most efficiently absorbed component. The authors attribute this high rate of carbohydrate absorption in high quality diets to an increase in the activity of certain carbohydrases to be found in the digestive gland. Protein absorption efficiency, however, remains unaffected by the quality of the diet. In our study, on the other hand, the biochemical component that is most efficiently absorbed by both species is protein, regardless of diet. This discrepancy may be due to interspecies differences between enzyme production in the digestive systems of cockles and clams, or also to the different biochemical composition of the microalgae used in the two studies. The high protein content (63.9%) of the microalgal portion of the diets assayed by Ibarrola et al. (1998) when compared to the protein content of the two diets used in the present study (40.5% for the natural diet and 7.0% for the carbohydrate-rich diet) may well account for the differences in protein absorption efficiency registered between the two studies.

The effect of diet on the feeding behavior of the two species in our study, i.e., the increase in ingestion and the resulting increase in absorption, allows *R. decussatus* to compensate for the nutritional deficiencies of the carbohydrate-rich diet, whereas *V. pullastra* seems unable to compensate fully for these deficiencies because it does not maintain the same level of protein absorption as observed in the natural diet. This latter level of absorption can be taken to be sufficient for this species, because it is a reflection of the conditions found in its natural habitat. If we consider that protein absorption is of fundamental importance for all organisms, because proteins are the source of necessary essential amino acids in the biosynthetic routes in the metabolism, this leads us to suppose that *V. pullastra* has a lesser capacity to respond to diets with a high carbohydrate content than does *R. decussatus*, which may

be an indication of the existence of different metabolic routes in the two species.

Studies that have been performed by our research group (reviewed by Labarta et al. 1997) in connection with feeding behavior, the biochemical composition of body tissues, and the nutritional requirements of the two species of venerids studied in the present work suggest that lipid demand is higher in *V. pullastra* than in *R. decussatus*, whereas carbohydrate demand is higher in the latter than in the former, provided that there is sufficient protein in the diet. This may be related to the mechanism by which each species adapts to its specific habitat: *R. decussatus*, which characteristically inhabits the inter-tidal zone and is subject to periods of emmersion as a result of the tidal cycle, would possess an anaerobic metabolism in which carbohydrates are a more appropriate source of energy than lipids. *V. pullastra*, on the other hand, a species that is permanently submerged because of its subtidal habitat, would not have these same nutritional requirements, which are more appropriate to an anaerobic metabolism, and would instead find lipids to be a more relevant source of energy, since they are the appropriate fuel for the aerobic routes of the metabolism. In our experiment both species were exposed to a completely unbalanced diet that contained a very high proportion of carbohydrates at the expense of protein and lipids. Both species responded in a similar manner, in qualitative terms, showing a considerable increase in ingestion which allowed them to counter the low protein content (proteins being an essential component of the diet) of the unbalanced diet. In quantitative terms, however, there are major differences between the two species: *R. decussatus* is able to maintain protein absorption levels, and even manages to double the quantity of lipids absorbed, whereas *V. pullastra* is unable to keep protein absorption at the same level, registering a 60% decrease, and is barely able to maintain lipid absorption. These results would appear to reinforce the previously mentioned hypothesis regarding metabolic differences between the two species.

ACKNOWLEDGMENTS

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THE PERSISTENCE OF NEW JERSEY'S OYSTER SEEDBEDS IN THE PRESENCE OF OYSTER DISEASE AND HARVEST: THE ROLE OF MANAGEMENT

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ABSTRACT New Jersey's Delaware Bay oyster fishery developed along a pathway common to many fisheries. Perennially large harvests led to depletion of the oyster resource, which led to increasing, but ineffective, harvest restrictions and cumbersome management. In the 1950s, two events altered the management structure. In the beginning of the decade, a university researcher dedicated himself to having oystermen and the state regulatory agency use information from research and monitoring programs directly in their decision making. He achieved limited success until a previously unknown oyster disease, eventually called MSX, occurred that threatened to drive the oyster fishery to extinction. The presence of MSX led oyster harvesters to become dependent on the information provided by the university. In addition, the regulatory agency and its regulations had to be responsive to short-term changes in the intensity and prevalence of disease. A tripartite management structure developed in which: 1) the oystermen, researchers, and state regulatory agency acted cooperatively and 2) flexible guidelines were developed that could respond to annual variation in oyster abundance and disease. Several aspects of this management arrangement could prove useful in other fisheries.

KEY WORDS: oyster, management, fishery

INTRODUCTION

Over the past decade, an increasing sense of urgency to develop effective, nontraditional approaches to fisheries management has developed. Too frequently, government-directed management has had problems sustaining fisheries resources at harvestable levels while providing economic and social stability for the fishery participants (McGoodwin 1990, Hannesson 1996). Alternative management models that have been suggested include adaptive management (Walters 1986), ecosystem management (Schramm & Hubert 1996), and responsible management (FAO 1995). All alternative management models suggested to date involve greater participation by fishery participants in the management decision processes, a management structure generally referred to as co-management. Many observers of and participants in fisheries are wary of including the principal users of the resource: they doubt that those who would gain immediate benefit from using a resource would sacrifice current profit for future sustainability (Jentoft et al. 1998). In contrast, Jentoft et al. (1998) have argued that there are numerous social and institutional elements that allow a more positive expectation of the outcome of co-management models.

Co-management has developed in some fisheries without a deliberate effort to develop a nontraditional management program (Jentoft & McCay 1995). Contingent needs can lead all participants in a fishery to search for an operating environment to solve certain problems. Such is the case with New Jersey's Delaware Bay oyster fishery. A detailed examination of the ontogeny and structure of this particular fishery provides several benefits. First, it allows those who are considering developing co-management programs to learn from the successes and failures of those who have already incorporated co-management. Co-management programs are emerging. For example, in the state of Maine, co-management has been legislated recently for the lobster fishery

(Acheson et al. 2000). Other fisheries in the region are expected to follow the same path. Second, the contingent need that had to be solved in New Jersey's Delaware oyster fishery was the presence of diseases that affected the resource. Apparently several populations of marine species have an increasing incidence of disease-induced mortality (Harvell et al. 1999). Managing in the presence of disease may be a more common feature of fisheries in the future. Accordingly, we present the following case study.

Historically low abundances of the eastern oyster, *Crassostrea virginica,* presently occur throughout much of the middle Atlantic US coast. Many factors have contributed to the decline of the large oyster populations that existed in Chesapeake and Delaware Bays, including management that failed to prevent overharvesting (Haven et al. 1978, Kennedy & Breisch 1983). A major factor contributing to recent declines of midcoast oyster populations and frustrating restoration efforts is the presence of one or more oyster diseases. Disease-induced mortalities have been so intense that in some areas oysters are rare and local oyster fisheries have become extinct (Bosch & Shabman 1989).

In Delaware Bay, the principal oyster disease organism for most of the past four decades has been the MSX parasite *Haplosporidium nelsoni.* Since 1990, a southern oyster parasite, *Perkinsus marinus,* which causes Dermo disease, has invaded the Bay becoming the principal disease agent affecting oysters. Epizootics produced by both parasites have caused extensive oyster mortalities in Delaware Bay; however, large numbers of oysters persist. Continued high abundances of oysters in Delaware Bay have been possible because many natural oyster beds occur in a spatial refuge from disease in the upper regions of the Delaware estuary. Salinities in this area frequently fall below levels necessary to sustain MSX infections. The Dermo parasite survives in these reduced salinities but does not produce lethal infections. The natural beds have been a primary source of seed oysters for the industry since the mid 1800s. Until recently, direct marketing from the beds had been prohibited. All seed oysters had to be transplanted to private leases in the lower bay where their growth and meat quality would be greatly enhanced before marketing. With the advent of Dermo

disease. movement of oysters into the lower Bay became uneconomical, and limited direct marketing from the beds began (Ford 1997).

Because the natural beds are located in the upper estuary, the seed resource would have survived the depredations of oyster disease without human intervention. However, a management scheme that developed shortly before the 1957–1959 MSX outbreak stabilized postepizootic yields from seed oysters. Oysters still had to be transplanted onto leased grounds where enhanced growth and fattening was now countered by higher disease pressure that increased mortality. After the outbreak of Dermo disease, an entirely new strategy had to be developed to sustain the industry in the face of a disease with very different characteristics. We believe that a description of New Jersey's management structure provides insights for those desiring an effective management structure for many fisheries. Below we describe the physical and biologic context of the seed oyster fishery in Delaware Bay. Next, we provide a brief history of the fishery and describe the development of the present management structure and how it functions. We then summarize important aspects of the role of oyster diseases and how the management scheme responded to challenges from the diseases. We conclude by highlighting the unique elements of the management structure that we feel led to its success. It is important to note that the authors were participants in many events described below and may be burdened with preconceptions as to the value and importance of different aspects of the management structure. However, our direct and extensive knowledge of the inner workings of the management structure allows us to place events and circumstances in a context that would not be available to an outsider.

Physical Description of Delaware Bay

Detailed descriptions of the physical and bathymetric characteristics of Delaware Bay are available elsewhere (Shuster 1959, Maurer & Watling 1973, Galperin & Mellor 1990a, 1990b). Delaware Bay is bounded on the north and east by New Jersey and on the south and west by Delaware (Fig. 1). The bay extends 75.2 km from its southeastern-facing mouth between Cape May and Cape Henlopen to the entrance of the Delaware River in its northwestern corner. The average depth is ca. 10 m with the greatest depths occurring near the central long axis of the bay. The eastern side of the bay has extensive tidal flats. The bottom consists largely of soft-substrates (sands and muds) with hard substrate limited to spatially discrete oyster beds and cobble aggregates.

Delaware Bay experiences predominately semi-diurnal tides with a 1–1.25 m tidal range near its mouth. Around 72% of the annual freshwater input enters the bay from the Delaware and Schuylkill Rivers. Salinity near the mouth ranges from 30–31 ppt and decreases with distance in a roughly uniform fashion up the bay to 0–4 ppt near Wilimington, DE. Water temperatures range from −1.8 to 29.0°C annually.

Oysters in Delaware Bay

Historically, natural oyster beds existed throughout Delaware Bay (Ford 1997). Before the mid-1800s, however, harvest practices and the distribution of oyster predators (primarily oyster drills, *Urosalpinx cinerea* and *Eupleura caudata*) eliminated beds in the lower bay. The geographic location of extant natural (seed-oyster) beds has remained fairly constant and predates the appearance of MSX in Delaware Bay (Engle 1953, Maurer et al. 1971). These oyster beds occur in several small rivers entering the bay
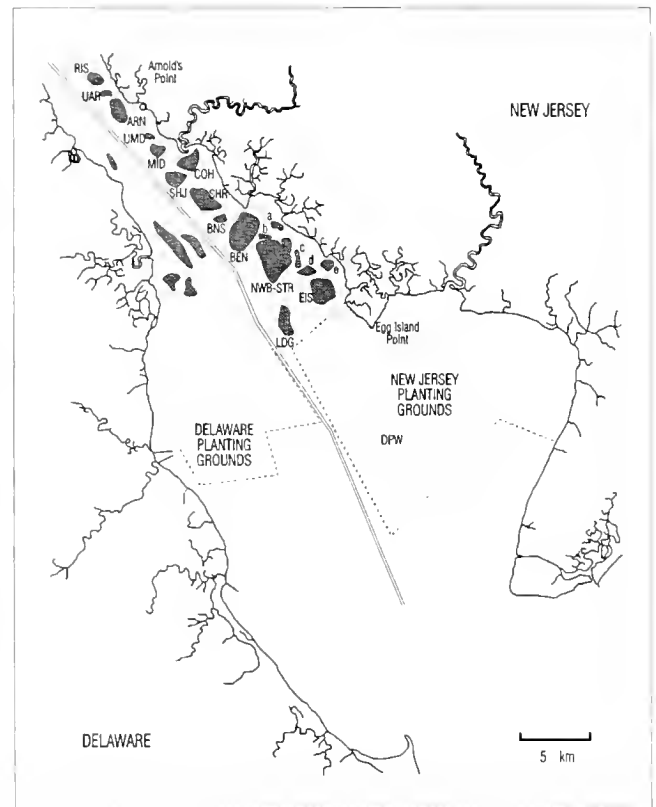
Figure 1. Location of the seedbeds (shaded areas) and the planting (leased) grounds (areas inside the broken lines) in Delaware Bay. The double line extending down the center of the bay represents the shipping channel. It separates the New Jersey and Delaware portions of the bay. Abbreviations for the seedbeds are the same as in Table 1. The labels of five small beds, located inland of EIS and NWB-STR, are indicated by letters (a—NPT, b—HGS, c—HWN, d—VEX, and e—BDN). DPW is the Deepwater site.

(Broadkill, Leipsic, Mispillion, Murderkill, and St. Jones Rivers in Delaware; Back, Cedar, and Nantuxent Creeks and Cohansey and Maurice Rivers in New Jersey) and in the bay itself between Egg Island Point in the south and Arnold's Point in the north (Fig. 1). Most of the beds are in the eastern or New Jersey half of the bay. Oyster beds vary in size from those that are a few m^2 in area ("lumps") to some that exceed 6×10^5 m^2. The density of oysters per unit area is highly variable within and between beds.

Salinity of the water immediately over the oyster beds varies with distance from the mouth of the bay (Engle 1953, Maurer & Watling 1973, Fegley et al. 1994). Over the lowermost beds (those closest to the mouth of the bay) bottom salinity typically ranges from 16.0–20.0 ppt. The uppermost beds generally experience a bottom salinity ranging from 7.0–15.0 ppt. Oysters experience reduced predation rates on all but the lowermost beds because the most abundant and effective oyster predators in Delaware Bay, the oyster drills, are inhibited by salinities less than 15 ppt (Engle 1953). In contrast to survival, oyster growth rates decline along the decreasing salinity gradient in Delaware Bay. Oysters transplanted from the lowermost beds have generally required a single growing season to reach marketable size whereas most oysters moved from the uppermost beds have needed to remain on the planting grounds at least 2 years before they could be landed.

Adult oysters spawn throughout the summer with most repro-

ductive activity occurring from mid June to mid July. The larvae remain in the water column from 10 to 20 d, depending on water temperature. Oyster spat set over most of the bay. The densest sets generally occur in the eastern portion of the bay south of Egg Island Point, where no beds exist and where oysters rarely survive to adulthood because of high predation, disease, and winter ice mortalities (Engle 1953, Ford & Haskin 1988).

Oyster Fisheries in Delaware Bay

Several detailed accounts of the history of Delaware Bay oyster fisheries exist (Miller 1962, Maurer et al. 1971, Ford 1997). The following description, based on these histories, concentrates on the New Jersey portion of the fishery.

In colonial times, natural oyster beds occurred throughout the bay although then, as now, most beds were located in the eastern (New Jersey) portion. Oysters were harvested directly from the beds and most were taken directly by ship to markets in Philadelphia. The concept of planting small "seed" oysters onto private leases for growth and fattening before taking them to market was introduced to Delaware Bay in the mid 1800s. Leases were established in the lower bay because the market quality of oysters was better there and because the local, natural beds had been largely destroyed by that time. Transplanted oysters were usually left on these relatively high-salinity leased grounds for 1-3 y before they were marketed. The seed oysters came primarily from the extant natural "seed" beds in the upper bay and in the creeks where low salinity protected small-sized oysters from predation. These beds remained a "public" resource. The practice of planting oysters was codified independently by laws in the States of Delaware and New Jersey. Until recently, planting seed oysters was the principal means of producing oysters in Delaware Bay. As planting became more widespread, the oyster fishery became dominated by companies that owned large schooners and used dredges to harvest oysters; hand tongers oystering from small boats have remained a marginal component of the fishery since that time (Fig. 2).

From 1900 to 1930, Delaware Bay oyster landings produced between one million and two million bushels annually (Ford 1997). After 1930 and until the mid 1950s, the productivity of the industry declined slightly and annual landings remained at or just below one million bushels (~40 million L, Fig. 3). Landings of this magnitude, although supplemented by planting of seed oysters collected from outside of the estuary (primarily Chesapeake Bay and Long Island Sound), removed tremendous numbers of oysters from the natural seeds. By the early 1900s, seedbeds near the planting grounds were reported to be out of production. Subsequent harvest practices (e.g., failure to return oyster shell to the seedbeds and the introduction of engines into the sailing schooners used to dredge seed oysters) and physical-biologic interactions (e.g., persistent droughts that increased the range and abundance of oyster drills) led to further degradation of the seedbeds. Finally, several years of poor recruitment onto the seedbeds and some unexplained mortalities of adult oysters in the 1940s and 1950s left oyster abundances on the seedbeds at historical lows.

Development of Oyster Seed Fishery Management

Legislation enacted by the States of New Jersey and Delaware during the 19th century attempted to regulate oyster fisheries in both states (Ford 1997). The overall goal was to preserve the oyster resource. Specific laws introduced culling (returning oyster shells to the bottom), restricted taking oysters from public seedbeds to a
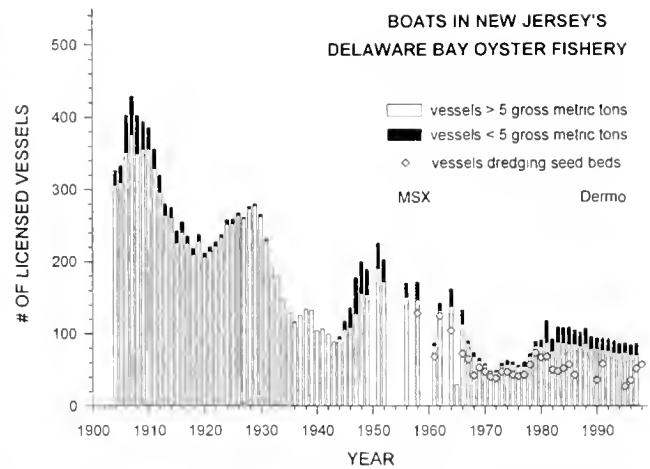
Figure 2. The number of vessels registered annually in New Jersey's Delaware Bay fishery. Open bars represent vessels greater than 5 gross metric tons in size. Shaded bars represent vessels less than 5 gross metric tons in size. From 1958 to 1991, the number of vessels participating in seedbed dredging each year is indicated by the diamonds. In the last 3 yr (1995 to 1997) the diamonds indicate the number of boats harvesting the seedbeds in the spring and fall for direct marketing of the oyster seed. The broken vertical lines indicate the first year the respective diseases were observed in the bay. No data were available in years when the beds were closed.

specific season, allowed the first private leasing of grounds, and created a variety of organizations to monitor and enforce the legislation. Enforcement was a perennial problem and, at the request of many oystermen, the State of New Jersey took control of both the public and private grounds in 1899 (the State of Delaware had done so in 1873, just two years after private grounds were developed there).

The principal regulation affecting the New Jersey seedbeds limited the period for oyster dredging to May and June. During this period, known as bay season, licensed vessels were permitted to take as many oysters as they could dredge and carry from the seedbeds for transplanting onto private leased grounds. Beyond limiting the length of bay season, there were no attempts to restrict the numbers of oysters taken from the beds. Prior to the 1950s, the seedbeds were closed to harvest only once, in 1928, to protect a large set of spat (newly settled oysters up to one year of age; Nelson 1929). During this time, information on year-to-year changes in oyster abundance on the seedbeds was not gathered. Few data were available to provide a basis for decisions by management.

Management of New Jersey's oyster resource can be traced to 1888. In that year Julius Nelson, a member of Rutgers University's New Jersey Agricultural Experimental Station, convinced the school to create the Department of Oyster Culture. Julius Nelson, and later his son, Thurlow, became leaders in the field of oyster biology and established a tradition of using scientific methods to produce information useful to the oyster industry (Nelson 1913, 1928, 1947). In the early 1950s, when oyster abundances on the Delaware Bay natural seedbeds reached historical lows, the Department of Oyster Culture, then under the direction of Harold Haskin, began studying the factors limiting oyster abundance on the seedbeds and gathering data that would suggest management strategies to rehabilitate the beds. The collection of data on oyster life-history in Delaware Bay in a regular and consistent manner
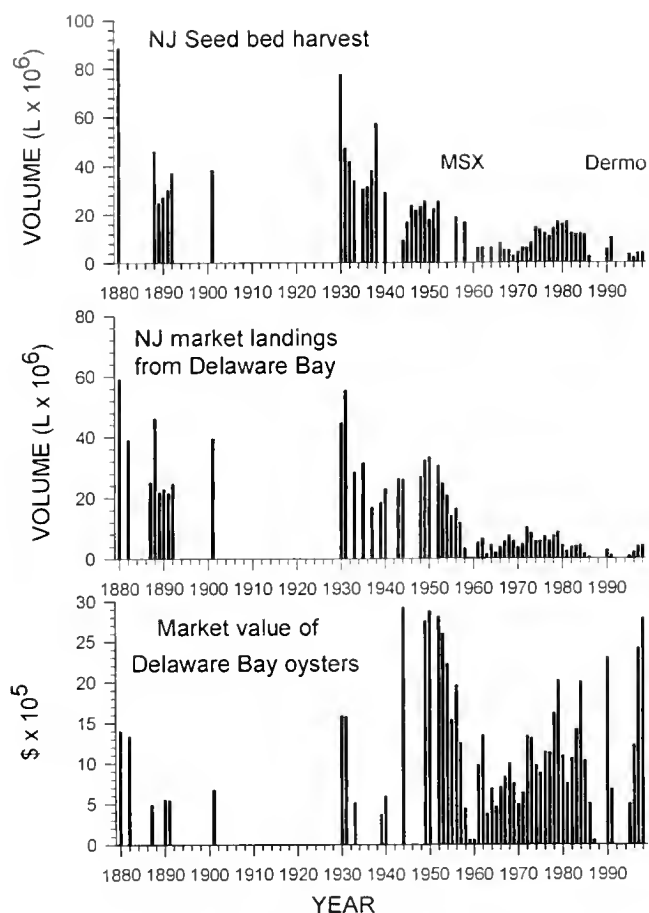
Figure 3. **Historical changes in seed bed harvest, market landings, and market value in New Jersey's Delaware Bay fishery. The broken vertical lines indicate the first year that the respective diseases were observed in the bay. The absence of bars prior to 1957 indicate that no data are available. The absence of bars after 1957 indicate 0 values for the respective measures. Harvests are mostly for direct marketing and not planting starting in 1996.**

has continued for 45 y and has provided the basis for what we believe has been an effective management scheme.

At its inception the seedbed rehabilitation program consisted of two key elements: gathering quantitative data on oysters (Research Component) and advocating the use of these data in making management decisions (Applied Component). The research component consisted of several studies conducted yearly including: (1) determining the temporal and spatial abundance patterns of oyster larvae, (2) determining the temporal and spatial patterns of oyster spat settlement and fouling organisms (invertebrates that compete with spat for space) onto artificial collectors, (3) detecting annual changes in the abundances of spat, yearlings, and older oysters on the seedbeds, and (4) estimating the volume of seed oysters transplanted from the beds. Much of the funding for monitoring in the early years derived from University sources, a condition that is uncommon in our experience. The applied component entailed a determined effort on the part of the Director of the Department of Oyster Culture (Haskin) to convince the state management agency and, more importantly, the oystermen of the need for additional restrictions on seed transplants and of the usefulness of scientifically collected data in decision-making. The use of scientifically collected data is now an accepted element. Both components have

had continued importance in the overall management of the resource.

Research Component

Of the several studies in the research component, two have been consistently of greatest use to management of the resource: collecting dredge samples from seedbeds and estimating the amount of oyster seed transplanted during bay season. Since the onset of Dermo disease, data on infection levels and oyster mortality rates have also been used on a regular basis in making management decisions.

For dredge sampling, several grids, each consisting of contiguous 275-m × 370-m rectangles (approximately 0.2 min of longitude by 0.2 min of latitude, respectively), were created for each of the 25 spatially largest seedbeds that had historically contributed the bulk of oyster production. Each year, generally between November and March, approximately 10% of the grids were chosen from each bed using a stratified random sampling design. Samples were taken from the middle of each grid. In the grid an oyster dredge (with a 71-cm tooth bar and a bag capacity of ~80 L) was towed on the bottom for one minute at constant boat speed (i.e. approximately constant effort) three separate times. Approximately 13–14 L of the contents of each of the three hauls were retained, pooled, and returned to the laboratory as a single sample. First, the volumes of live oysters (adults, yearlings, and spat), cultch (oyster shell with no live oysters attached), and debris (sponges, algae, wood, etc.) of each sample were estimated. Then the following quantitative attributes were determined by direct examination: (1) the number of oysters older than 1 y, (2) the number of "yearlings" (oysters that were about 1 y old), (3) the number of spat, (4) the number of "boxes" (articulated but empty oyster valves), (5) the number of "gapers" (recently or nearly dead oysters that do not fully close their valves when handled), and (6) the number of dead spat and, if any distinctive drill or crab valve damage was apparent, the source of spat mortality.

Estimates of seedbed yields were made by research crews every day that dredging occurred on the seedbeds from 1956 to 1991. How many and which boats dredged, which beds the boats dredged, and estimates of the volume of oysters moved to the planting grounds at the end of the day were obtained by direct inspection. Estimating the volume of oysters harvested was done by noting the size of the pile on the deck and the position of the water line on the oyster boat. In several years research crew estimates were compared with estimates of seed oyster volume made by the boat captain and by direct measurements. Remote observer estimates were generally within 10% of the captain's estimates and of direct measures.

Estimates of the percent composition of commercial dredge samples were also made during bay season. On Thursday (usually) of each week of seed planting season unculled 40 L samples of oysters and shell were taken directly from the decks of oyster boats. Boats were selected on the basis of which beds they dredged. The beds of interest were those that had experienced the greatest amount of dredging activity during the week or that had begun the week with relatively low percentage (by volume) of oysters. On shore a committee composed of industry members, managers, and laboratory personnel sorted the samples into oyster (live adults, yearlings, and spat) and shell (anything without an oyster attached) and estimated the relative volumes of the two portions. This information was then used to decide whether to

close some of the beds or to end the seed transplant early. If the average percent of oysters by volume was less than 40% for a bed the committee gave serious consideration to closure.

The 40% value was a "rule of thumb" benchmark that was never supported by statute or regulation. It was not supported by scientific evidence. When the seedbed rehabilitation program began the approximate percent oyster on many beds was around 40% and many felt that it should not go lower. The industry members understood this measure (as opposed to more complex statistical indices) that required simple math and that they could derive on their own via examination of dredge hauls. Also, when percent oyster did drop much below 40% harvesting oysters became prohibitively expensive for boats using manual culling. Use of the 40% rule was flexible. Depending on other factors (abundance of oysters elsewhere, number of spat in the sample, perceived economic needs of the oystermen) a bed could be closed before the percent oyster measure reached 40% or at a considerably lower percentage (as low as 20% in a few cases).

Applied Component

A shellfish council, officially consisting of industry members appointed by the Governor, had long been in place to advise the state agency in charge of the seedbeds (the council also supervised the private leases approving transfers, vacancies, boat licenses, etc.). In the mid 1950s incorporating research results into the council's decision-making proved difficult. The concept of managing oyster beds with recently collected data was foreign to both the state agency (NJ Bureau of Shellfisheries) and the oystermen. However, the greatly depleted condition of the beds indicated that restrictions on seed transplants would be austere for some time to come. The patent threat to the fishery by the condition of the seedbeds and the persistent efforts of the Director of the Department of Oyster Culture advocating the utility of research results led to the development of a tripartite management scheme. An independent source of information, Rutgers University, was added, in an informal advisory role, to the shellfish council and state regulators (Fig. 4). This system remains in effect today.

In late winter, several months prior to the beginning of bay season, data collected from the seedbeds by the university researchers are presented to the shellfish council and representatives of the state management agency. The primary concerns are the
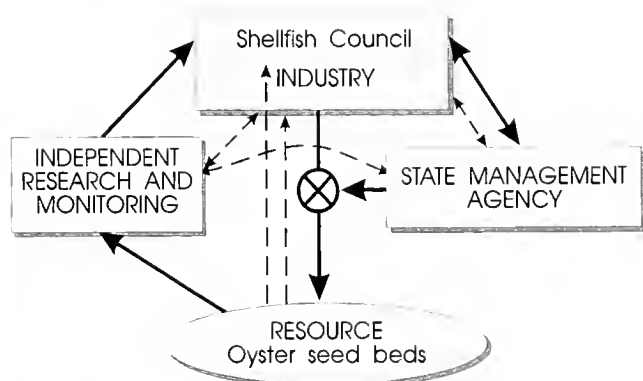
Figure 4. Diagram illustrating the relationships among the components of the New Jersey Delaware Bay oyster fishery. Solid lines indicate formal informational pathways. Broken lines indicate informal informational pathways. The X represents the control of industry access to the seedbeds via state management.

relative compositions of dredge samples taken from the seedbeds (percent oyster) and the seedbed spat abundances. An oral presentation of these data (usually supplemented with a written summary) is made to the shellfish council members who use this information and, in some years, their own direct observations of the beds, to decide (1) whether there will be a bay season, (2) how long the season will be, and (3) whether any beds will be excluded from fishing. The council's recommendations are then submitted to the state management agency (specifically the Commissioner of New Jersey Department of Environmental Protection who directs the Bureau of Shellfisheries), where they have generally been approved.

Onset of MSX Disease

In the spring of 1957, widespread mortalities of oysters planted the previous year occurred on the New Jersey leased grounds. Within two years the epizootic had killed over 90% of the oysters on the planted grounds and almost half of those on the seedbeds (Haskin et al. 1966). The causative agent, H. nelsoni (popularly referred to as MSX), was identified in 1958 and has remained enzootic in the estuary (Ford 1997). Since 1957, dockside landings of oysters from Delaware Bay have remained well below a half a million bushels (~20 million L) of oysters annually (Fig. 3), although significant underreporting of these landings may be occurring (Haskin & Ford 1983).

Uninfected oysters residing in salinities greater than 15 ppt can become infected with H. nelsoni from June to early November. The disease progresses to a lethal stage within several weeks in susceptible oysters. Mortalities are delayed in Delaware Bay native stock; it has developed a degree of resistance to the disease (Ford & Haskin 1987). Some oyster deaths occur in late summer or fall of the first year of planting, but these are usually tolerably low (Ford & Haskin 1982). Mortalities are cumulative, however, and become unacceptably high if oysters are not marketed within a year. The large oyster mortalities produced by MSX on the planted grounds altered the practices of the Delaware Bay oyster fishery. First, importation of oyster seed from other regions ended. Second, only relatively large oyster seed could be transplanted from the seedbeds to the planting grounds because only a single growing season was likely to be available to growers. It was no longer possible for small oysters to survive in the lower bay for the two to three years necessary to reach market size. Planters could not stockpile oysters on their leases anymore. Third, oystermen concentrated their planted oysters in a relatively small area of the bay less prone to disease. Leased bottom was made available that encroached onto the lower seedbeds in an attempt to provide less saline and less disease-ridden planting grounds. Fourth, oyster boats decreased operating costs by using automatic culling machines instead of manual labor to separate oysters from cultch. Fifth, regulations were changed to permit marketing oysters earlier in the year. This allowed planters to land oysters as soon as they reached market size instead of waiting until 1 September as they had previously. Sixth, a limited fishery based on boat size was established in 1981 to prevent a large influx of participants during good times who had no commitment to preservation of the resource.

The onset of MSX disease initiated a long-term monitoring program that followed the spatial and temporal patterns of the disease in the bay and consequent oyster mortality (Ford and Haskin, 1982). Results garnered from this effort helped interpre-

tation of data acquired from the annual seedbed sampling program. At approximately one month intervals, oysters were dredged, using the same device described above, from the larger seedbeds and several locations on the planting grounds. The dredge samples were taken only from the most productive grids on the seedbeds. In contrast to the fall/winter seedbed sampling procedure, several successive dredge hauls were conducted until a bushel (~40.7 L) containing only live oysters, gapers, and boxes was obtained. All gapers and boxes were examined for evidence of shell damage that could be attributed to crabs, drills, or dredging. Gapers and boxes with undamaged valves were assigned to the nonpredation mortality category. The interiors of the boxes were further inspected to determine whether any fouling organisms had recruited onto the inner surfaces of the valves. Boxes with no fouling on the inner valve surfaces were considered "recently dead." Spatial and temporal variation in the rates of valve fouling were estimated by placing clean valves in the field at regular intervals and examining them at subsequent intervals for the presence of fouling organisms. Seasonal "fouling intervals" ranged from 2 to 3 wk in the summer and up to 10 wk in the winter (Ford & Haskin 1982). Estimation of the annual mortality from predation and nonpredation sources were made by accumulating mortalities determined over short intervals.

MSX disease prevalence and intensity was determined via histologic procedures in live oysters and gapers collected during the mortality sampling. After sectioning and staining, the abundance and location of MSX parasites in the tissues were determined via microscopic examination. In local infections (nonlethal at the time of collection) the parasites occur only in the gills. In systemic infections parasites are distributed through all oyster tissues. Systemic infections are found in 90% of oysters that die of MSX disease (Ford & Haskin 1982).

Onset of Dermo disease

Infections by the southern oyster parasite, *P. marinus*, causative agent of Dermo disease, had been historically of little consequence in Delaware Bay. During the mid 1950s light infections were found in planted oysters after parasitized seed was imported from Virginia where the disease was endemic. Dermo infections became rare in the bay after importation of seed ended. The water temperatures in Delaware Bay were generally believed to be too cold for Dermo disease to persist (Ford & Haskin 1982) and sampling specifically for Dermo disease ended in 1963. In the late summer of 1990, oyster mortalities that did not fit the pattern associated with MSX disease were documented in several locations in Delaware Bay (Ford 1996). The causative agent was quickly identified as *P. marinus*. Since 1990, Dermo infections have been persistent, widespread, and responsible for continuing oyster mortality in the bay.

In contrast to the pattern of MSX distribution, Dermo infections have extended onto the seedbeds and caused substantial mortalities of seed oysters. *P. marinus* is much more tolerant of low salinity than *H. nelsoni*. It survives on most of the seedbeds, even though it does not cause many lethal infections on the uppermost beds. Parasites proliferate rapidly in oysters transplanted to the planting grounds in spring, stimulated by both high temperature and high salinity. Under these conditions, transplanted oysters typically die before the fall market season. The consequences of a mortality pattern quite different from the delayed mortalities induced by MSX disease was forcefully demonstrated to the planters shortly after the onset of the Dermo epizootic. Planters were advised of the

presence of Dermo disease in Delaware Bay immediately after it was identified in the summer of 1990. During the remainder of the summer and fall, the disease spread to all planting areas and to the lower seedbeds, but caused relatively little mortality and yields from planted oysters were the best in many seasons. The following year, the abundance of oysters on the seedbeds was the best since the early 1980s and nearly 300,000 bushels (1.2×10^7 L) were moved to the planted grounds. A large majority of these oysters were already infected with *P. marinus*, which quickly proliferated. Despite advisories about relatively high infection levels by researchers (including warnings by oyster disease researchers from institutions other than Rutgers University expressed in a special public meeting), most planters, remembering the profitable results of the previous year, chose to leave their oysters on their leases rather than to harvest early. Mortalities, when they began, were severe and only about a quarter of the oysters survived to the fall market season.

The MSX surveillance program was severely diminished after the mid 1980s because of funding limitations and an expressed hesitation by university administrators to commit to long-term monitoring programs. The advent of Dermo disease, however, raised enough concern within the industry that limited monitoring was resumed. It centered primarily on disease diagnosis in oysters collected during the fall seedbed survey, which provided information on the spatial distribution and intensity of Dermo disease on the natural beds at a time of peak prevalence and intensity (Ford & Tripp 1996). Because it is likely that oysters rarely, if ever, completely rid themselves of *P. marinus*, even under the low temperature and low salinity conditions that are unfavorable to the parasite (Ragone-Calvo & Burreson 1994, Ford et al. 1999), the fall sampling provided a good estimate of what percentage of oysters are infected on each bed. Subsequent sampling in the spring before bay season, provided additional information on infection intensity, which typically decreases over the winter in proportion to temperature and fresh-water influx. Infection intensity in oysters likely to be transplanted provided a rough measure of whether infections would progress to the lethal stage relatively sooner or later after planting.

The results from the Dermo disease surveillance program and from the earlier MSX program were presented to the shellfish council and to individual planters. In recent years, mailings to all lease holders describing the most recent levels of oyster mortality and disease prevalence were made.

Delaware Bay Oyster Fishery Activity, 1953 to 1991

Seed dredging has occurred in most years since the first MSX epizootic (Fig. 5). Generally all of the beds were open, but oystermen concentrated their efforts in just a few beds. The 1960s harvests were relatively small and came primarily from the uppermost beds. By the end of the 1960s most oyster seed came from the beds in the middle of the seedbed region. Four beds, Cohansey, Shell Rock, Bennies, and New Beds, produced 68.2% of the oyster seed from 1958 to 1991. These are among the largest beds and perennially have relatively high abundances of moderately large oysters (Table 1).

As would be expected, samples collected for the weekly estimation of relative oyster volume during bay season were taken from where most of the harvest activity occurred. During the 1960s and early to mid 1980s the relative volumes of oysters in the samples were generally less than 40% (Table 2). Only a quarter of these samples (11 of 42 instances) was less than 30% and in only

ANNUAL MEAN PERCENT VOLUME OF OYSTERS
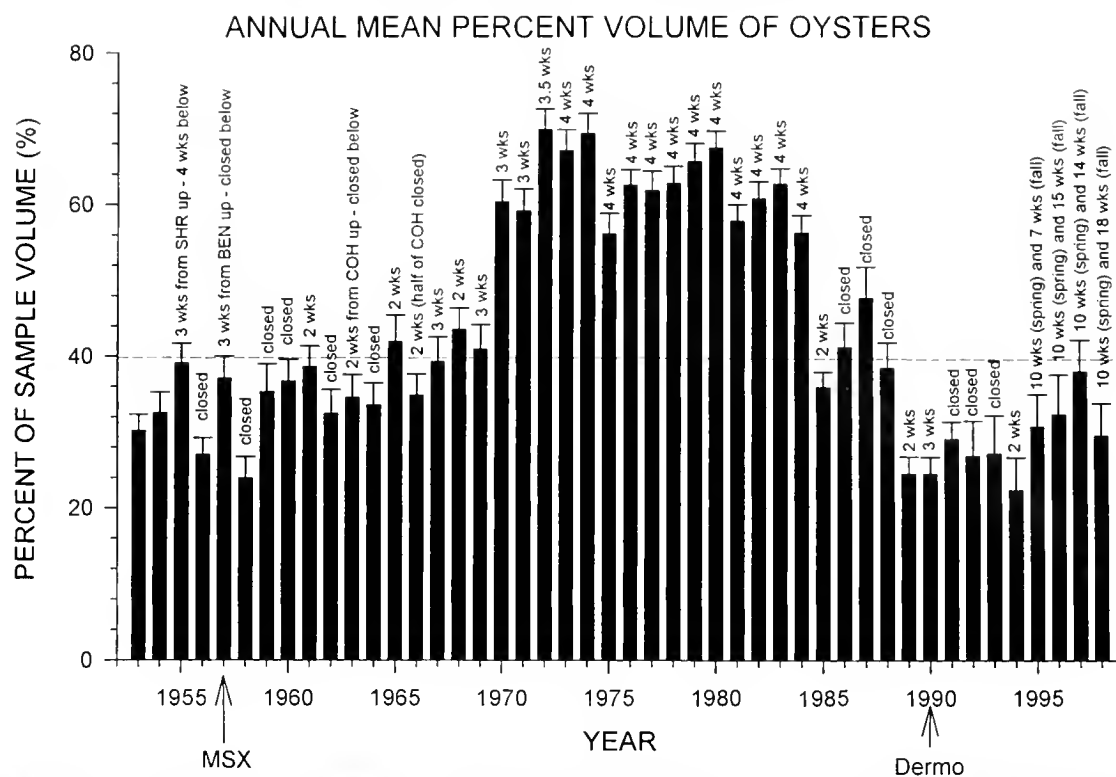
Figure 5. Average (±1 SE) percent volume of oysters in dredge samples for each year. The average is calculated across all beds; *n* ranges from 60 to 120 for any given year. The arrows indicate the first year the respective diseases were observed in the bay. The horizontal broken line indicates a percent volume of 40%. Text above each bar describes the length of the subsequent bay season. Abbreviations for seedbeds are the same as in Table 1.

three instances were the proportions less than 20%. During the mid to late 1970s the relative oyster volume frequently exceeded 40%, but these data were never used to extend a seedbed harvest season beyond the length that had been agreed upon earlier in the year. Individual seedbeds were closed before the end of bay season only four times (Shell Rock Bed, 1961; Cohansey Bed, 1967; Shell Rock Bed, 1972; and Bennies Bed, 1974). Low percent oyster was the reason in half of these closures, while protection of spat led to the other early closures.

The fishery benefited from very successful recruitment in 1972 although relative abundances of oysters on the beds were increasing before this year (Fegley et al. 1994). The large 1972 set provided oysters until the early 1980s. The persistence of harvestable oyster seed for almost a decade after the 1972 set was aided by the management and harvest practices of the fishery participants. For instance, despite large abundance of oysters in 1974 the length of bay season was not extended to take immediate advantage of this bounty either that year or in any successive year. Within years, the efficiency of seed harvest (actual harvest/potential harvest × 100%) remained near 60% throughout the period (the potential harvest was based on estimates of the total abundance of oysters present on the seed bed large enough to be suitable for transplant). The observed efficiency was most likely a function of boat harvest limitations rather than conscious efforts of the harvesters. Oyster recruitment onto the seedbeds was relatively low in the years after 1972; another "large" set (only a third the size of the 1972 set) did not occur until 1986. Restrained harvesting of the large 1972 set by the fishery, combined with average or above-average annual Delaware River flow into the bay, remains the most likely explanation

for the continued presence of oysters on the seedbeds into the late 1970s and early 1980s.

In the mid 1980s seedbed harvests began to decline. During this time there were increased prevalences and intensities of MSX disease throughout the bay (Fig. 6); widespread mortality of oysters followed. This was the first time since the mid 1960s that the seedbeds exhibited such high levels of disease and predation. The mid 1980s were also the first time since the mid 1960s that the annual mean Delaware River flow remained below the long-term average for several successive years (Fegley et al. 1994). No seed dredging occurred for 3 yr (1987–1989). During this protracted closure of the fishery there were modest increases in the abundances of oysters on the beds and seed transplants began again in 1990. Unfortunately that was also the first year of a Dermo disease epizootic.

The effects of Dermo disease upon the New Jersey oyster fishery have been substantial. Data provided by university researchers informed oystermen that most of the oysters they would plant were infected with the Dermo parasite and would not survive long after planting. Based on this monitoring information, the shellfish council voted to close the seedbeds in 1992, 1993, and 1994. By 1995, after 3 yr without a planting season, it was obvious that the traditional transplant scheme would work no longer.

In 1995, a new strategy was agreed upon and tried for the first time that allowed direct marketing from the seedbeds. Up to this time, all oysters removed from public seedbeds had to be transplanted onto private grounds before they could be marketed. In the new scheme, which was developed by the Bureau of Shellfisheries and agreed to by the Shellfish Council, each licensed vessel re-

TABLE 1.

Some characteristics of the seed beds related to seed harvest. The area of the seed bed includes nonproductive bottom. Mean percent oyster is based on dredge samples taken in the random sampling program (1953–1991). Mean individual size is estimated by dividing the volume of a dredge sample consisting of oysters by the number of oysters present. The harvest data are the total volume of seed removed from each bed between 1958 and 1991. The five largest values in each category appear in boldface. The names of the beds, which are listed from those uppermost in the bay to those that are lowermost, are given below.

Bed*	Area (Hectare)	Rank	% Oyster (±1 SD)	Rank	Indiv. Size (mL)	Rank	Harvest L × 10³	Rank
RIS	162	13	**73.6 (13.5)**	**2**	37	18	3,066	13
UAR	121	15	**74.1 (14.3)**	**1**	51	16	787	17
ARN	232	9	**70.7 (18.4)**	**3**	49	17	8,249	10
UMD	20	18	49.9 (27.7)	13	63	15	1,855	15
MID	374	7	**64.9 (17.2)**	**5**	70	13	**17,617**	**5**
COH	**545**	**3**	62.2 (17.5)	6	78	8	**43,735**	**2**
SHJ	**454**	**4**	**66.5 (18.5)**	**4**	74	10	16,251	6
SHR	**404**	**5**	61.7 (20.6)	7	85	6	**42,784**	**3**
BNS	101	17	59.9 (22.3)	8	**91**	**5**	9,335	8
BEN	**636**	**2**	48.0 (25.2)	14	**98**	**4**	**40,941**	**4**
NPT	212	11	53.0 (22.8)	11	69	14	2,476	14
HGS	111	16	46.8 (23.8)	15	76	9	11,270	7
NWB-STR	**829**	**1**	53.4 (26.9)	10	85	7	**62,496**	**1**
HKN	202	12	55.0 (19.4)	9	71	12	1,121	16
BDN	293	8	43.4 (25.9)	17	**135**	**2**	203	18
VEX	162	14	52.8 (20.1)	12	74	11	4,051	11
EIS	394	6	43.6 (25.4)	16	**112**	**3**	8,945	9
LDG	222	10	33.0 (23.0)	18	**146**	**1**	3,479	12

* RIS, Round Island; UAR, Upper Arnold's; ARN, Arnold's; UMD, Upper Middle; MID, Middle; COH, Cohansey; SHJ, Ship John; SHR, Shell Rock; BNS, Bennies' Sand; BEN, Bennies'; NPT, Nantuxent Point; HGS, Hog Shoal; NWB, New; STR, Strawberry; HKN, Hawk's Nest; BDN, Beadon's; VEX, Vexton; EIS, Egg Island; LDG, Ledge.

ceived a quota (of equal size, regardless of boat size). Vessel owners were required to buy a tag costing $1.25 per bushel for each bushel they expected to harvest up to their quota. A time period was set in which the quota was to be used. After this period the status of the resource, markets and other factors were evaluated, and another quota decision was made. In most cases the quota per boat was increased. This activity has generated a considerable amount of revenue (Table 3). Purchase of tags alone totaled $374,615 (through the fall of 1998). This money was deposited into an "Oyster Resource Development Account" that is used for shell planting and moving oysters from upper to mid bay beds.

Direct marketing from public beds goes against a trend towards privatization, which is generally considered more efficient than public fisheries (Haven et al. 1978). Because direct marketing does not require maintaining leased private grounds nor capital investment in moving oysters from the public to the private grounds there is a possibility that new participants could more easily enter the fishery. Yet, direct marketing from the New Jersey Beds has been the only obvious use of the resource under prevailing disease conditions. For instance, in 1991 and 1995 (the beds were closed from 1992 through 1994), a total of 390,000 bushels was taken from the seedbeds and transplanted to the leased grounds, but because of high subsequent mortality, only 63,000 bushels (2.6 × 10^6 L) were landed, producing a total return of $1,189,190. For each bushel removed from the seedbeds, direct marketing has returned nearly seven times more in dockside value compared with typical planting returns during periods of high Dermo disease (Table 4).

The presence of Dermo disease has increased the reliance of oystermen and state officials on the results of university research

and monitoring. In the past, information about MSX prevalence was of secondary importance to the shellfish council when they were deciding whether to have a bay season (MSX was generally uncommon on the seedbeds). In contrast, the high prevalences of Dermo disease in oysters on the seedbeds raised concerns about transplanting infected oysters, which could result in rapid proliferation of the disease and high oyster mortalities before they could be marketed. Data on Dermo disease prevalence has been the primary information leading to limited seed transplanting in the past few years. A 4-week bay season was agreed to in 1995, but the shellfish council closed the beds after two weeks. Shellfish council deliberations cover a range of issues when the council makes decisions on closures (Appendix 1).

DISCUSSION

Aspects of the Delaware Bay Management Structure

Management of the Delaware Bay New Jersey oyster fishery has the elements common to many fishery management structures. It consists of a management agency, an industry, a means of data collection and evaluation, an industry council, and a set of statutes and regulations. The difference between this system and other fishery management structures is the way these entities relate to each other. Although these special relationships cannot, by themselves, be credited with the continued persistence of harvestable oyster populations in Delaware Bay, we believe their implementation has developed an atypical management program.

There are at least six basic differences—some obvious, others subtle—between the Delaware Bay New Jersey management scheme and many others. First, as for several estuarine shellfish-

TABLE 2.

Weekly estimations of average percent oyster volume during seed bed harvest season. Values below 40% are shaded. Bed designations are the same as in Table 1. ND = no data.

Year	RIS	ARN	MID	COH	SHJ	SHR	BNS	BEN	OB	NPT	HGS	NWB	VEX	LDG	EIS	AVG
1958			33.5	36.8	33.4	30.7		34								33.7
ALL SEED BEDS CLOSED TO HARVEST (1959–1960)																
1961			35.6	40.3	37.1	24.1										34.3
1962			32	29.8	31.7	30.3										30.9
ALL SEED BEDS CLOSED TO HARVEST (1963)																
1964			17.3	28.2	15.9											20.4
ALL SEED BEDS CLOSED TO HARVEST (1965)																
1966		74.3	38.6	33.5		23.7				39.5	48.7					43
ALL SEED BEDS CLOSED TO HARVEST (1967)																
1968																ND
1969																ND
1970																ND
1971											45.5					45.5
1972				64.4		50.8						56.3				57.2
1973				58.6	56	55.3					47	57.1				54.8
1974			85.5	71	75.4	69.9		63				66.5			52.5	69.1
1975			79	81.9	71	85.8		78.1			55	78				75.5
1976				74				68.2				64	78.5		65	69.9
1977								52.6				57	71	64.3	39	56.8
1978						66.4	69.7	49.4	32			43.8	53.9			52.5
1979						51.7		37.4				43.5				44.2
1980						54.9		46.8	35		45.5	61.3				48.7
1981																ND
1982	64	36.3		36.2		36.6		28.3				31.3			37.3	38.6
1983								32.2				44			35	37.1
1984			36	49.7	30.7	63.5	58.7	52.2				31.8			27	43.7
1985						59	54.8	30.8				31.8			35	42.3
1986	60.7					19.2	25.9	23.6				25				30.9
ALL SEED BEDS CLOSED TO HARVEST (1987–1989)																
1990						52.4	52									52.2

eries, there is no formally written and passed management plan, nor has there ever been one. Only a number of very basic provisions are encoded in State statute and regulations. Second, the fishery has been closed to new entries since 1981. Third, all of the major participants are housed in close proximity to each other and have been for nearly a century (the Haskin Shellfish Research Laboratory is located at the home port of the New Jersey oyster fleet and the State Bureau of Shellfisheries has an office in the Laboratory building). Fourth, these three groups have worked, and continue to work, together closely in various combinations. Fifth, a tripartite relationship exists in which each entity has a specific role: the industry is represented by the Delaware Bay Shellfish Council, the State Bureau of Shellfisheries provides the administrative support for the Shellfish Council and the Commissioner of the Department of Environmental Protection makes final decisions based on Council recommendations, and an independent group (in this case the Haskin Shellfish Research Laboratory) collects and provides data to the other two. Sixth, formal, informal, and personal information exchange between all three parties takes place on a regular basis. The actual importance of these six differences in the development, evolution, and execution of the management strategy is not easily evaluated; however, the salient features of each are described below.

1. The lack of a written plan provides flexibility. The process required to make changes can be adapted to the situation at hand and, with the exception of those portions that are en-

coded in law, most issues are settled in Council meetings. All decisions are made openly (regularly scheduled shellfish council meetings are advertised in the paper, anyone may attend the meetings and express their opinions to the gathering, minutes are taken and distributed at the next meeting, newspaper journalists generally attend and publish articles on decisions within one to two days, and special unscheduled meetings are held after all industry members have received notification by direct mailings.). The decision process however is not burdened by regulatory needs for formal hearings, published notices, comment periods, etc. If all three parties (industry, State, and the Laboratory) agree, even major changes can be accomplished relatively rapidly. The change to harvest practices brought about by Dermo disease provides an example of this flexibility. This disease caused such high losses in oysters that by 1995 it was obvious that the traditional movement of oysters from the seedbeds to the planted grounds in spring was neither commercially viable nor biologically desirable. Discussion began in the fall Council meetings about harvesting directly from the seedbeds. This was in direct opposition to over 100 y of practice and the proposal generated a great deal of heated debate. In general, the older members of the fishery were opposed and the younger members thought that the new approach should be tried. At the end of March, after five to six meetings and an industry evaluation of the seed-
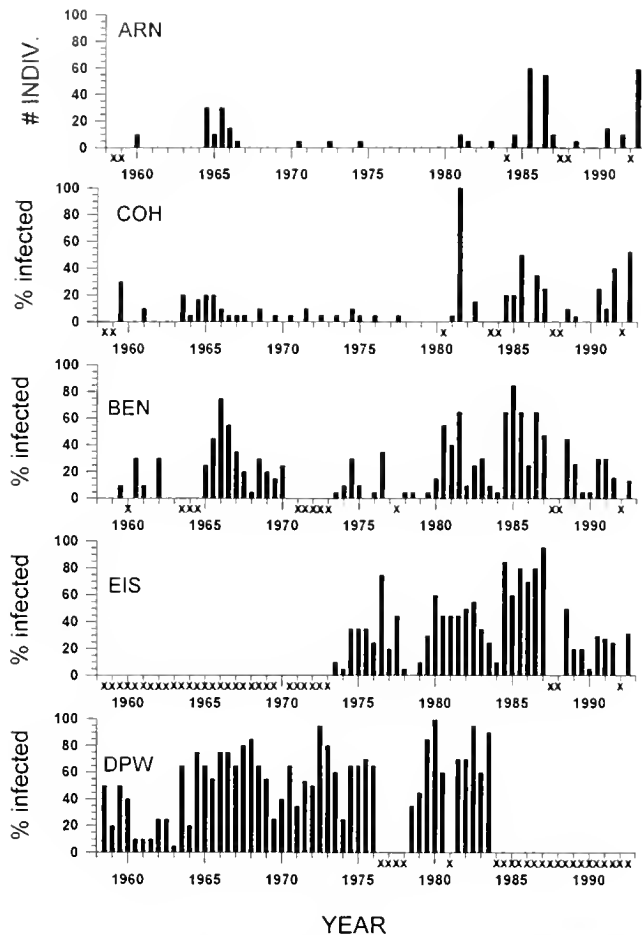
Figure 6. Percentages of live oysters infected with either MSX or Dermo disease on several seedbeds and one site located in the planting grounds (DPW, Deepwater). The absence of a symbol indicates no samples were taken from that location in that year. The sites are arrayed from the least saline (ARN) to the most saline (DPW). The abbreviations for the seedbeds are the same as in Table 1.

beds in mid-March, general agreement was reached that a direct harvest should take place beginning in mid-April, with each boat limited to 1000 bushels instead of the normal unlimited transplantation. This change in statute was introduced into the New Jersey State legislature with three provisions: 1) a limited direct market program should be attempted, 2) if oysters were to be harvested from the beds, a per-bushel fee should be imposed with the proceeds used for bed rehabilitation, and 3) the harvest would have to be actively managed to be successful; recommendations for opening and closing the season should be in the hands of the Council, with final authorization being given by the Commissioner of the Department of Environmental Protection (Appendix 2). Once these aspects were "agreed upon", legislation was drafted, introduced into the NJ Legislature in May, passed and signed by the Governor by the first week in September. Fall direct harvest from the seedbeds began one week later.

2. The closed nature of the fishery means that all participants are known and readily contacted for regular or special meetings. Mailings of informational bulletins are easily accomplished. Contact is uncomplicated even though out-of-state

interests control a significant part of the industry because they have local representatives who act in much the same fashion as other local fishery participants.

3. The importance of all groups having significant on site representation cannot be overemphasized in fostering the flow of information and appreciation of differing outlooks. The close proximity permits daily contact among the parties, but more importantly, nurtures a sense of community. It allows each individual and group to become aware of the other's point of view and to understand their biases. This does not mean all groups agree on every issue, but it does allow interested participants to evaluate what is being said in a context broader than that of a formal meeting.

4. Working together in various capacities is partly an outgrowth of the close proximity of the different parties and adds to their overall ability to understand and communicate with each other. For instance, since 1989 the industry has donated a boat and captain for the Laboratory's annual survey of the seedbeds. Without this donation continuation of the annual shellbed survey would have not occurred given the existing University resources during that time. The State often collects samples for the Laboratory, has collected samples of interest to the industry, and often allows industry members to sample the beds "out of season." Laboratory representatives regularly attend Shellfish Council meetings where they present results of ongoing projects or simply answer questions on issues of immediate interest.

5. The tripartite scheme, with a party independent of the management authority collecting basic data, holds in check the belief common to many fishermen that data obtained by management agencies are biased, or that the interpretation of those data is biased. In the current scheme, both the management agency and the industry are free to criticize data collection and/or evaluation in any way they see fit. This provides a check and balance, somewhat equivalent to "peer review" on the data collection and presentation process. In addition, a research organization can use funds from competitive funding sources to support research that does not have an immediate interest to management or the industry. However, these "pure" research projects can occasionally provide new information to the attention of the industry and the management agency that they would not have otherwise.

6. The formal, informal, and personal relationships, as with the close physical proximity, allows communication and information exchange to take place on many different levels. What is said in private conversations is often not representative of the positions presented in public meetings. This is because each group has personal views that may not be appropriate for expression in a formal meeting. For instance, the formal role of the researchers is to present the facts and to elucidate potential biologic risks. Their opinion on management alternatives is frequently sought, and they may endorse certain options, but they generally refrain from advocating a specific action. These scientists may have views on whether the industry is making optimal economic use of the resource, but this would be not be expressed in a formal presentation of the data on the status of the resource. Similarly, an individual in the industry may think the resource is being exploited too heavily, but because of social relationships in a small community, not wish to express this view in

Time Period	Number of Bushels Landed (vol. in L)	Approximate Value of Bushels Landed	Value of Tags Sold
Spring 1996 (10 weeks)	17,828 (7.3×10^5)	$320,904	$22,285
Fall 1996 (7 weeks)	42,570 (1.7×10^6)	$893,970	$52,213
Spring 1997 (10 weeks)	27,479 (1.1×10^6)	$577,059	$34,349
Totals	87,877 (3.6×10^6)	$1,791,933	$108,847

Time period includes the length of the dredging season. We present the harvest in the fishery's traditional bushels but we also convert those volumes into L.

a formal meeting. The industry members look to the Laboratory or the State to present this view in the formal context.

Two obvious characteristics of management for the New Jersey seed oyster fishery has been the high degree of cooperation and mutual respect among the oystermen, State officials and university researchers. In formal Council discussions each entity generally honors each other's expertise and role. The relationship has been uneasy, particularly when the resource was scarce. In recent years, however, the restrictions on the fishery imposed by severe oyster disease have been important in maintaining a mutual dependency of the three parties. Scientific data have become recognized as being more significant than ever in the management process. Cooperation of all parties has been crucial in implementing and testing new practices. The persistence of disease and its potential to kill oysters has forced the industry to proceed cautiously and to husband the oyster resource thoughtfully. The industry may have acted in an equally prudent way in the absence of the existing management structure, although pre-1950s fishery practices suggest otherwise. We believe that the interactive management structure, described above, has fostered effective decisions about the use of the oyster resource in the presence of disease.

Scientific Data: Formal Use

Critical to the management structure has been the availability of current population data, collected in a consistent manner over a prolonged period. Although the data are clearly used, the manner of use has varied, depending on the status of the resource and the industry at the time. Below, we provide instances where the biologic data can be shown to have influenced Council decisions, others where more informal uses of the data are evident, and still others where the data were generally ignored.

Prior to 1991 (when the Dermo disease epizootic became a decisive factor) the abundance of oysters and spat, and to a lesser degree MSX disease prevalence, were considered when decisions

TABLE 4.

Comparison of returns per bushel of oysters removed from the seedbeds by planting (in 1991 and 1995) and by direct marketing (1996–1997) during periods of high Dermo disease.

Seedbed Oysters Fate	Bushels (vol. in L)	Total Sales	Average Return per Bushel
Leased grounds	390,000 (1.6×10^7)	$1,189,190	$3.05
Direct marketing	87,877 (3.6×10^6)	$1,791,933	$20.39

We present the harvest in the fishery's traditional bushels but we also convert those volumes into L (vol. in L).

were made about the length of seedbed season. A general, direct relationship of these measures and the resultant occurrence or length of the season is apparent (short or no season when percent oyster <40%, longer seasons when percent oyster >40%; Fig. 5). On specific occasions, the data clearly influenced decisions. In 1972 Bennies Bed was closed to dredging. At that time the relative abundance of oysters was over 40% and the proportion of oysters infected with MSX in the preceding two springs was low; however, oysters were available on other beds and the opportunity to allow previous good sets on Bennies Bed to mature undisturbed by dredging was realized. The usefulness of this decision was never formally tested because in 1972 that bed and the remainder of the bay experienced another, even larger, recruitment event that proved to be an important source of oysters for years to come.

Data use has been amply illustrated since 1991 when it was recognized that planting oysters infected with the Dermo parasite would likely result in unacceptably high losses of planted oysters and loss of shell from the seedbeds. This realization closed the seed fishery for 3 consecutive years despite lost income to the fishery and the opposition by some industry members. The desire of these members to continue to plant as usual was muted because most participants in the fishery shared beliefs that restrained the degree of risk that the fishery as a whole would take. The shared beliefs included the following: 1) that the "disease problems" would eventually lessen (as they did with MSX), making preservation of the resource until that time an important and common goal; 2) that data gathered and presented by the "third-party" researchers were accurate and unbiased (although conclusions about the data were not always widely shared); and 3) that the experience of oystermen concerning when and where to plant, and when to harvest, were important in making decisions about the advisability of dredging seed oysters.

Scientific Data: Informal Use

There is no clear correlation in the long-term data between MSX prevalence and oyster mortality on private leases. A major reason is because the total mortality on a particular ground is only partly a function of disease levels. It is also influenced by decisions of the lease holders who transplanted oysters. Oystermen frequently solicited information about MSX prevalence and intensity from the Laboratory. If MSX prevalence and intensity seemed to be increasing on the leased grounds, some planters would equip extra boats to harvest oysters to insure they retrieved all marketable individuals before they died (L. Jeffries, pers. comm., 1995). Not all lease owners availed themselves of the data or, if they did, acted on them. Oystermen were free to ignore the monitoring data and gamble that the disease would be less destructive than expected.

Little or No Data Use

The "40% rule" was ignored on several occasions during the 1960s and late 1980s (2 and Fig. 5). The industry was still reeling from the financial losses caused by the initial MSX epizootic in the 1960s and was severely stressed again in the 1980s because of a drought that stimulated renewed MSX activity. Economic pressures clearly predominated over the biologic data; however, the data were not entirely ignored because the length of bay season was restricted to only 2 wk in most of these years.

Economic and Financial Pressures

Economic considerations continually threatened this management strategy. Oystermen had to maintain cash flow during prolonged periods when oyster harvests were small or impossible; most responded by diversifying their activities. Boat owners who also owned shucking houses kept the houses active by shucking oysters from other locations (primarily Connecticut, but also from the Gulf Coast) or by processing surf (*Spisula solidissima*) and mahogany clams (*Arctica islandica*). Some oystermen moved boats into the Atlantic surf clam fishery or used them to harvest finfish, blue crabs (*Callinectes sapidus*), whelks (*Busycon* spp.), or horseshoe crabs (*Limulus polyphemus*) in Delaware Bay. Others diversified economically by direct marketing of multiple seafood products or managing marinas. Some of the older oystermen possessed sufficient cash reserves to temporarily retire. Many younger participants left the industry; the on-again off-again nature of the fishery restricted their ability to reenter. The large costs of preparing a boat to work in the fishery when economic return was so uncertain resulted in a *de facto* limited entry fishery prior to the establishment of a regulatory limited fishery. Only those who could risk substantial financial losses could continue to participate.

CONCLUSION

New Jersey's management of the Delaware Bay oyster seed fishery demonstrated an ability to respond relatively quickly to both threatening and promising changes in the dynamics of oyster populations and oyster mortality sources. Despite this flexibility and the fact that management is largely in the hands of the industry itself, the resource has been generally well conserved. In fact, the impact of seed dredging on the oyster population cannot be statistically measured (Fegley et al. 1994). We suggest that the primary reasons for the persistence of the resource include (1) the high degree of communication among the three parties involved in the management strategy, (2) the presence within the industry of a few individuals who took a long-term and relatively conservative management view and who were generally respected by others in the industry; and (3) the perception of a shared risk among industry members, which also constrained their activities.

Not all aspects of the Delaware Bay management system may apply to other fisheries. For example, the fishery has relatively few participants who operate in a geographically constrained area. Part of the resource lies within an area where diseases and predators are absent or reduced by prevailing environmental conditions. Both of these conditions reduced the scale of management complexity in the present case. However, several characteristics of this fishery and its management structure could be exported to other locations. We argue they include the following: Human harvest activities on some parts of the resource need to be limited. Long-term, reliable, third-party monitoring of the resource, diseases, and harvest activities should be integrated as a consistent part of the decision processes of the management structure. Continued personal contact through meetings, discussions and working together is essential in transmitting information. Last and most importantly, the participants in the fishery should agree on the basic goals of the program and all must play a role in the management of the bed and its dependent fishery. Participating groups must agree on their respective formal roles, restrain themselves from "stepping beyond" their areas of expertise, and respect the role and viewpoints of the other participants.

APPENDICES

I. An Example of Shellfish Council Deliberations Before the Advent of Large-Scale, Direct Marketing from the Seedbeds

The following account describes deliberations by the Delaware Bay Shellfish Council during bay season of 1995. Oyster planters, representatives of the New Jersey State management agency, Rutgers University personnel, and shellfish council members participated in what was often a chaotic discussion. However, a consensus was reached. A cursory description is presented here to provide an example of the issues considered when making decisions and of how biologic information provided by the University was integrated with economic realities faced by planters.

Bay season had begun on 10 April and was scheduled to last for a minimum of two weeks. A decision on the closing date was to be made near the end of the second week. On 20 April 1995, the shellfish council met to examine dredge samples that had been collected from the beds that day and to consider extending bay season. By that date approximately 3000 bu of oysters had been marketed directly from the beds at approximately $15–$17 per bushel. A little more than 20 boats harvested (seed for planting plus direct market) a total of about 100,000 bushels. Most of the harvest was from New, Bennies, and Bennies Sand Beds. Some harvest was from Ledge Bed. Sampling to determine percent oyster on the beds was conducted on 13 April and 20 April from Bennies Sand and New Beds, and from New Beds on 20 April. Mean percent oyster was high on both dates (Bennies Sand = 61% and New Beds = 63% on the 13th and New Beds = 62% on the 20th).

Although there was general agreement that plenty of oysters remained on all of the beds, several other concerns were discussed. First, prices for oysters marketed directly from the beds were low and only 3' oysters were acceptable. This meant that a good deal of costly on-board sorting was required to produce a marketable product. Second, as nearly all seed was infected with *P. marinus*, any oysters planted on the leased grounds would have to be marketed before July to avoid mortality. Third, the season had been

good so far. Transplanting more oysters to the planting grounds would likely lead to decreases in profit because summer prices are usually low and the cost of moving oysters might not be recovered if subsequent mortality was high. Fourth, if oysters were not moved and they died on the seedbeds, at least the shells would remain as cultch. Fifth, if the beds remained open, everyone would keep fishing in spite of the economic risk. After listening to all these issues the shellfish council opted for a conservative strategy and decided to close the seedbeds for the season.

H. An Example of Shellfish Council Deliberations After the Advent of Direct Marketing from the Seedbeds

Direct marketing of oysters from the seedbeds has had mixed results. This process provided $4.3 million in revenues to the industry for harvests in 1996, 1997, and the spring of 1998, and allowed the industry to maintain a presence in the markets and maintain boats. Tag fees provided for an enhanced shelling effort. The down side to this form of landing was that the industry was restricted to the time period agreed to and could not stockpile oysters on the planted grounds to satisfy markets at other times. Because the oysters were harvested from lower salinity waters, the meat quality was not as good as in oysters from farther down bay and the price received for the product was not as high as it might have been. Chiefly because of these latter conditions, some industry members wished to plant oysters.

The State achieved direct revenue ($1.25/bu) from oysters removed from the seedbeds for market, but would only receive payment on planted oysters once they were landed. Thus in the former case the State (and directly the oyster industry accounts) received payment up front, while in the latter case the State took on the majority of the risk. If the oysters died on the planted grounds the resource would not be paid for, the shell would no longer be on the seedbeds, and no funds would have been generated to replace it.

In 1997 the State and industry agreed to a spring direct harvest followed by an evaluation of the seedbeds to determine if a planting season could be allowed in the summer of 1998. The chief reason for the planting would be to allow meat quality to improve during the late summer and fall. The chief worry was the level of the oyster disease Dermo. University researchers sampled for Dermo and reported to the council in an open meeting. Samples were removed in July from the five beds deemed by the industry to have the greatest probability of being harvested. The samples revealed that oysters on all beds were heavily infected with Dermo. The summer had been hot and dry and the forecast was for a continuation of these conditions.

The discussion in the August 1998 council meeting was heated because some segments of the industry wished to move oysters anyhow, while others were reluctant to risk the resource. The latter group said that the resource would remain for later harvest if it was not moved. The group finally agreed to wait and monitor conditions further. Laboratory researchers took samples in August. Conditions had not improved and the Council deferred a seed move and decided to allow direct market harvest to begin (1500 bu/license) beginning on 17 August. The council requested a September sample of disease prevalence: it remained high. The council decided to have a 5-d transplant in an 8-d period beginning 7 October. To participate each boat would have to participate in a one day intermediate transplant (5 and 6 October) in which oysters from up bay would be moved to an intermediate bed. Direct market harvest would cease when the transplant began. A meeting was scheduled for 1 October to make final adjustments to this plan. In October the direct market program allocation was increased by 1000 bu/license, otherwise the transplant program was to occur as decided earlier.

As of the November council meeting the direct market program had landed approximately 73,000 bu: 10,000 bu were moved in the intermediate transplant and 58,800 bu were transplanted to the leases.

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INFLUENCE OF TIMING OF BROODSTOCK COLLECTION ON CONDITIONING, OOCYTE PRODUCTION, AND LARVAL REARING OF THE OYSTER, *CRASSOSTREA GIGAS* (THUNBERG), AT SIX PRODUCTION SITES IN FRANCE

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ABSTRACT Gametogenic development and response to conditioning procedures of six samples of oysters *Crassostrea gigas* (Thunberg) collected in the Bassin d'Arcachon, each cultivated at a different production site along the Atlantic coast of France were compared simultaneously from December 1998 to July 1999. Oysters were conditioned with and without food (fed oysters and unfed oysters, respectively). Samples at northern production sites (Baie des Veys, Aber Benoît, and Baden) initiated gonadal development and spawning about one month earlier than those at southern production sites (Bouin, La Tremblade, and Arcachon). Three conditioning experiments (December 1998 to February 1999, February to April 1999, and April to June 1999) favored Baie des Veys and Aber Benoît oysters, because these resulted in higher body component indices and higher proportions of mature oocytes in the three conditionings that produced more gametes than the other samples in all experiments. Unfed oysters from Baie des Veys and Aber Benoît produced viable gametes and larvae in all the experiments. No significant difference was observed in larval culture (growth and mortality) among samples, of both fed and unfed animals. Differences in the timing of gametogenesis and response to conditioning among northern and southern samples seem adaptive and non-genetic in nature, since all oysters were collected from the same population in the bay at Arcachon. Nutrient recycling seems to have been an important regulating factor for gametogenesis in the northern samples. The occurrence of oysters in different locales having differences in the timing of gametogenesis and response to conditioning has implications for spat production in hatcheries.

KEY WORDS: conditioning, *Crassostrea gigas*, gametogenesis, larvae, oocytes

INTRODUCTION

The Pacific oyster *Crassostrea gigas* (Thunberg) was introduced to France to replace the Portuguese oyster *C. angulata* that was decimated by a virus in the 1970s (Héral 1989). The Pacific oyster exhibited high survival rates and adequate growth. Since 1982, spat importation from Japan and progenitors from British Columbia were no longer needed because the collection of juveniles in French bays and lagoons became sufficient to sustain oyster cultures (Mann 1983). The oyster industry expanded extensively throughout this time and France became the fourth largest oyster producer in the world in 1994. At the present time, there is great inter-annual variability of spat setting at collection sites caused by collector overcrowding and hydroclimate variations (Robert & Gérard 1999). Moreover, the demand for spat increased continually over time, but hatcheries produced only 10% to 15% of the juveniles required by oyster farmers. Under these circumstances, national management programs are directed at studying reproductive factors affecting this species under hatchery conditions to improve current spat production procedures.

The culture of *C. gigas* in France is conducted in four stages: (1) spat collection; (2) intermediate culture; (3) culture; and (4) fattening (Cochard 1990). Juveniles (12 to 18 mo) are transferred from collection areas at Marennes–Oléron embayment and Arcachon lagoon to production sites, where they continue development until reaching commercial size (24 to 36 mo later, depending on the region). In France, *C. gigas* reaches first sexual maturity in 12

to 18 mo (Soletchnik et al. 1997). This means that oysters can complete two reproduction cycles before the end of the harvest period at production sites. Since oysters are exposed to fluctuations in temperature, photoperiod, and quality and quantity of suspended fine particulate matter (seston) that affect their physiology and growth (Barillé et al. 1994, Goulletquer et al. 1996), we would expect important geographic variations of gametogenesis along the French coast.

Under laboratory conditions, many factors including those affecting gametogenesis and broodstock conditioning influence larval development in both early and late juvenile stages (Martínez et al. 2000). Le Pennec et al. (1998) pointed out that pectinid egg development and consequent larval production are extremely variable in hatcheries and that results are not reproducible from one year to the next. For *C. gigas*, Lannan et al. (1980) demonstrated that this variation is related to gonadal development of parental oysters and that this involved environmental and heritable components. Seasonal studies have shown that environmental factors, such as temperature and food availability, are closely related to reproductive performance in bivalves (Ruiz et al. 1992). For instance, the quantity of phytoplankton in temperate and high-latitude seas varies seasonally, producing cyclical changes in availability of nutrients (Gabbott 1975, Abad et al. 1995). Regulatory substances with gonadotrophic action vary periodically, and they play important roles in spawning and in maturation of oocytes and adults (Deridovich & Reunova 1993).

To obtain gametes in hatcheries in an optimum state of development, it is essential to know the gonadal stage of the parents at the time of conditioning, as well as the rate of gametogenesis during the conditioning intervals (Lannan et al. 1980). Moreover, during the conditioning of broodstock from different localities, seasonal variations in gonadal development must be identified

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(Chávez–Villalba et al. 2002). The objective of this study is to discover the response to artificial conditioning procedures of oysters originating in Arcachon, but cultivated in six different geographic regions of France. This was accomplished by simultaneously comparing conditioning with and without food, oocyte production, and larval rearing during three consecutive periods of oyster cultivation from Baie des Veys (BV), Aber Benoît (AB), Baden (BA), Bouin (BO), La Tremblade (LT), and Arcachon (AR).

MATERIAL AND METHODS

Experimental Conditions

At the end of November 1998, 150 oyster samples were taken from each of six production areas along the Atlantic coast of France, where they had been cultured in plastic bags on tables for almost two years. These oysters had been collected in the Bassin d'Arcachon, grown at Baden until they were 18 mo old, and distributed in March 1997 to the various culture sites. Two sites are situated along the English Channel: Baie des Veys (BV) in Normandy and Aber Benoit (AB) in Brittany. Four are located along the Bay of Biscay on the Atlantic coast: Baden (BA), Bouin (BO), La Tremblade (LT) and Arcachon (AR) (Fig. 1). Specimens were obtained at the same time from all sites and transported immediately to the IFREMER center at Brest, where they were placed in a flow-through seawater system for one week. Temperatures in the tanks were maintained in close proximity to those found at the production sites at the time of collection; 10°C in December, 11°C in February and 12°C in April. The procedure was conducted three times: December 98 (first conditioning), February 99 (second conditioning), and April 99 (third conditioning). Additionally, oysters were collected in June and July 99, but they were not conditioned because they were mature (Lango–Reynoso et al. 2000).

After acclimation, each sample was divided into two groups for conditioning and transferred to seawater maturation tanks, where the temperature was increased 1°C per day until 19°C (heating period) and the photoperiod was adjusted to 16 h of daylight.

Oysters were subjected to two conditions: with and without food. The fed groups had a diet used commonly for conditioning in experimental hatcheries: a mixture of two microalga species (10^9 cells of each species/day/animal) from monospecific cultures of *Isochrysis aff. galbana* Green (Clone T-iso; Tahitian *Isochrysis*) and *Chaetoceros calcitrans* Takano. The samples recovered in June were tested for histology only, and the samples collected in July were tested for histology and stripped immediately (see larval culture).

Sampling

Upon arrival at the laboratory, 20 oysters were chosen randomly from each sample for biometrical measurements. Weights of whole animals, empty shells, and soft tissue were determined to within ±1 mg using a digital balance. The soft tissues of 10 oysters were freeze-dried during 48 h, and dry weights were measured. The body component index of Walne and Mann (1975) was calculated:

$$WMI = \frac{DSTW * 1000}{DSW}$$

where *WMI* is the Walne–Mann index, *DSTW* is the dry soft tissue weight in grams, and *DSW* is the dry shell weight in grams.

Only fed oysters were considered for histologic study. Two samples of 10 oysters each were taken from each group during the conditioning experiments. The first sample was obtained before the heating period, and the second sample was taken at the end of the conditioning period. For individuals collected in June and July, the histologic samples were taken immediately after biometric measurements.

Semi-quantitative Histologic Analysis

Oysters used for histology were opened, and a section of approximately 1 cm³ visceral mass was taken from above the pericardial area, and fixed in Bouin's solution for at least 48 h. Samples were dehydrated with a series of ethanol treatments of
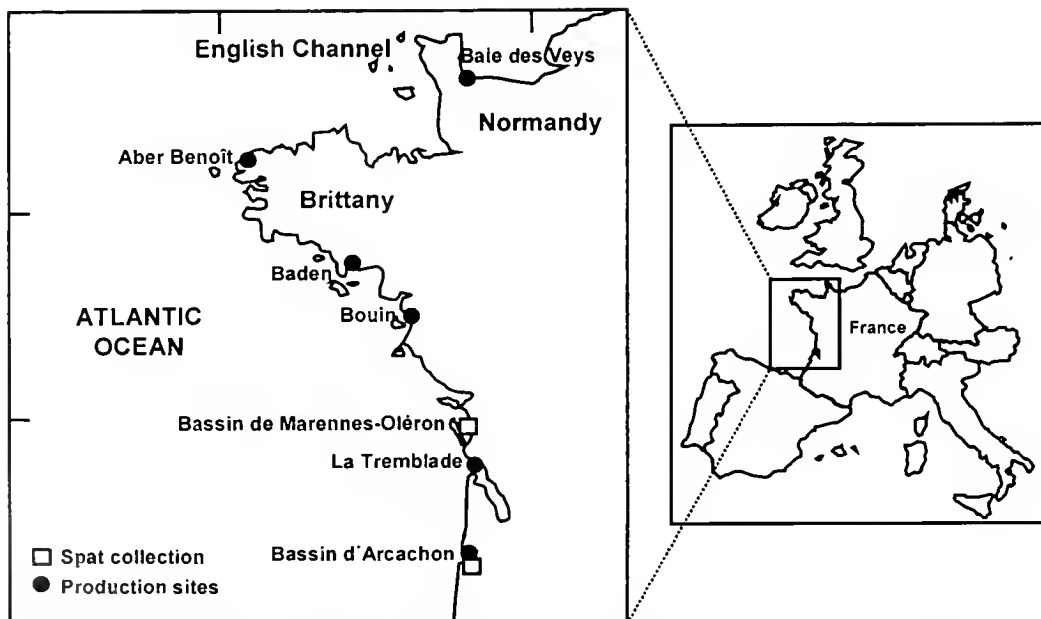

Figure 1. Location of the six *Crassostrea gigas* production sites studied. Spat collection and production sites are also marked.

increasing concentration, cleared in toluene, and embedded in paraffin following a standard procedure. Sections (5 μm) were cut, mounted on glass slides, and stained with Groat's hematoxylin and eosin Y solution (Martoja & Martoja–Pierson 1967). The histology slides were examined under a microscope connected to a video camera to determin oocyte size and frequency, and gametogenic activity. Recorded images were processed by digital image analysis.

Oocytes were measured and histology classified following the description by Lango–Reynoso et al. (2000). These operations were conducted on 100 randomly chosen oocytes per oyster, and measurements followed a standard bias reduction procedure for selecting measurement fields. Transects of gonad preparations were oriented to maximize coverage of the larger vertical or horizontal oocyte field axis. All oocytes with a well-defined germinal vesicle in a field were measured, and every oocyte measured was assigned to a reproductive stage based on diameter and histologic characteristics of the gonad (Table 1).

Larval Rearing (larval yield estimation)

Both fed and unfed oyster groups were considered for larval rearing. Following seven weeks of conditioning, oysters were opened and their sex was determined by observing a fresh smear sample from the gonad under a microscope. After this procedure, females and males were separated and gametes from both sexes were recovered using the scarification technique described by Allen and Bushek (1992). Gonads of all oysters were scarified by a light incision of the gonadal tegument. Oocytes were collected in beakers by rinsing the gonad with filtered seawater. Then the oocytes were passed through a 60-μm sieve to eliminate undesirable material. Mature oocytes were retained in a 20-μm sieve. These were rinsed several times and placed in 2 or 5-L beakers. To determine oocyte production, three 50-μL samples per group were examined, and counted under a profile projector. Males underwent the same procedure, but spermatozoa suspensions were examined under a microscope for mobility. Batches of spermatozoa of low mobility were discarded. A minimum of three batches was mixed together and diluted 10- to 20-ml l^{-1} for fertilization. Oocytes were fertilized in 5-L beakers, and checked for normal progress 0.5 to 1 h later (Robert & Gérard 1999).

After fertilization, an equal number of embryos from all oysters of each group were pooled together and placed one group per tank in 150-L experimental tanks at concentration 33 embryos per ml. After 48 h the tanks were emptied and the larvae recovered by sieving. Three 50-μL larvae samples from each tank were taken for larval yield estimation: number of D larvae after 48 h of culture/initial number of embryos.

Standard methods were used during larval rearing. Tank seawater temperature was maintained at 20°C throughout the experiment, and larval diet consisted of a mixture of three microalga species: 40% *Chaetoceros pumilum*, 40% *Isochrysis aff. galbana* Green (Clone T-iso, Tahiti *Isochrysis*), and 20% *Pavlova lutheri*. Feeding increased from an initial concentration of 80,000 cells/ml to a final concentration of 150,000 cells/ml. Seawater in the tanks was renewed every two days, and larvae were recovered by sieving. Larvae were measured during the second, ninth, and sixteenth days of culture by sampling 1 or 2 mL of seawater containing larvae from each experimental tank after sieving. Larval samples were placed on microplates and fixed with formaldehyde (5%). Two or three pictures of each sample were taken using a Scioncorp frame grabber and processed by image analysis for size evaluation (Scion Image for Windows). Larvae length was deemed equivalent to that of the major axis of the best-fitting ellipse.

Data Analyses

The oocyte proportion corresponding to each reproductive stage was calculated according to Lango–Reynoso et al. (2000), and arcsine transformed (Snedecor & Cochran 1972) for each oyster. The logarithms of oocyte production data were calculated. The transformed proportions and logarithms were compared using the Kruskal–Wallis test. A two-way ANOVA test was used to examine the effect of conditioning and sample on (1) early, growing, and mature oocyte categories; (2) the Walne–Mann index; (3) oocyte production; and (4) the D larval yield. A three-way ANOVA test was run to analyze the effect of conditioning, days of culture, and origin (sample) on larval culture. Statistics were analyzed at significance level $\alpha = 0.05$.

RESULTS

Gametogenesis

The results of oocyte evolution from December 98 until July 1999 are presented here without regard to conditioning experiments. The mean oocyte size for each sample is shown in Figure 2. Oysters from the six samples had the same oocyte diameter distribution except the AB sample that had degenerating oocytes in December 1998. In this sample we detected that the proportion of oocytes in the early gametogenesis stage increased significantly (36.4–92.4%) at the same time as that of degenerating oocytes decreased significantly (44.8–0.0%) from December to February.

TABLE 1.

Reproductive scale for *Crassostrea gigas* proposed by Lango–Reynoso et al. (2000). Each reproductive stage is based on an oocyte diameter (μm) interval. Cytological characteristics corresponding to each stage are included.

Stage	Interval (μm)	Histologic Description
Early gametogenesis	3.0–12.0	Follicles are elongated and often isolated in the abundant connective tissoe, with walls consisting of primary oocytes of homogeneous size.
Growing	12.1–30.0	Start of oocyte growth. A large range in oocyte size at all gametogenic stages can be observed, including some free oocytes. Interfollicular connective tissue disappears.
Mature	30.1–41.0	Follicles of homogeneous size completely filled with mature oocytes with distinct nucleus.
Degenerating	41.1–60.0	Follicles containing degenerating oocytes, often elongated in shape, sometimes broken. Obvious redevelopment indicated by increased number of primary oocytes.
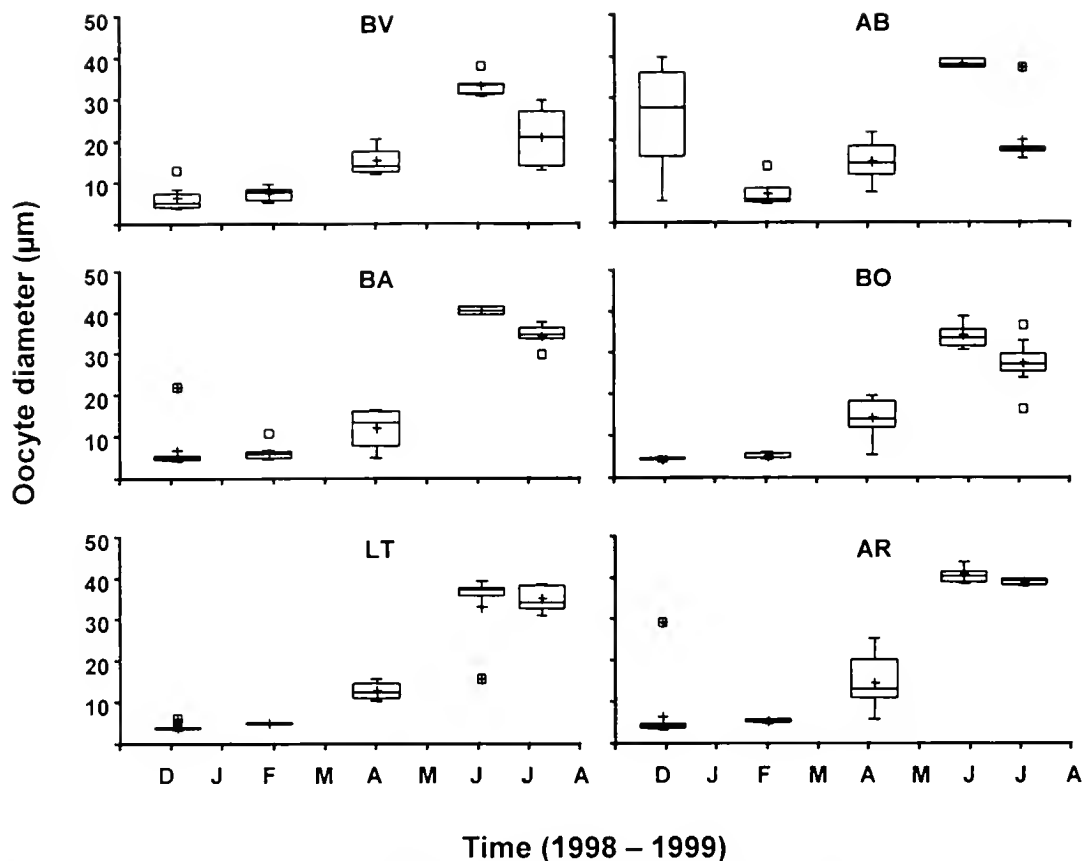
Figure 2. Evolution of oocyte diameters (mean ± SD) over time in *Crassostrea gigas* specimens collected at six production sites on the Atlantic coast of France: BV (Baie des Veys), AB (Aber Benoît), BA (Baden), BO (Bouin), LT (La Tremblade), and AR (Arcachon).

In the other samples, oocytes at this stage did not change significantly during the same period. In February, growing oocytes were observed only in the oysters from BV, AB, and BA (4.0, 8.0, and 3.0% respectively). In all samples, oocytes in growing and mature stages increased significantly from February to April, and from April to June, respectively. The BV, AB, and BA samples, which contained growing oocytes in February, were the same samples in which mature oocytes decreased significantly from June to July. The oocytes in the early gametogenesis and growing stages increased significantly in the BV (2.4–28% and 18.6–47.6% respectively) and AB (0.0–33.2% and 1.3–49.0% respectively) samples during the same period. In the other samples (BA, BO, LT and AR), no significant change was detected from June to July.

Conditioning

The results of the three conditioning procedures performed in this study are presented in Figure 3. In the BV sample, the proportion of growing oocytes increased significantly in the first (3.0–18.8%) and second (3.7–30.7%) conditionings, and in the last experiment the proportion of oocytes in this category decreased significantly (57.0–7.0%). The proportion of mature oocytes increased significantly in this sample in all the experiments. By the end of the first conditioning, degenerating oocytes were no longer observed in the AB sample, and there was no significant change in growing oocytes, but the proportion of mature oocytes increased significantly in the three conditionings. For the BA and BO samples, the same pattern as for the AB sample was observed, except during the second conditioning experiment when the pro-

portion of growing oocytes increased significantly (2.9–18.0% in BA, and 0.0–27.2% in BO). The proportion of growing oocytes also increased significantly in the second conditioning for the LT sample (0.0–37.2%), but that of mature oocytes increased significantly only in the second (0.0–41.5%) and third (0.0–89.7%) experiments. Finally, the proportion of AR sample growing oocytes increased significantly during the first (0.0–39.5%) and the second (0.0–21.8%) conditionings but the proportion of mature oocytes increased significantly only in the last experiment (7.0–88.2%).

The two-way ANOVA test on early gametogenesis, growing, and mature oocytes showed that the effect of the sample was not significant in any conditioning experiment, but the effect of conditioning was evident on the proportion of early gametogenesis stage oocytes, which increased significantly in the first and the second conditionings. No significant conditioning effect was detected on oocytes at the growing stage during the three experiments. Finally, conditioning increased the proportion of mature oocytes significantly during the second experiment.

Index

Values of the Walne–Mann index (WMI) are presented in Figure 4. The WMI increased significantly in BO (3rd experiment), LT (2nd and 3rd experiments) and AR (1st and 3rd experiments) by the end of conditioning. In the other samples no significant difference was detected between the start and end of conditioning. The two-way ANOVA test concerning the effect of conditioning showed that WMI values in the third experiment were significantly higher than those in the two previous conditionings, but these

Figure 3. Temporal variation in oocyte diameter of specimens conditioned with food, from six sites of *Crassostrea gigas* production during December 1998 to February 1999 (first conditioning); February to April 1999 (second conditioning); and April to June 1999 (third conditioning). Each line represents one individual. S (start of conditioning), E (end of conditioning), BV (Baie des Veys), AB (Aber Benoît), BA (Baden), BO (Bouin), LT (La Tremblade), and AR (Arcachon).

values were significantly lower than those of oysters collected in July. The WMI values of oysters from the AB sample were significantly higher than those from the other samples, except BV.

Gamete Production

The highest gamete production was observed in oysters collected in July except for those of BO, conditioned in April to June (Fig. 5). The two-way ANOVA test showed that there were significant conditioning and sample effects on gamete production. We observed that gamete production increased significantly from the first to the second experiment, and also from the second to the third conditioning. No difference between the last conditioning and that of oysters collected in July was observed. The AB sample produced significantly more gametes than that of BO.

We observed that only the unfed groups of AB and BV produced gametes during the first conditioning, and that the quantity was significantly higher in AB oysters. In the second conditioning, four groups produced gametes, and are ranked by gamete quality as follows: AB, BV, BA, and BO. The AB and BV groups produced significantly more oocytes than the BA and BO groups. In the third conditioning, two-way ANOVA revealed significant effects of conditioning and sample on gamete production. All groups produced significantly more gametes except LT, which produced no oocytes during the three conditioning procedures. AB oysters produced significantly more gametes than any other group (Fig. 5).

D Larval Yield

The two-way ANOVA test showed no significant effect of conditioning or sample on D larval yield of either fed or unfed oysters. D larval yield for fed and unfed oysters during the three

conditioning experiments, as well as that for animals collected in July are presented in Figure 5. During the first conditioning, the highest larval yield (80%) was observed for fed oysters from BV, while the lowest corresponded to those of AB and BA (28 and 22%, respectively). In the second conditioning, the BO group had the highest percentage (90%), while the lowest (51%) was observed for LT oysters. In the third conditioning, and for oysters collected in July, we found that larval yield was homogeneous ($\approx60\%$) for all groups. We had technical problems with unfed oysters during the second experiment, so the D larval yield was not measured and consequently, no larvae were reared. Nevertheless, we observed no significant difference in larvae yields between fed and unfed oysters during the first and third conditionings.

Larval Growth

Three-way ANOVA demonstrated significant effects of conditioning and time on size of larvae produced by both fed and unfed oysters (Fig. 6). Larvae from fed oysters, were significantly larger in the third conditioning than in the first or second conditionings. The first conditioning of unfed animals in groups BV and AB were larger also. There was no significant sample effect on larval size of fed or unfed animals. We compared the size of larvae from fed and unfed oysters, and those of oysters collected in July on the last day of culture (16th day), and found no significant difference.

DISCUSSION

The gametogenic development of *Crassostrea gigas* in this study is similar to that reported by Lango–Reynoso et al. (2000) for two populations in Brittany and one in Marennes–Oléron. We observed that the gametogenic cycle (December 1998 to July
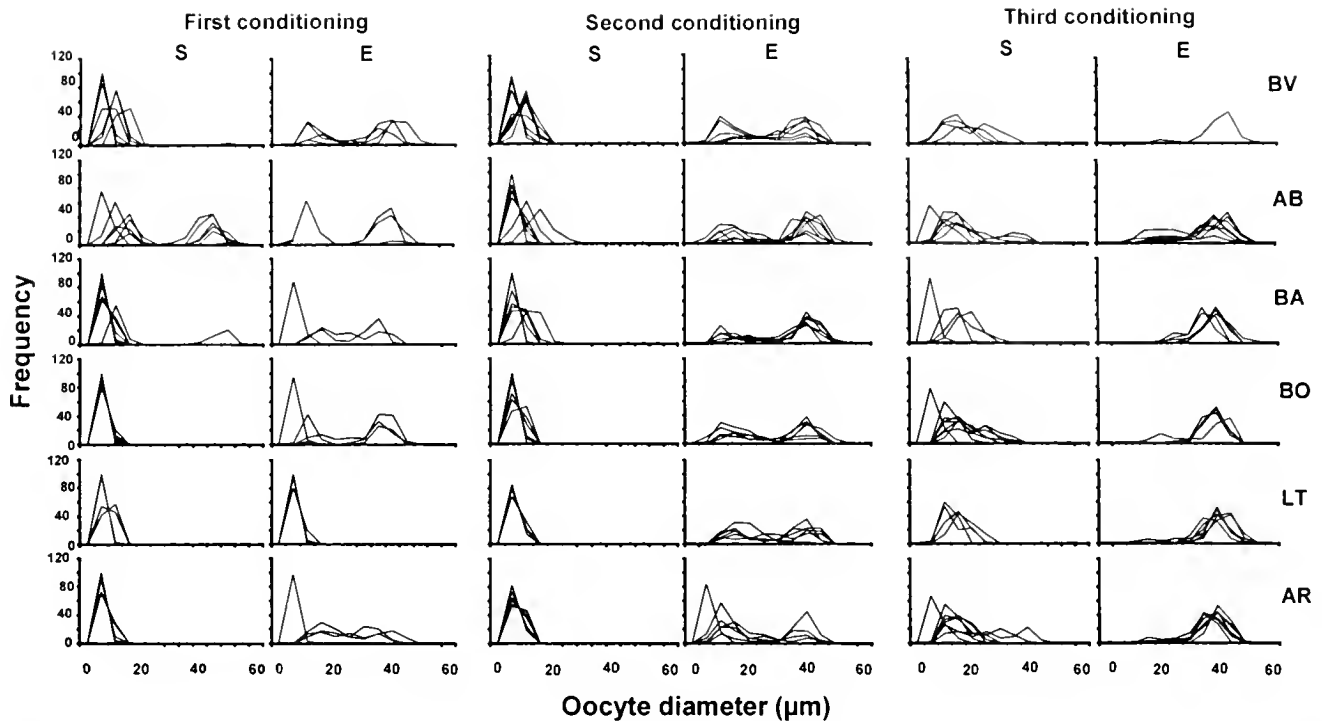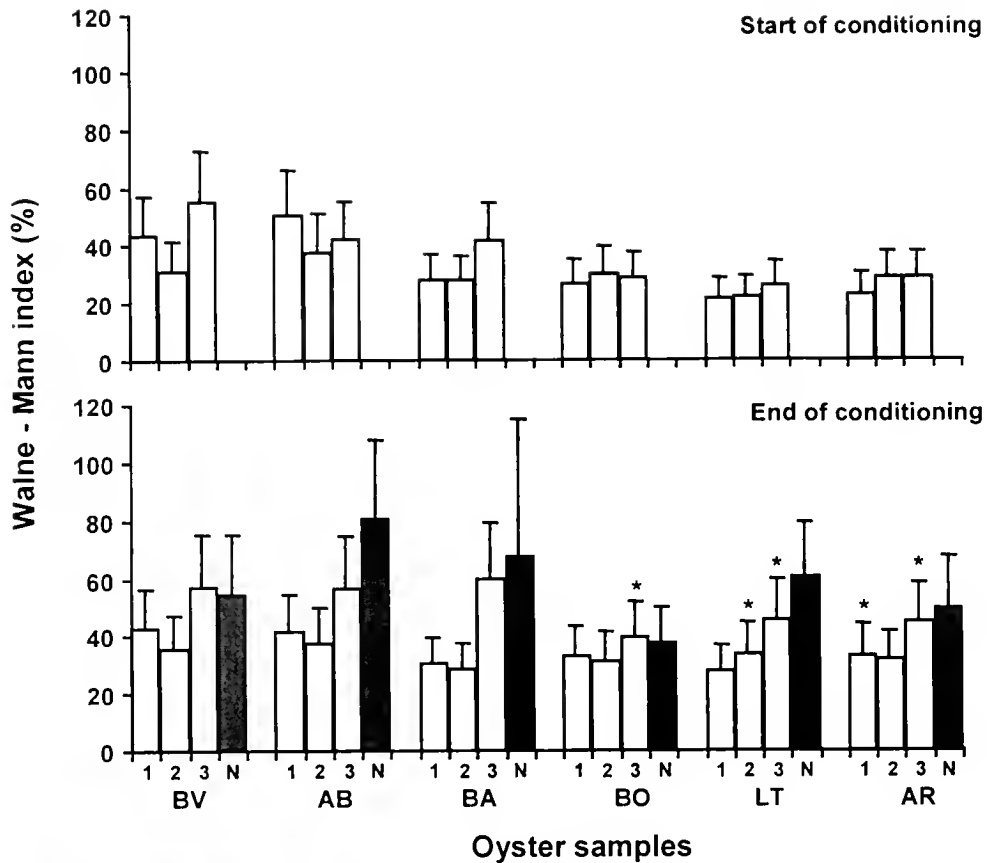
Figure 4. Walne–Mann indices (WMI) of specimens conditioned with food, from six sites of *Crassostrea gigas* production during December 1998 to February 1999 (1), February to April 1999 (2), April to June 1999 (3), and July 1999 (N). BV (Baie des Veys), AB (Aber Benoît), BA (Baden), BO (Bonin), LT (La Tremblade) and AR (Arcachon). An asterisk (*) represents a significant difference between WMIs at the start and end of conditionings in a Kruskal–Wallis test (*P* < 0.05).

1999) of all samples examined in this study followed the same pattern. Primary oocytes were evident from December to February. In the Aber Benoît sample, a large proportion of degenerating oocytes (45%) were detected in December, but not in February. Degenerating oocytes occur because the oysters at this site have partial spawnings from September to January, and gametes in the gonad are reabsorbed very slowly (Chávez–Villalba et al. 2001). Histologic observations show that only northern samples BV, AB, and BA had growing oocytes in February. Oocytes grew in all groups from February until maturity in June, and there were always more than 75% mature oocytes. Histologic observations show that northern oysters spawned only partially between June and July, and that the proportions of mature oocytes were 25%, 18%, and 52%, respectively. Moreover, we detected primary and growing oocytes in the gonads of these samples during the same period, indicating the development of a new oocyte generation. In contrast, the proportion of mature oocytes of southern samples BO, LT, and AR continued above 80%. Lango–Reynoso et al. (2000) found that oysters at northern sites initiated gonad growth, achieved maximal gonad development, and began spawning about one month earlier than oysters from Marennes–Oléron. The results of this study and those of previous experiments in our laboratory (Chávez–Villalba 2001) were consistent with the observations of Lango–Reynoso et al. (2000). Differences between northern and southern oysters in the timing of gametogenesis during the conditioning experiments revealed that northern samples performed best

in laboratory conditions. These oysters in the three conditionings presented higher Walne–Mann index values and higher proportions of mature oocytes and produced more oocytes than other samples in all experiments. Moreover, unfed BV and AB oysters produced viable gametes and larvae in all experiments. This demonstrates that differences between northern and southern sites in environmental influences regulate the initiation or completion of gametogenesis.

Differences between populations in the term and extent of gonad growth, apart from genetic differences, suggest the existence of environmental factors regulating gonad development (Barber et al. 1991). The differences found in this study should not be considered genetic since all juvenile oysters were collected in the Bassin d'Arcachon. Thus, we believe that there is intraspecific variation in gametogenesis of *C. gigas* in France that is an adaptation to different local environmental factors. Dinamani (1987) stated that the pacific oyster shows flexible reproductive behavior that includes changes in timing and length of gametogenesis depending on the environment in various regions of the world. It is known that water temperature is a principal environmental factor affecting gonad development in marine bivalves (Loosanoff & Davis 1963). Goulletquer and Héral (1997) pointed out that the temperate climate in France is affected by the Gulf Stream, with a geographic barrier around Brittany, limiting the distribution of marine species between the coldest regions in the north and warmest in the south. The fact that oysters from northern locations

Gamete production x 10⁶
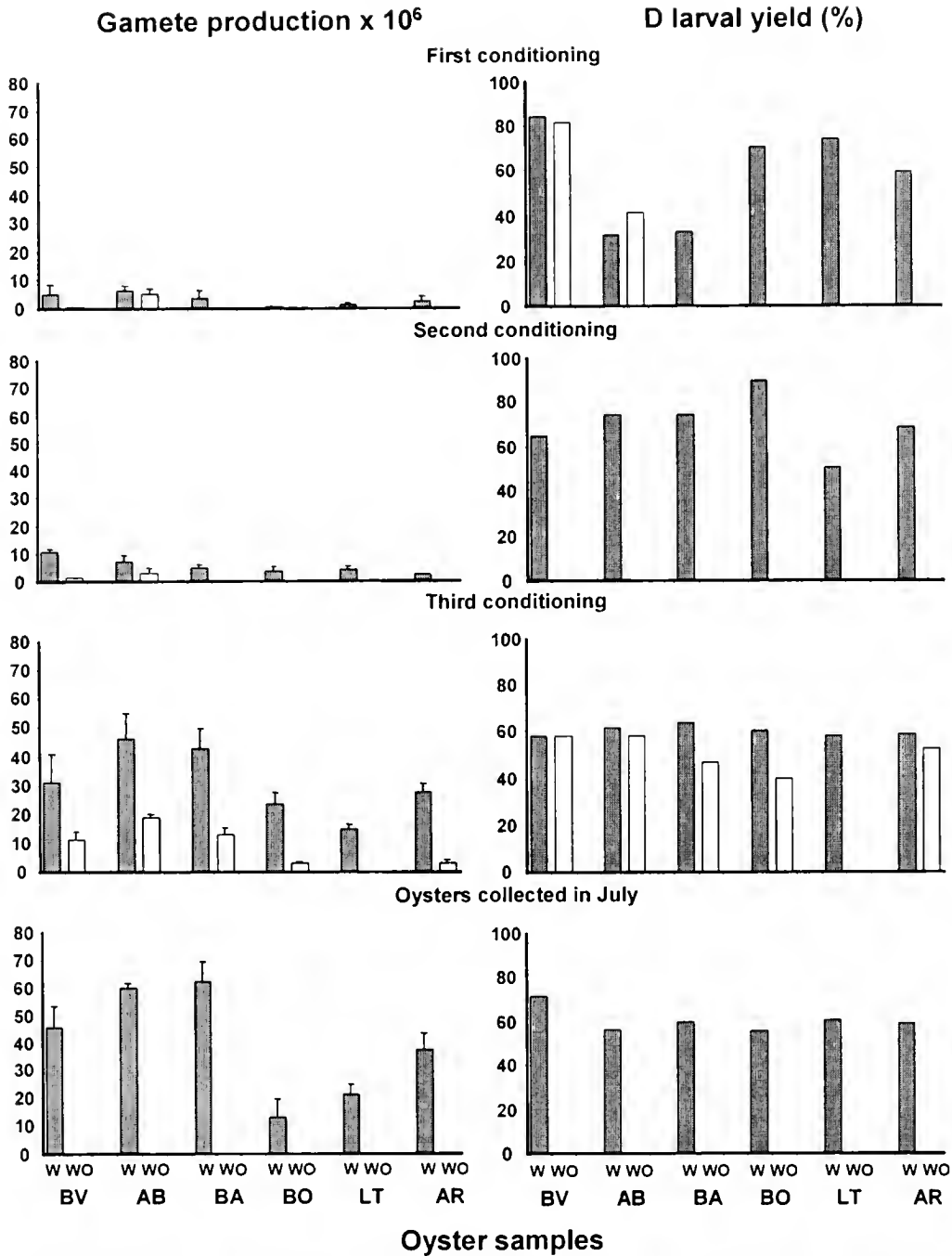
D larval yield (%)

Figure 5. Gamete production and D larval yield of specimens collected at the end of each conditioning with (W) and without (WO) food, at six sites of *Crassostrea gigas* production during December 1998 to February 1999 (first conditioning), February to April 1999 (second conditioning), April to June 1999 (third conditioning), and July 1999. BV (Baie des Veys), AB (Aber Benoît), BA (Baden), BO (Bouin), LT (La Tremblade), and AR (Arcachon).

acclimated to lower temperatures than those from the south and began gonad growth earlier, eliminates temperature as the single regulator of gonad development in *C. gigas*. Goulletquer and Héral (1997) indicated that another difference between northern and southern locations is variations in trophic conditions caused by tidal effects. Tidal cycles can vary markedly in the quality and amount of suspended particulate matter (Pastoureaud et al. 1996). We believe that differences detected in this study result from variation in stored reserves that depend on food availability (Thompson

et al. 1996). This view is supported by MacDonald and Thompson (1988), who reported site-specific variation in the gonad development of *Placopecten magellanicus*, due to adaptation to local variations in environmental factors, most notably food availability.

There is evidence that periods of reserve accumulation and gamete production are temporally separated in temperate species (Emmett et al. 1987, Thompson & MacDonald 1990). Berthelin et al. (2000) found that reserves in *C. gigas* are constituted during the autumn and the winter, and that these reserves are used later in
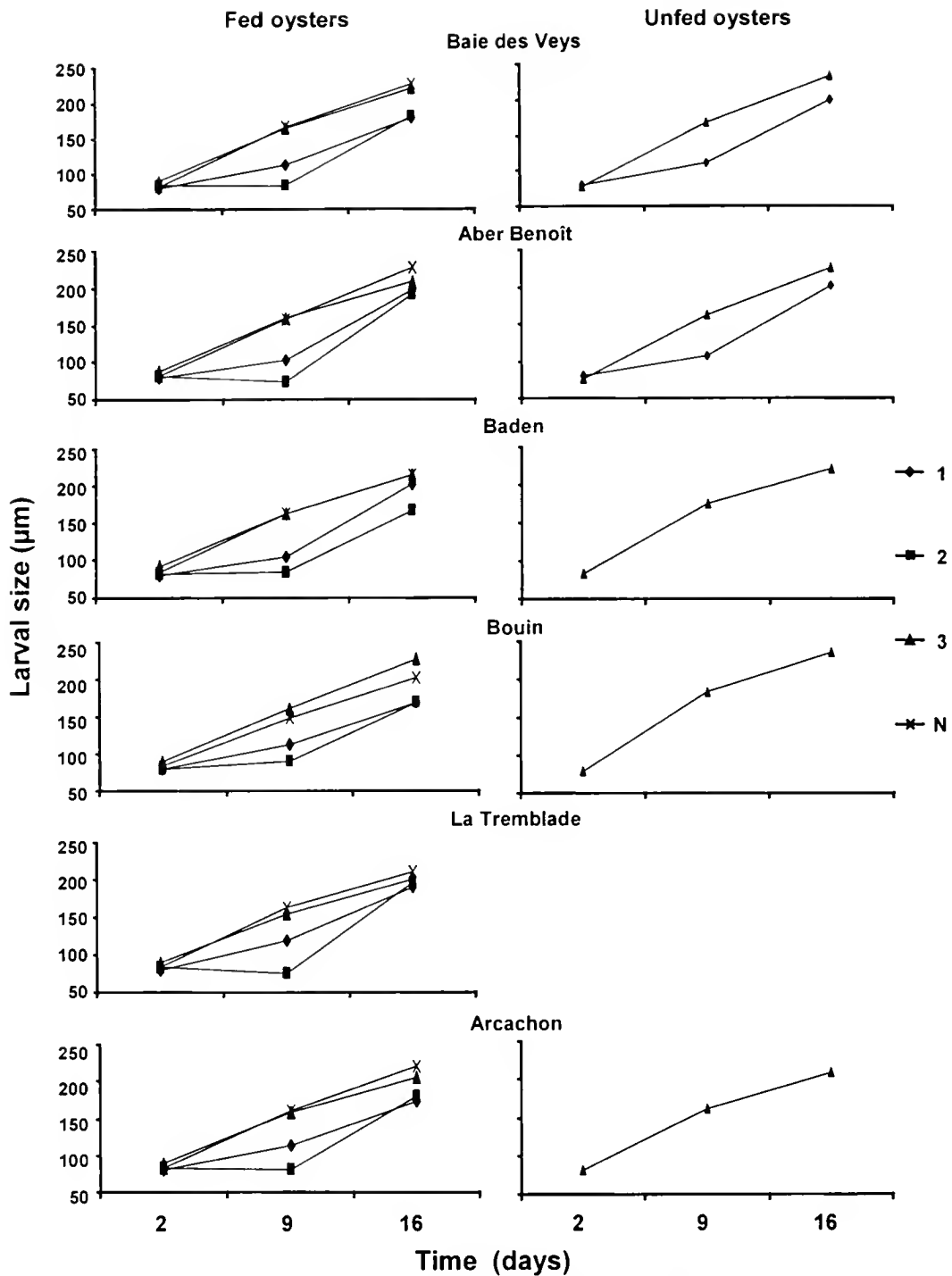
Figure 6. Change in larval size until day 16 of culture of specimens conditioned with and without food, from six sites of *Crassostrea gigas* production during December 1998 to February 1999 (1), February to April 1999 (2), April to June 1999 (3), and July 1999 (N).

gametogenesis. This suggests greater food accessibility that favored nutrient accumulation in oysters from northern locations. Some considerations for assuming this are for example, that the region of Baie des Veys is a high carrying-capacity ecosystem (Goulletquer et al., 1996) and that the national production program of *C. gigas* in France gets the highest meat yield per year from the oysters of Aber Benoît (Goyard 1997). In contrast, Bouin oysters have poor growth rates and low biologic yields compared with the rest of French oyster production (Gérard, 1995). Héral et al. (1986)

found evidence of biologic overload in the Marennes–Oléron basin (La Tremblade) produced by a huge oyster biomass (95,000 tons), and Pastoureaud et al. (1996) indicated low seston quality encountered by oysters in this bay. Barber and Blake (1983) suggested that potential food supply for the scallop *Argopecten irradians* decreases with latitude and that metabolic rate increases with temperature. The metabolic rate in the Japanese oyster increases with temperature (Bougrier et al. 1995). Previous observations suggest that the metabolic rate of *C. gigas* increases with decreasing lati-

tude, but there is less food that results in less energy for reproduction.

It was significant that the best laboratory performance coincided with partial spawning in nature. Ropert (1999) reported that oysters in Baie des Veys have a partial spawning during their reproductive cycle, and Chávez–Villalba et al. (2001) found that oocytes left from the incomplete spawning of Aber Benoît oysters are slowly reabsorbed from September to January. This led us to think that apart from the advantage of ambient food at northern sites, it is possible that nutrient recycling from reabsorption of unreleased gametes within the gonad is a regulating factor in the timing of gametogenesis. Post-spawning reabsorption has been observed in *C. gigas*, in which gametes remaining after spawning are reabsorbed (Steele, 1998). Beninger and Le Pennec (1991) suggest that reabsorption of residual gametes leads to nutrient recycling in scallops. Le Pennec et al. (1991) found evidence for lipid catabolism during reabsorption of unreleased gametes in *Pecten maximus* and they suggested that the products of these catabolic activities could be stored as glycogen. This was confirmed by several investigators, including Berthelin et al. (2000), and could indicate that nutrients in northern oysters could be recycled from residual gametes during the autumn–winter period and then used for gametogenesis. It would be interesting to compare the conditioning response of northern oysters maintained in natural conditions throughout the year with those returned to natural conditions in the autumn or winter after artificial spawn in July or August.

We observed greater gamete production in fed oysters than unfed. Robinson (1992) found comparable results when comparing gamete production of *C. gigas* oysters maintained with and without food. However, in our study the D larva yield of animals conditioned with and without food was close, in particular for northern oysters. Moreover, we found that there is no difference in larval growth without considering broodstock culture conditions. It seems that these oysters maintain oocyte quality by reducing their number when there is not enough food.

Gametogenic cycles in bivalves are strongly tied to glycogen stocking cycles and to ultimate synthesis, *de novo*, of lipids during spring vitellogenesis, which depends on stocked glycogen (Gabbott 1975). Interruption of these cycles, due to artificial conditioning at high temperature, might force oocyte development before sufficient glycogen has been accumulated for lipid synthesis. The consequence might be production of few gametes with low biochemical quality (Gallager & Mann 1986). Our observations suggest that the stocking reserve in unfed oysters allows production of fewer gametes of high quality. It seems that viability and survival of reared larvae are directly related to the initial quantity of lipids during gamete emission (Holland & Spencer 1973, Gallager &

Mann 1986). Apparently, unfed BV and AB oysters maintain their lipid stock during conditioning, probably due to large glycogen reserves, which assures not only lipid synthesis but also gamete development.

When comparing larval development in the three conditioning experiments, we observed that larval growth of fed and unfed oysters is significantly inferior during the first two experiments. Although Lannan et al. (1980) showed the importance of season in the timing of broodstock collection for artificial conditioning, they had no explanation concerning mechanisms that govern egg quality and the variability of survival during larval rearing. However, Gallager and Mann (1986) noticed that growth and survival of *Mercenaria mercenaria* and *C. virginica* larvae were associated directly with the initiation and duration of conditioning. Berthelin (2000) found that glycogen stores in the gonads of *C. gigas* during autumn and the beginning of winter remained low in spring, while proteins and lipids increase significantly from March to April, coincident with the first phytoplankton blooms. Results of conditioning during December to February suggest that reserves used for larval growth in fed and unfed oysters were accumulated in autumn and winter, and reserve allocation during spring increased fecundity and larva growth but not necessarily D larval yields.

Knowledge of the general condition of animals before exposure to experimental conditions is important to obtain gametes in the optimum state of development. This study shows that the stored reserves of northern oysters allow them to perform better during conditioning than southern oysters. The existence of oysters having distinct gametogenic development and therefore distinct responses to conditioning has implications for oyster spat production in hatcheries. Broodstock from northern locations can be conditioned starting in December because they mature after six weeks of exposure at elevated temperatures (19°C). According to Chávez–Villalba et al. (2002) this occurs because 60% of oocytes in the gonad are mature after conditioning. The response of these oysters to artificial conditions can be maintained throughout gametogenic development, whereas the oysters from southern locations mature only after commencing conditioning in April. These conditioning experiments suggest that using oysters from northern locations in hatchery operations should result in substantially increased hatchery production.

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APPEARANCE AND PATHOGENICITY OF OVARIAN PARASITE *MARTEILIOIDES CHUNGMUENSIS* IN THE FARMED PACIFIC OYSTERS, *CRASSOSTREA GIGAS*, IN KOREA

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ABSTRACT The ovarian parasite *Marteilioides chungmuensis* that infects the ovaries of Pacific oyster *Crassostrea gigas* has increased in frequency in farmed oysters on the southern coast of Korean peninsula since the early 1990s. The appearance and pathogenicity of the ovarian parasite in the farmed oyster in Jinhae Bay, Korea, were investigated in 1996 and 1997. Infection by *M. chungmuensis* was highest during spawning (from June to August) and gonadal regenerating season of the oysters (from September to October), with prevalences ranging from 13.3 to 57.1% in 1996 and from 28.6 to 61.5% in 1997, respectively. The surveyed oysters showed signs of recovery from the infection after October. Glycogen levels were considerably lower in *M. chungmuensis*-infected oysters those that of uninfected oysters. A rapid accumulation of glycogen was observed in uninfected oysters together with the gonadal regeneration after the summer spawning. By contrast, no increase in glycogen content was found in infected oysters until the end of the investigation. Lipid levels were slightly higher in the infected oysters than in the uninfected oysters. Serum protein concentrations were significantly lower in the infected oysters than in the uninfected oysters. Also, the increase of serum protein concentration after the summer spawning was apparent in the uninfected oysters but not in the infected oysters. These results indicate that the infections by *M. chungmuensis* may have an adverse impact on metabolic recovery after spawning of the oysters.

KEY WORDS: Pacific oyster, *Crassostrea gigas*, ovarian parasite, *Marteilioides chungmuensis*

INTRODUCTION

The ovarian parasite *Marteilioides chungmuensis* is found in the ovaries of the Pacific oyster, *Crassostrea gigas*. The parasite is a Protozoan belonging to the Phylum Ascetospora Sprague (Comps et al. 1986, Park & Chun 1989). It is found in the cytoplasm of the ovum and measures 3–3.5 μm in diameter (Comps et al. 1986).

The effects of the ovarian parasite on the growth of oysters have been studied for the last 10 years. Park et al. (1999) reported that the parasite could induce ovary necrosis and prohibit normal growth of fertilized eggs. Oysters infected by this parasite show grossly visible ovary deformations, with lump-like hypertrophy that renders them unmarketable. Therefore, the parasite is considered one of the most serious problems for oyster production in Korea. The parasite has been found in oysters from almost all oyster culture areas of Korea (Chun 1970, 1979, Park & Chun 1989, Park et al. 1999), although prevalence and intensity of infection vary with region and season. Infection levels along the southern coast have increased since 1990 and the oyster industry in this area is facing increasing production challenges as a result of poor seed collection.

Studies of *M. chungmuensis* have concentrated on histopathology, infection dynamics, and transmission pathways, but little work on the effects of the parasite on the physiology of the oyster has been conducted. To better understand the effects of this ovarian parasite on oyster aquaculture, this study examined the relationship between *M. chungmuensis* infection on the oysters and gonad regeneration and its related physiologic parameters.

MATERIALS AND METHODS

Collection of Oyster Broodstock

Oysters were collected from the culturing sites around Chilcheon Island in Jinhae Bay of Korea (Fig. 1). Although many oyster beds still operate in this bay, sufficient seed for stocking purposes produced no longer in this formerly productive seed collection area since 1990. For broodstock sampling, one oyster string was divided into three sections (upper, middle, and bottom), and 30 individuals were collected monthly from each section.

Examination of M. chungmuensis *Infection Levels*

Sampled broodstock were washed with clean seawater and shucked by hand. A 3-mm thick dorsoventral cross section through the anterior third of the soft tissues was fixed in Davidson solution and processed for light microscopy. Paraffin-embedded sections (4 μm) were stained with Harris' hematoxylin–eosin for microscopic examination. Because early stages of infection are difficult to detect by light microscope, additional tissue smears were made from ovarian tissues and stained with eosin-methylene blue (Fig. 2).

Evaluation of Infection Levels and Oyster Condition Factors

The oyster samples were divided into two groups, infected and uninfected, based on gross evidence of *M. chungmuensis* infections, to evaluate infection effects on specific oyster condition parameters. To have an accurate gross indication of infection, oysters were collected from May to September when infections are most obvious to the naked eye (Fig. 3). Infection levels were divided into two levels by observation of slides that were smeared with reproductive tissues and stained with eosin-methylene blue (H group: heavy infection of >50% prevalence; M group: moderate infection of <50% prevalence). Of condition parameters, glycogen content (excluding ovarian tissues) was measured using the method of Whyte and Englar (1982); biochemical composition of the meat using the AOAC method (1990); and serum protein concentrations using the Lowry method (1951).

RESULTS

M. chungmuensis *Infection Levels*

Prevalences of infection from 1996 to 1997 ranged between 0.0 to 61.5% (Fig. 4). Infection levels of *M. chungmuensis* were high-

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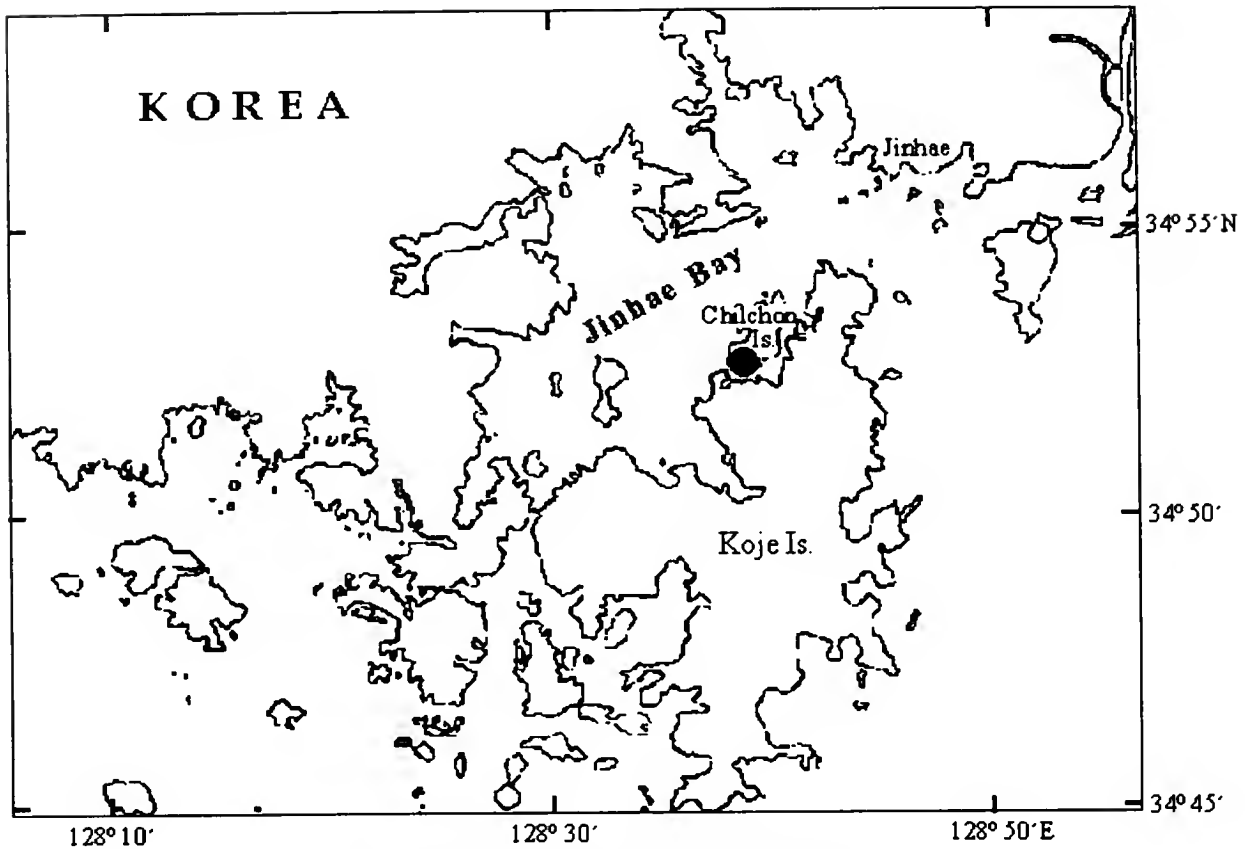
Figure 1. Map showing sampling site in Jinhae Bay, Korea.

est in September 1996 (57.1%) and in August 1997 (61.5%). No infections were detected from January to April 1996. In 1997, the parasite was detected all the year round, with highest prevalences in August.

Correlation Condition Factor to Levels of M. chungmuensis *Infection*

Glycogen

Monthly mean glycogen levels ranged from 2.0 to 14.8%, from 2.1 to 16.0%, and from 4.0 to 20.2% in H group, M group, and uninfected oysters, respectively (Fig. 5). Glycogen levels in all the three groups were maxima in May and minima in August. Glyco-

gen levels in the infected oysters were similar between H and M groups but consistently lower than in uninfected oysters (analysis of variance, $P < 0.01$ for all the sampling months, Table 1). The glycogen level increased abruptly in September when the gonadal tissues were regenerated (Fig. 5). However, recovery of glycogen level after summer spawning was observed in the infected oysters.

Biochemical Analysis of Oyster Tissue

Lipid levels fluctuated in the narrow ranges from 11.5 to 14.0%, from 10.0 to 13.0%, and from 5.0 to 9.0% in H group,
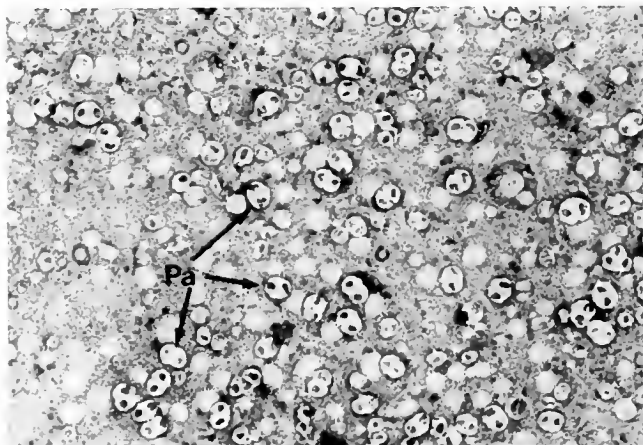
Figure 2. Photomicrograph of a smear preparation from a heavily infected ovary. Pa, parasite. Eosin-methylene blue (×200).
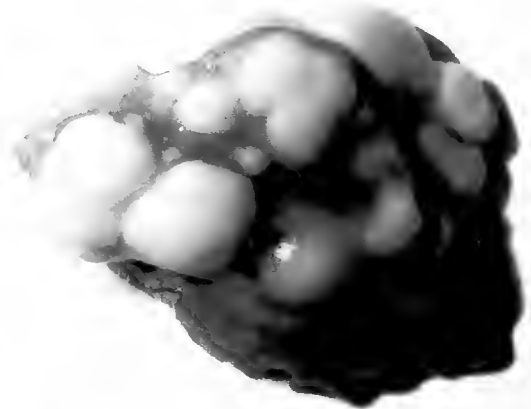
Figure 3. The external view of an oyster with an advanced *M. chungmuensis* infection associated with ovarian hypertrophy, rendering the meat "lumpy" in appearance.
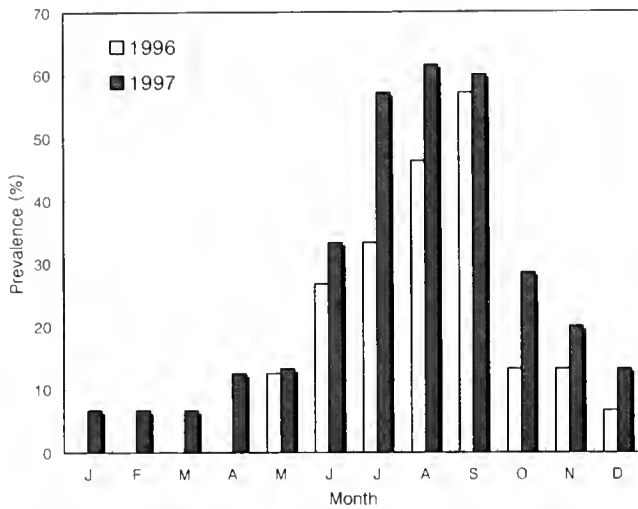
Figure 4. Monthly variations of the % prevalence of the ovarian paraste *M. chungmuensis* of the Pacific oyster, *Crassostrea gigas*.
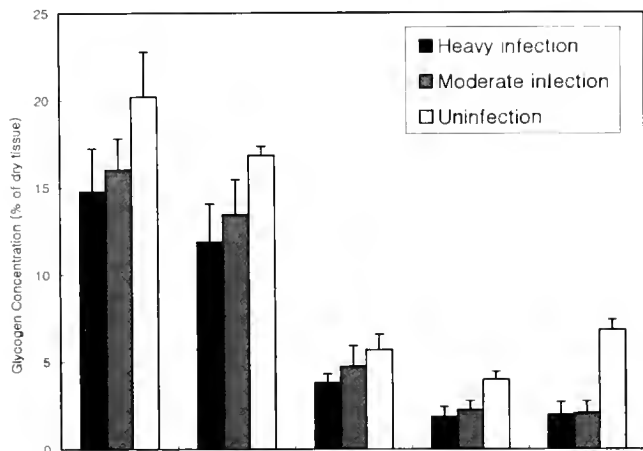
Figure 5. Monthly variations of levels (% of dry tissue weight ± 1 SD) in the infected and uninfected oysters from May to September.

M group, and uninfected oysters, respectively (Fig. 6). The lipid contents in the infected oysters were similar between H and M groups (paired *t*-test, $P = 0.174$) and showed slightly greater levels than those in the uninfected oysters (paired *t* test, $P = 0.06$ for both infected groups). Protein levels ranged from 56.4 to 62.0%, from 55.1 to 58.9% and from 53.2 to 55.8% in H group, M group, and the uninfected oysters, respectively. No apparent differences were found between infected and uninfected oyster (paired *t*-test, $P = 0.111$ between H and M groups, $P = 0.562$ between H-group and the uninfected oysters, and $P = 0.673$ between M group and the uninfected oysters). Carbohydrate levels ranged from 4.2 to 17.0%, from 9.1 to 20.1%, and from 8.7 to 25.6% in H group, M group, and the uninfected oysters, respectively. Because glycogen levels accounted for most of total carbohydrate levels, temporal variations of carbohydrate levels paralleled those of glycogen with maxima in May and minima in August (Fig. 6). Ash levels ranged from 11.5 to 27.7%, from 16.0 to 25.1%, and from 10.7 to 25.6 in H group, M group, and the uninfected oysters, respectively, with maxima in August and minima in May. No pronounced correlation to infection was found for ash content (paired *t*-test, $P = 0.930$, $P = 0.384$, and $P = 0.085$, respectively, for the same paired variables as the statistical treatment of protein content).

Serum Protein

Mean serum protein concentration ranged from 3.1 to 4.0 µg/µL, from 3.7 to 6.2 µg/µL, and from 5.1 to 11.4 µg/µL in H group, M group, and the uninfected oysters, respectively (Fig. 7). Serum protein concentrations in the infected oysters were significantly higher in H-group than in M-group (analysis of variance, $P < 0.001$ for each sampling month except September). Serum protein concentrations were then significantly lower in infected oysters then in uninfected oysters (analysis of variance, $P < 0.001$ for all the sampling months. Serum protein concentration in uninfected broodstock was highest in May and lowest in August, with an obvious increase after the summer spawning of the oysters. However, in H-group oysters, the low concentration of mean ≤ 4.0 µg/µL remained constant from May to September. In M group oysters, the serum protein concentration was highest in May and lowest in August–September. Finally, no increase in the serum protein concentration after the summer spawning was observed in both groups of the infected oysters.

TABLE 1.

Results of ANOVA and Tukey post-hoc test ($\alpha = 0.05$) for absolute values of tissue glycogen and serum protein in each sampling month.

Parameters	Month	H Group		M Group		Uninfected Oysters	P
Tissue glycogen (% of dry tissue)	May	14.8 ± 2.5	=	16.0 ± 1.8	<	20.2 ± 2.6	<0.001
	June	11.9 ± 2.2	=	13.5 ± 2.0	<	16.8 ± 0.5	<0.001
	July	3.8 ± 0.5	=	4.7 ± 1.2	<	5.7 ± 0.9	<0.001
	August	1.8 ± 0.6	=	2.2 ± 0.5	<	4.0 ± 0.5	<0.001
	September	2.0 ± 0.7	=	2.1 ± 0.7	<	6.8 ± 0.6	<0.001
Serum protein (µg/µL)	May	4.0 ± 0.7	<	6.2 ± 0.7	<	11.4 ± 0.5	<0.001
	June	3.5 ± 0.4	<	6.2 ± 1.3	<	8.3 ± 0.4	<0.001
	July	3.7 ± 0.5	<	4.8 ± 0.3	<	7.2 ± 0.5	<0.001
	August	3.1 ± 0.5	<	4.1 ± 0.5	<	5.1 ± 0.8	<0.001
	September	3.1 ± 0.7	=	3.7 ± 0.7	<	6.5 ± 1.0	<0.001

Data represent mean ± 1SD.

Heavy infection

Moderate infection
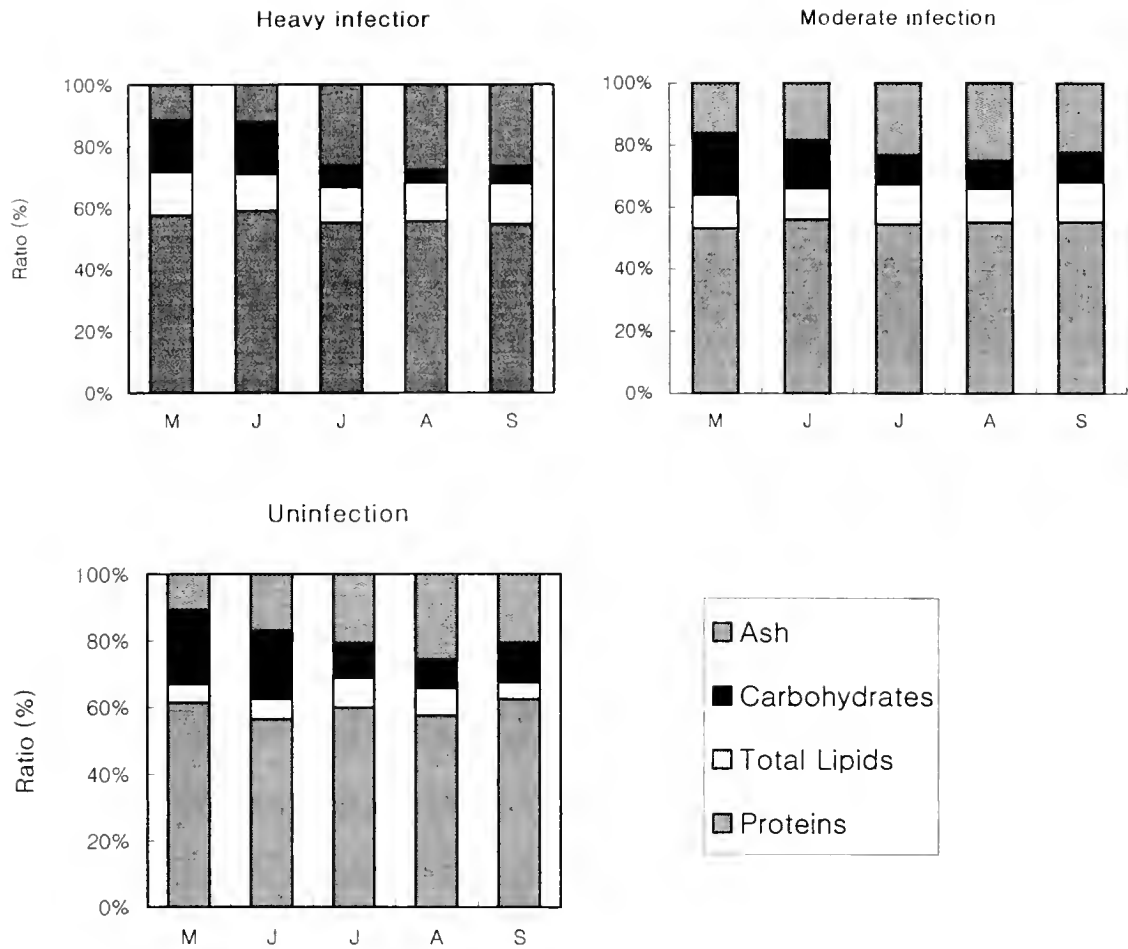
Uninfection

Figure 6. Monthly variations in the biochemical composition of infected and uninfected oysters.

DISCUSSION

Park and Chun (1989) reported *M. chungmuensis* infection prevalences of 5.3–6.7% between 1986 and 1987 in the oyster growing area of Hansan-Geoje Bay in the southern coast of Korea.
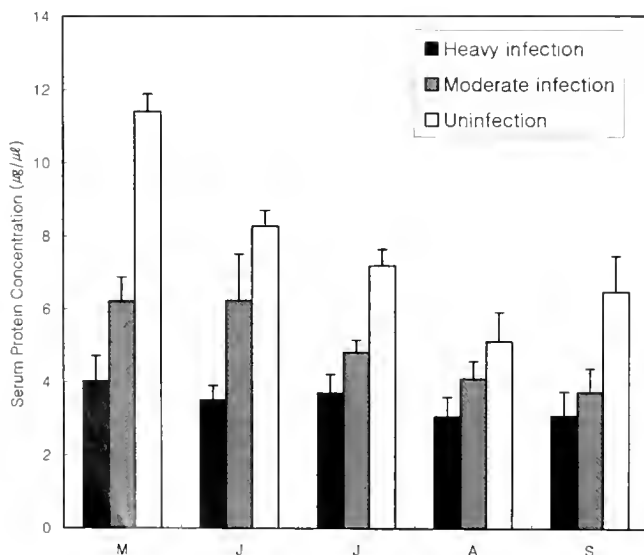
Figure 7. Monthly variations of serum protein concentrations (μg/μL ± 1 SD) in the infected and uninfected oysters from May to September.

The parasite was detected from June to October. Park et al. (1999) reported prevalences of 15.0–18.6% in oysters from Goseong Bay and Geoje Bay in 1993, with infections detected from May to September. During a subsequent survey in 1997, the parasite was detected all the year round, with an average prevalence of 26.6%. Therefore, the prevalences of *M. chungmuensis* have been increased annually during last decade in Korea.

M. chungmuensis only infects oyster ovarian tissues, inducing necrosis of the ova and massive hypertrophy of the gonad. The parasite impedes development of the fertilized eggs (Matsuzato et al. 1997, Matsuzato & Masumura 1988, Park & Chun 1989). Park et al. (1999) compared infected eggs with uninfected eggs and concluded that the infected eggs did not undergo fertilization. And also uninfected eggs isolated from infected oysters could be fertilized but their growth pattern was abnormal. More than 80% of the fertilized eggs which were from infected oysters showed abnormal shapes and died during the early umbo stage. To date, there is no clear evidence that the parasite *M. chungmuensis* induces mortality of the mature oyster.

Glycogen reserves have been considered to be the main energy reserves both for the formation of gametes of marine bivalves, especially under conditions of nutrient stress and also for the maintenance during nutritional stress (Beninger & Lucas 1984, Encomio & Chu 2000). It is accumulated in storage tissues of the digestive gland, gonad, and mantle (Berthelin et al. 2000). Glycogen reserved in the gonad and mantle is used for gamete matura-

tion. Lower content of glycogen in infected broodstock indicates that the parasite may directly reproductive success. Serum protein increased after the summer spawning in the uninfected oysters, but decreased in infected oysters. Reduced serum protein as well as reduced glycogen content may exacerbate morbidity, but there is no clear evidence to date that this has a direct correlation to mortality of oyster broodstock. Protein may not be used for gametogenesis, but is considered to be an essential metabolic requirement (Berthelin et al. 2000).

Increased serum protein after spawning is normal; however, this did not occur in infected oysters examined. Thus, as with tissue protein, the effects of infection may have a significant metabolic impact, whereby the drop in prevalence of infection in October was caused the death of heavily infected oysters, rather than recovery from infections.

The protistan parasite *Perkinsus marinus* has been responsible for high mortality of eastern oyster *Crassostrea virginica* in the United States. The physiologic effects of *P. marinus* infection are most apparent as a reduction in growth rate as well as reproductive capacity (Barber and Mann 1994, Paynter 1996, Dittman et al. 2001). The physiologic effects of *M. chungmuensis* infection on *Crassostrea gigas* may reduce reproductive capacity of oyster population in Korea.

ACKNOWLEDGMENTS

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MOLECULAR PHYLOGENETICS OF FIVE *CORBICULA* SPECIES DETERMINED BY PARTIAL 28S RIBOSOMAL RNA GENE SEQUENCES

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ABSTRACT Partial 28S ribosomal RNA (rRNA) gene sequences of five species (*C. fluminea, C. papyracea,* and *C. leana* from Korea, *C. japonica* from Japan, *C. largillierti* from China) in the genus *Corbicula* were investigated for their genetic divergence. Neighbor-joining analysis on the alignment of 412 base pairs of *C. fluminea, C. largillerti, C. papyracea, C. leana* and *C. japonica* (with *Polymesoda maritima, P. caroliniana* and *Sphaerium corneum* chosen as an outgroup) provides a robust molecular phylogeny for the genus; (*C. japonica, C. papyracea, C. largillierti, C. leana, C. fluminea, P. maritime, P. caroliniana,* and *S. corneum*). The results of this study provide potential use of 28S rRNA gene sequence for phylogenies in the family Corbiculidae.

KEY WORDS: Corbiculoidea, *Corbicula* spp., 28S rRNA, phylogeny, China, Korea, Japan

INTRODUCTION

Corbicula is conservative, possessing few morphologic characters useful for species discrimination and displaying a broad range of subtle variability, especially with respect to shell form and color. The genus *Corbicula* is present in freshwater, brackish water, and estuaries in southeastern Asia, Africa, the Indian subcontinent, the Pacific Islands, and South America, where it is an important component of benthic communities in both lentic and lotic environments (Leveque 1973, Britton & Morton 1979). In Korea, six species, *C. fluminea, C. leana, C. fenouilliana, C. papyracea, C. colorata* and *C. portentosa,* are recognized based on shell form (Kwon et al. 1993).

Corbicula species can be categorized as 3 major groups based on reproductive characters and ecology (Miyazaki 1936); the species belonging to Group 1 are monoecious, viviparous, and incubatory. They have nonswimming planktonic veliger larvae and live in freshwater; the species belonging to group 2 are dioecious, oviparous, nonincubatory, and also live in freshwater regions; the species belonging to group 3 are dioecious and oviparous. They do not incubate the young, has free-swimming planktotrophic larvae and live in brackish waters. The phylogenic relationship among these three groups cannot fully be clarified with these taxonomic characters alone. Recently, the chromosome numbers and the degrees of genetic differentiation from a limited number of species of the genus *Corbicula* have been investigated (Okamoto & Arimoto 1986, Lee & Kim 1997, Park et al. 2000).

Within the tandemly repeated rRNA gene complex, coding sequences for small (18S) and large (5.8S + 28S) subunit rRNA components are flanked by nontranscribed and internal transcribed spacer regions. As a result of functional constraints within the ribosome, coding regions are in general more conserved than the spacer regions (Raué et al. 1990, Mulvey et al. 1998). The 28S rRNA gene contains "conserved" core regions interspersed with more variable "expansion segments" or domains, designated D1 to D18 (Raué et al. 1988). Hillis and Dixon (1991) reported that, if chosen carefully, many divergent domains in the gene coding for large subunit ribosomal RNA are useful for reconstructing recent events. Sequence data from the 28S rRNA gene have been successfully used for intergeneric resolution within the Corbiculoidea (Park & Ófoighil 2000). This study is base on analysis of sequences from 5' end 28S rRNA gene five common *Corbicula* species.

MATERIALS AND METHODS

Sample Collection and DNA Extraction

Five *Corbicula* species *C. fluminea* (Cheorwon, Gangwon Province); *C. papyracea* (Yeongwol, Gangwon Province); *C. leana* (Wanju, Chunbuk Province) from Korea, *C. japonica* (Iwaki, Fukushima Province) from Japan and *C. largillierti* (Tung-ting lake, Hunan Province) from China were analyzed and nucleotide sequences were applied to five specimens in each species (Fig. 1). *Polymesoda maritima* (Corbiculidae, GBDB Accession no. AF131010), *P. caroliniana* (Corbiculidae, GBDB AF131011) and *Sphaerium cornium* (Sphaeriidae), GBDB AF131013) were also analyzed as an out group. Voucher specimens of the *Corbicula* species used in this study have been placed in the Department of Parasitology, Kwandong University College of Medicine, Korea.

Genomic DNA was isolated from fresh tissues using DNeasy Tissue Kit (Qiagen #69504) following manufacturers instructions. The 28S gene regions were amplified by the polymerase chain reaction (PCR) from 20 to 40 ng of genomic DNA. For the 28S, primers used were forward 5'-GATTACCCGCTGAACTTAAG-CATAT-3' and 5'-GCTGCATTCACAAACACCCCGACTC-3' reverse and D1F and D6R were used (Park & Ófoighil 2000). PCR amplification was conducted over 40 cycles using the following conditions; 1 min at 95°C, 1 min at 54°C, and 1.5 min at 72°C with a final extension of 7 min at 72°C. The PCR products were purified
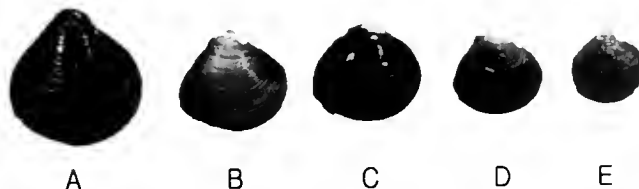
Figure 1. Shells of *Corbicula* species. A, *Corbicula fluminea*; B, *C. leana*; C, *C. papyracea*; D, *C. japonica*; E, *C. largillierti*.

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1. C. fluminea      AACCAGGATTCCCCCAGTAACGGCGAGTGAAGCGGG-AAGAGCCCAGCACCGAATCTCCC

2. C. largillierti  ············································································

3. C. papyracea     ···········································G································

4. C. japonica      ············································································

5. C. leana         ···················T·····················G································

6. P. maritima      ···A·········TT·····················-··A································

7. P. cariliniana   ···A·········T·····················-··A································

8. S. corneum       ···A·········TT···········································CT·
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1. GGCCTGACGGGCGGCGAGAAATGTGGTGTATAGGCGGCCGATTGTTGCCGGGTCCGGCGCTCAA-GTCCTCCTGATCGTG

2. ·····CG···································································A············

3. ·····CG················································································

4. ·····CG················································································

5. ·······························································-························

6. ····ATG·······································CC····TT·············-················

7. ····ATG···························C·····················CG·········-················

8. ·A··ATG···C····AG·············C···AA······GC··AGTC··G·T·C···G··A··················
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1.  GCCTTGCCCAGAGCGGGTGTCAGGCCCGT---GGCGGCGCTGGAGACGGCGGCTTCGAGCCTCCTTGGAGTCGGGTTGTT

2.  ·······················---------·······A································

3.  ·······················---------·······A································

4.  ·······················---------·······A································

5.  ·······················---------········································

6.  ·····················T----········AT···T··T································

7.  ·····················T----····G···TG···T································

8.  ···A·A···················G·ATC····CT·GAC·CGG··-----·AA·····T····C································
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1.  TGGGAATGCAGCCCAAAGCGGGTGGTAAACTCCACCTAAGGCTAAATACTGGCACGAGTCCGATAGCGGACAAGTACCGT

2.  ····································································A································

3.  ················································································

4.  ················································································

5.  ················································································

6.  ····································································A································
```

by gel extraction (Qiagen Co.) and ligated into a T cloning vector (Novagen Co.). Clones were generated by transforming *Escherichia coli* NovaBlue competent cells provided in the T cloning vector kit, according to the protocol of the supplier. The recombinant plasmid was screened using isoprophy-β-thiogalactoside (IPTG) and X-gal. DNA from positive recombinants was purified using the QIAprep spin plasmid kit (Qiagen Co.). DNA sequencing was performed using the dideoxy chain termination method and an automated DNA sequencer (Applied Biosystems, Model 373A, Perkin Elmer). At least two clones were sequenced per

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7.    ·······································A··························
8.    ··············T·············································
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1.    AGGGAAAGTTGAAAAGAACTTTGAAGAGAGAGTTCAAGAGTACGTGAAACCGCATAGAGCCAAACGGGTGGATCCGCAG
2.    ···············································G···············
3.    ···························································
4.    ···································T······················
5.    ···························································
6.    ···································T··········G············
7.    ···································T··········G············
8.    ·············································A····GT··············
```

```
1.        AGTCGACCCGGGGAATTCAGCCCGGCGGGTGCC
2.        ·································
3.        ·································
4.        ·································
5.        ·································
6.        ······················GA··T····
7.        ······················GA··C····
8.        CAG···G···T····················
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Figure 2. Aligned of 5′ 28S rRNA gene sequences of Corbiculoidea. Dashes represent gaps in the alignment.

isolate, and additional clones were sequenced as necessary to resolve ambiguous sites.

Sequences Analyses

Nucleotide sequences were aligned using Clustal X (Thompson et al. 1997). Phylogenetic analyses were performed by a distance method, using Kimura 2-parameters distance, to obtain a neighbor-joining tree (Saitou & Nei 1987) and using the MEGA v1.01 program. (Kumar et al. 1993). Gaps were considered as an additional character state in pairwise comparisons. The statistical confidence of a particular cluster of sequences was evaluated by the bootstrap procedure (1000 replicates).

RESULTS

The alignment of the partial 28S rRNA gene sequences of *C. fluminea, C. papyracea, C. leana, C. japonica, C. largillierti, Polymesoda maritima, P. caroliniana,* and *Sphaerium corneum* is shown in Figure 2. Nucleotide sequence data reported in this study are available in the GenBank database under the accession numbers: *C. fluminea* (AY052553), *C. largillierti* (AY052554), *C. papyracea* (AY052555), *C. japonica* (AY052556) and *C. leana*

(AY052557). The 28S sequence was 412 base pairs, which included gaps in length. Nucleotide sequence differences for the various pairs of Corbiculoidea are presented in Table 1. For this gene segment, interspecies differences from recognized species within any species where clones from the different sequenced isolates (*C. fluminea, C. papyracea,* and *C. leana*). Interspecies variation within the genus *Corbicula* was detected at a low level of 0.73 to1.70% (from 4–22 nucleotides). Among the genus, however, *Corbicula, Polymesoda,* and *Sphaerium* exhibit more varia-

TABLE 1.

Nucleotide sequence differences between pairs of *Corbicula* taxa for 28S rRNA region.

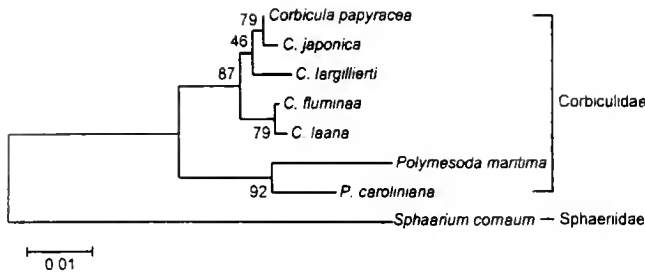| Species (Origin)	2	3	4	5	6	7	8
1. *Corbicula fluminea* (Korea)	6	6	4	3	22	18	56
2. *C. largillierti* (China)		5	4	7	18	16	57
3. *C. papyracea* (Korea)			4	6	22	20	57
4. *C. japonica* (Japan)				6	19	17	57
5. *C. leana* (Korea)					22	18	56
6. *Polymesoda maritima* (USA)						12	62
7. *P. caroliniana* (USA)							56
8. *Sphaerium corneum* (Germany)							

Figure 3. Tree dipicting relationships among genus *Corbicula* inferred from 28S rRNA gene sequence data using *P. maritina*, *P. caroliniana*, and *S. corneum* as an outgroup. A distance matrix was calculated using the Kimura-2-paramater model and the tree constructed using the Neighbor-Joining method.

tions (1.70–15.1%, from 22–62 nucleotides). The distances between the genus *Polymesoda* and *Sphaerium* and the various *Corbicula* species are significantly greater than some interspecies distances with the *Corbicula* genus. The phylogenetic tree shows relationships among the interspecies based on the 28S sequences (Fig. 3). Analyses using *P. maritima*, *P. caroriniana* and *S. corneum* as outgroups supported the monophyly of genus *Corbicula*. Also, in the neighbor-joining tree, monophyly was strongly supported for both the families Corbiculidae and Sphaeriidae.

DISCUSSION

In the family Corbiculidae, there are three genera: *Corbicula*, *Batissa* and *Polymesoda*. Of these, only *Corbicula* has a significant number of freshwater and brackish-water species. The other genera are dominantly brackish-water clams and characteristically have reducing sediments in tropical mangrove swamps. There are marked ecologic and reproductive differences between interspecies of Corbiculoidea (Table 2). Geographic variation in physiology, sex determination, and reproduction are undefined. There are references in the literature to a single species (*C. fluminea*) pos-

sessing different sexual strategies (e.g., protandry, protogyny, separate sexes) in different parts of its range (Morton 1982). Reproduction in these species must be by parthenogenesis, but mature sperm are found in the gonads.

The earliest corbiculid (mid-Jurassic) and dreissenid (Eocene) fossils were clearly marine (Keen & Casey 1969, Nutall 1990) and all dreissenid and some corbiculid species retain an indirect mode of development involving broadcast spawning and a pelagic veliger larval stage (Morton 1985, Morton 1989, de Severeyn et al. 1994). A planktonic veliger larva is considered to be nonadaptive in riverine freshwater environments because it lives in colonies at upstream habitats (McMahon 1991). Some freshwater corbicuid species have evolved parental care of young in association with a greatly reduced (*C. fluminea*) (King et al. 1986) or completely absent (*Neocorbicula limosa*) (Ituarte 1994) pelagic larval ontogeny. From the comparisons of the chromosome numbers and karyotypes in three species, Okamoto and Arimoto (1986) assumed that the ancestral species of the hermaphroditic species including *C. leana* originated from the ancestral species of *C. sandai* that had originated from the ancestral species of *C. japonica*. Lee and Kim (1997) reported that the genetic similarity coefficient of *C. fluminea*, *C. leana*, and *C. colorata* in freshwater was very closed (Rogers S ≤0.970), whereas *C. japonica* in brackish-waters was genetically distant (S = 0.873) from them. In this study, despite widespread geographic origins of the Corbiculidae, their percentage sequence variation in the 28S rRNA was low, <5.3%. Phylogenetic tree calculated using neighbor-joining method is shown in Figure 3. Instead of a single monophyletic *Corbicula* lineage, *C. papyracea*, *C. japonica*, and *C. largillierti* are members of a separate clade distinct from that shared by *C. fluminea* and *C. leana*; (i.e., within the Corbiculinae there are two sister groups) both of which contain species currently assigned to *Corbicula*. Based on the 28S rRNA data, the genus *Corbicula* is indistinguishable by biologic habitat features; *C. japonica* live in brackish-water, while other species live in freshwater. Partial 28S rRNA gene sequences provide useful data for resolving phylogenies within the *Corbicula* species groups.

TABLE 2.

Habitats, reproduction, and chromosome numbers in 12 species of the superfamily Corbiculoidea.

Species	Habitats	Reproduction	Chromosomes 2n	References
Corbiculidae				
C. fluminea	Freshwater	Hermaphrodite	54	Park et al., 2000
C. papyracea	Freshwater	Hermaphrodite	54	Park et al., 2000
C. leana	Freshwater	Hermaphrodite	54	Okamoto & Arimoto, 1986
C. colorata	Freshwater	Dioecious	38	Park et al., 2000
C. japonica	Brackish-water	Dioecious	38	Okamoto & Arimoto, 1986
C. sandai	Freshwater	Dioecious	36	Okamoto & Arimoto, 1986
Sphaeriidae				
Pisidium coreanum	Freshwater	Hermaphrodite	190	Park et al., 2002
P. casertanum	Freshwater	Hermaphrodite	ca. 150, 180	Barsiene et al., 1996
			ca. 190	Burch et al., 1998
Sphaerium corneum	Freshwater	Hermaphrodite	36	Keyl, 1956
S. occidentale	Freshwater	Hermaphrodite	ca. 209	Burch et al., 1998
S. striatinum	Freshwater	Hermaphrodite	ca. 68–98	Woods, 1931
			ca. 152	Lee, 1999
Musculium secures	Freshwater	Hermaphrodite	ca. 247	Burch et al., 1998

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DOMINANCE OF THE ASIATIC CLAM, *CORBICULA FLUMINEA* (MÜLLER), IN THE BENTHIC COMMUNITY OF A RESERVOIR

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ABSTRACT *Corbicula fluminea* dominated the benthic community of Lake Nacogdoches, East Texas, composing 97% of the total biomass of benthic invertebrates. *C. fluminea* appears to be restricted to the littoral zone. Lower depths have lower oxygen, especially during the stratified period, which may restrict the distribution of *C. fluminea*. *C. fluminea* was found only down to a depth of 4 m and had and extremely patchy distribution. The greatest density within a patch was found at 1 m depth (35.8 ± 13.8 m^{-2}) and the greatest biomass within a single patch was at 2 m (137.17 ± 69.21 g · m^{-2}). *C. fluminea* density differed significantly among substrate types. The maximum density (43 ± 14 m^{-2}) was found in sediments with dead *C. fluminea* shells and course detritus, and the lowest density (3.6 ± 3.6 m^{-2}) was found in silt. The spatial distributions of *C. fluminea* and three species of unionids were similar both in depth and across substrates in the reservoir. We found no correlation between the densities of *C. fluminea* and other benthic invertebrates. Finally, we contrasted the effect of *C. fluminea* on benthic communities to what is known about the impacts of another invasive bivalve, the zebra mussel.

KEY WORDS: Corbicula fluminea, benthic community, *Hydrilla*, invasive species

INTRODUCTION

Asiatic clams [*Corbicula fluminea* (Müller)] are native to Southeast Asia and have been successfully invading North American water bodies since the beginning of the 20th century. They currently occur in 36 states in the United States and northern and central Mexico; however, they are not found in Canada (McMahon 1982, McMahon 1999, McMahon & Bogan 2001). *C. fluminea* invaded Texas in the 1960s and has now spread statewide (Howells 1992). *C. fluminea* is a simultaneous hermaphrodite that is ovoviviparous. Fertilized eggs are brooded in the interlamellar spaces of the gills through the trochophore and veliger stage and released at the nonswimming pediveliger stage (McMahon 1999). Because of a high reproductive potential (<68,000 pediveligers adult^{-1} y^{-1}), *C. fluminea* can rapidly increase in population density within a short period of time (Aldridge & McMahon 1978, McMahon 1991, McMahon 1999, McMahon & Bogan 2001). *C. fluminea* is infaunal, usually burrowing in soft sediments. Adults can grow to 50–70 mm in size and can live for 3–4 y (reviewed in McMahon 1999). One of the reasons for its success may be the ability of *C. fluminea* to feed both from the water column (using siphons; Cohen et al. 1984, Boltovskoy et al. 1995), and from the sediments (using the foot to pedal-feed; Reid et al. 1992, Hakenkamp et al. 2001)

Carried into raw water systems on intake flows, *C. fluminea* nonswimming pediveligers and juveniles may settle in places with water currents below 1.2–1.5 m sec^{-1} and form adult populations >20,000 m^{-2} (McMahon 1999). The total damage caused by *C. fluminea* for US industries in 1986 was estimated at $1 billion (Isom 1986). *C. fluminea* can also play an important role in aquatic ecosystems as a benthic–pelagic coupler (Lauristen 1986, Hakenkamp & Palmer 1999). *C. fluminea* can reduce phytoplankton levels (Cohen et al. 1984), seston concentration (Leef et al. 1990), particulate phosphates (Greer & Zeibell 1972) and chlorophyll *a* levels (Beaver et al. 1991). Water clarification by clam filtering

favors the growth of rooted macrophytes, shifting primary production from planktonic to benthic communities (Phelps 1994, McMahon 1999). As a consequence, *C. fluminea* is becoming a major component of benthic communities in freshwater environments across North America (McMahon 1983, Counts 1986, Poff et al. 1993, McMahon 1999).

C. fluminea may also influence bottom fauna as a result of pedal-feeding via bioturbation of sediments or consuming benthic fauna directly (Hakenkamp & Palmer 1999, McMahon 1999, Hakenkamp et al. 2001). Although there are some reports that the Asiatic clam can compete with native unionid bivalves (Kraemer 1979, Leef et al. 1990, Howells 1992), there are no data about the impact of this invasive bivalve on biodiversity and functioning of the macroinvertebrate community or productivity and food web interactions. Hakenkamp et al. (2001) found that an increasing abundance of *C. fluminea* was negatively associated with the abundance of benthic bacteria and flagellates but had no apparent effect on other benthic protists or meiofauna. This contrasts with studies of another invading bivalve, the zebra mussel, *Dreissena polymorpha* (Pallas) (reviewed in Karatayev et al. 1997, Karatayev et al. 2002).

We determined the abundance and distribution of *C. fluminea* along depth gradients and among substrate types and their role in the benthic community, especially possible impacts on native fauna including unionid bivalves. We also compared patterns of the distribution of *C. fluminea* and its impact on bottom invertebrates with those found for zebra mussels.

METHODS

Study Area

Studies were conducted at Lake Nacogdoches, a monomictic reservoir in East Texas (31°37′N, 94°49′W). Lake Nacogdoches is the municipal water supply reservoir for the city of Nacogdoches, Texas. The dam-forming Lake Nacogdoches was completed in July 1976. The reservoir has a surface area of 8.94 km^2, maximum

storage capacity of 49.7 million m³, maximum depth of 13 m, and an average depth of 5.6 m.

The upper shallow (<5 m depth) and more eutrophic part of the reservoir is situated north of an island in the lake and constitutes approximately 40% of the water body (Fig. 1). Bottom sediments in this shallow part are mainly silt and a mixture of silt and clay. The lower part of the reservoir is less eutrophic, deep (up to 13 m), and has a variety of substrates, including sand, gravel, clay, shells, course detritus, and silt, as well as various combinations of these. The drainage area of the reservoir is 231 km², and Loco Bayou is the primary tributary (Prater 1991). During December to March, there is a long period of homeothermy. In spring, summer, and fall the water column of the reservoir is stratified. A lack of mixing and high productivity in the reservoir cause complete oxygen depletion below the thermocline by late spring. As a result, the oxygen content at depths greater than 6 m never exceeds 1 mg L⁻¹ from May to August (Taylor 1980).

In the early 1980s, *Hydrilla verticillata* (l.f.) Royal was accidentally introduced into the Lake Nacogdoches and by 1989 covered approximately 45% of the reservoir (Prater 1991). *H. verticillata* spread mainly in the upper shallow part of the water body, where it completely covered the reservoir. In contrast, in the lower part less than 3% of the reservoir is covered with *H. verticillata* (Fig. 1).

Sampling Protocol

To determine the distribution of *C. fluminea* and its effect on the benthic community of Lake Nacogdoches, a total 96 bottom
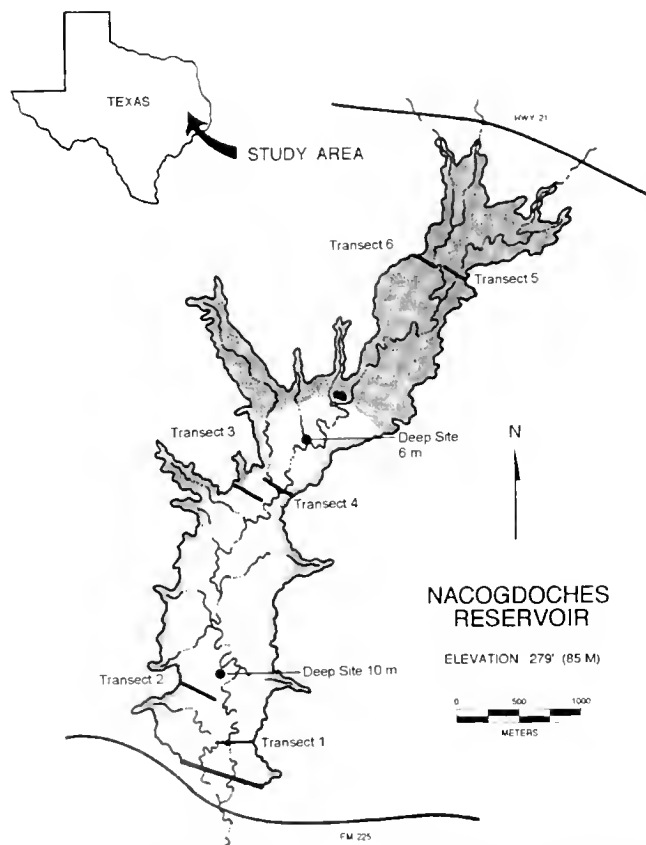

Figure 1. Location of transects sampled in Lake Nacogdoches. Shaded area represents the extent of the reservoir covered by *Hydrilla verticillata*.

samples were taken in September (transect 1) and October (transects 2–6) 2001 (Fig. 1). For each transect, samples were collected from 1, 2, 3, 4, 6, and 8 m, except for transects 5 and 6, where samples were collected at depths of 1, 2, 3, and 4 m. These last two transects were situated at the upper shallow part of the reservoir with a maximum depth less than 5 m. In addition, the deep (profundal) part of the lake was sampled separately (6 and 10 m depth). Three or more replicate samples were taken at each depth with an Ekman grab (sampling area = 0.0233 m²) and washed through a 550-μm mesh. At each sampling point, water transparency, bottom temperature, pH, oxygen, and conductivity were recorded (Table 1). After sampling, all macroinvertebrates were transferred to containers with 10% neutral-buffered formalin and labeled. All macroinvertebrates were identified to the genus or species level, counted, and weighted to the nearest 0.0001 g after being blotted dry on absorbent paper (wet mass). For oligochaetes, only *Branchiura sowerbyi* Beddard and *Stylaria lacustris* (Linnaeus) were identified to species level. All *C. fluminea* and unionids were cut open with a scalpel to remove water from the mantle cavity, measured, weighed (wet mass), and identified to species. The average mass of individual *C. fluminea* in a sample was calculated by dividing the total mass by the number of clams in the sample. Because several samples contained no *C. fluminea* (density = 0), we used nonparametric Kruskal–Wallis test to analyze the data. When multiple statistical tests were conducted on the same data, we used a Bonferroni correction to determine the critical alpha for significance.

RESULTS

Corbicula fluminea *Distribution*

During our September sampling, the reservoir was still well stratified for temperature and oxygen to around 6 m depth (Table 1). In October, the lake was well mixed and both temperature and oxygen did not vary appreciably with depth. Oxygen content was low only at the deepest sampled site (10 m depth). Water pH and conductivity did not show sharp changes across the thermocline (Kruskal–Wallis test, $P = 0.20$).

C. fluminea was found only in the lower part of Lake Nacogdoches (transects 1–4; Fig. 1). We did not find any live *C. fluminea*, or even their dead shells, in the upper part of the reservoir, which was covered with *H. verticillata* (transects 5 and 6).

We found a significant difference in some chemical parameters between regions of the lake with *C. fluminea* (transects 2–4, 1–4 m) and the area of the lake with *H. verticillata*, where we did not find clams (transects 5–6, 1–4 m). The pH was slightly higher in the upper region (7.96 ± 0.009, $n = 12$) vs. 7.86 ± 0.011 ($n = 7$; Kruskal–Wallis test, $P = 0.0005$). Dissolved oxygen was slightly lower in area covered with *H. verticillata* (9.26 ± 0.06 ($n = 7$) vs. 9.76 ± 0.15 mg L⁻¹ ($n = 12$); Kruskal–Wallis test, $P = 0.016$), but this difference was only marginally significant (critical alpha with the Bonferroni Correction = 0.012). Conductivity was lower in the upper part of the reservoir (93.11 ± 0.28 ($n = 7$) vs. 95.66 ± 0.53 m Siemens cm⁻¹ ($n = 12$); Kruskal–Wallis test, $P = 0.002$). Transect 1 was not included in these analyses as it was sampled 20 days earlier.

The average (± SE) *C. fluminea* density and biomass in the lower portion of the reservoir (transects 1–4, depths 1–8 m) was 15.6 ± 5.3 m⁻² and 71.9 ± 18.8 g m⁻², respectively. There were no significant differences in density or biomass of *C. fluminea* between the four transects (Kruskal–Wallis test, $P > 0.44$). In addi-

TABLE 1.

Oxygen concentration, temperature, conductivity and pH in Lake Nacogdoches.

Parameter	Depth (m)						
	1	2	3	4	6	8	10
Transect 1							
Oxygen, mg · L^{-1}	9.72 (1)	9.57 (1)	9.40 (1)	9.26 (1)	3.03 (1)	0.45 (1)	—
Temperature, °C	27.8 (1)	27.7 (1)	27.6 (1)	27.6 (1)	26.3 (1)	24.3 (1)	—
Conductivity, mSiemens cm^{-1}	96.9 (1)	95.3 (1)	94.3 (1)	94.2 (1)	97.7 (1)	116.7 (1)	—
pH	7.87 (1)	7.90 (1)	7.90 (1)	7.87 (1)	7.76 (1)	7.60 (1)	—
Transects 2–4							
Oxygen, mg · L^{-1}	10.24 ± 0.36 (3)	9.62 ± 0.39 (3)	9.64 ± 0.30 (3)	9.52 ± 0.25 (3)	7.83 ± 0.40 (3)	6.25 ± 2.15 (2)	—
Temperature, °C	23.10 ± 0.17 (3)	22.69 ± 0.30 (3)	22.83 ± 0.12 (3)	22.82 ± 0.12 (3)	22.45 ± 0.05 (3)	22.30 ± 0 (2)	—
Conductivity, mSiemens cm^{-1}	96.27 ± 1.77 (3)	95.2 ± 0.95 (3)	95.2 ± 0.68 (3)	95.97 ± 1.06 (3)	95.70 ± 0.20 (3)	97.65 ± 2.25 (2)	—
pH	7.87 ± 0.03 (3)	7.86 ± 0.02 (3)	7.86 ± 0.01 (3)	7.84 ± 0.02 (3)	7.84 ± 0.03 (3)	7.80 ± 0.02 (2)	—
Transects 5–6							
Oxygen, mg · L^{-1}	9.30 ± 0.20 (2)	9.20 ± 0 (2)	9.33 ± 0.12 (2)	9.15 (1)	—	—	—
Temperature, °C	22.30 ± 0.20 (2)	22.31 ± 0.16 (2)	22.41 ± 0.27 (2)	22.7 (1)	—	—	—
Conductivity, mSiemens cm^{-1}	92.60 ± 0 (2)	93.5 ± 0.50 (2)	93.55 ± 0.75 (2)	92.50 (1)	—	—	—
pH	7.96 ± 0.03 (2)	7.95 ± 0.04 (2)	7.96 ± 0.005 (2)	7.97 (1)	—	—	—
Profundal							
Oxygen, mg · L^{-1}	—	—	—	—	8.45 (1)	—	0.85 (1)
Temperature, °C	—	—	—	—	23.4 (1)	—	21.3 (1)
Conductivity, mSiemens cm^{-1}	—	—	—	—	93.6 (1)	—	123.0 (1)
pH	—	—	—	—	7.14 (1)	—	7.80 (1)

Transect 1 was sampled in September; all other transects were sampled in October. Transects 5 and 6 were in an area of the lake covered with *Hydrilla verticillata* and no *Corbicula fluminea*. Transects 2–4 had *C. fluminea* and no *H. verticillata*. In addition, we sampled two sites deep in the lake (profundal, 6 and 10 m). Average values ± standard errors of mean are given, sample sizes are in parentheses, — = no data.

tion, density and wet mass did not differ significantly with depth down to 4 m (Kruskal–Wallis test: density: $P = 0.29$; biomass: $P = 0.40$; Figs. 2 and 3). This lack of significance is probably caused by the high degree of patchiness (Index of Dispersion Test; $I = 59.7$; $\chi^2 = 1 * (n - 1) = 59.7*(48 - 1) = 2805$. $\chi^2_{0.025, 47} = 67.8$, $P < 0.001$). Many samples had no *C. fluminea*, which resulted in an increase in the variance in observed means. The mean density of *C. fluminea* was greatest at 1 m ($35.8 ± 13.8$ m^{-2}), and the maximum biomass was at 2 m ($137.17 ± 69.21$ g m^{-2}). The maximum density of *C. fluminea* in a single sample was 172 m^{-2} (transect 4, 1 m) and maximum wet mass (soft body + shells) in a single sample was 770 g m^{-2} (transect 2, 2 m).

The average individual mass for *C. fluminea* (total sample mass/density) differed significantly with depth (ANOVA,

Figure 2. Density of live and dead shells of *Corbicula fluminea* at different depths in Lake Nacogdoches. Averages, standard errors of mean, and sample sizes are given.

Figure 3. Total wet mass (left axis) and average individual wet mass (total wet mass divided by the number of clams in the sample, r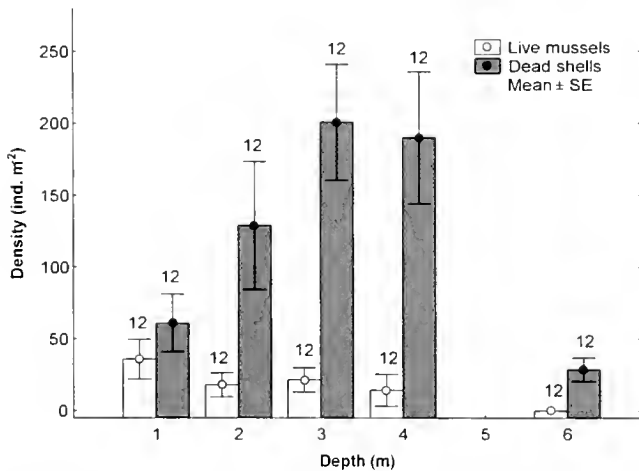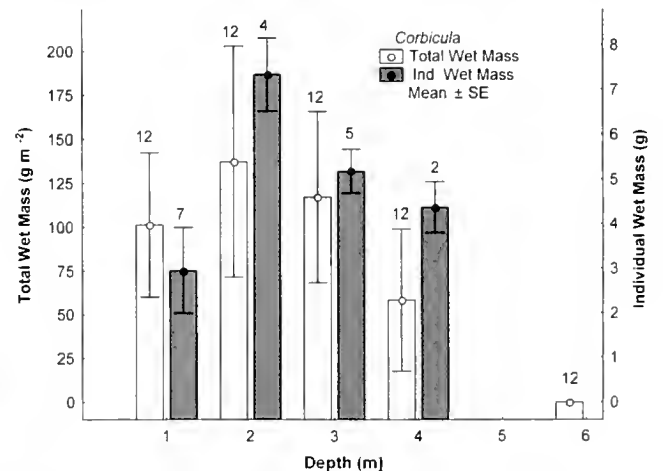ight axis) of *Corbicula fluminea* at different depths in Lake Nacogdoches. Averages, standard errors of mean, and sample sizes are given.
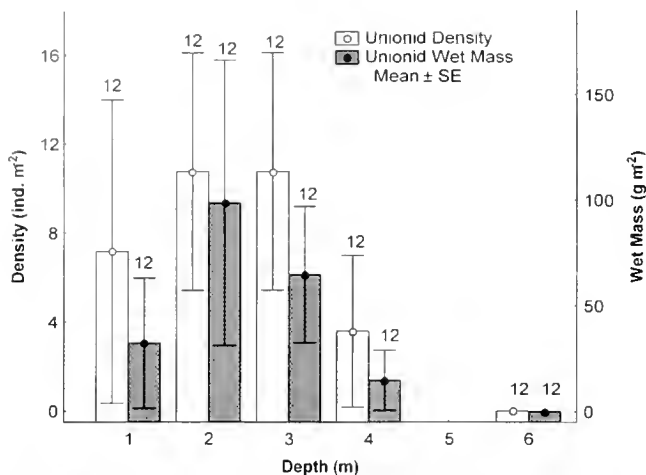
Figure 4. Density (left axis) and total wet mass (right axis) of unionids at different depths in Lake Nacogdoches. Averages, standard errors of mean, and sample sizes are given.

$P < 0.029$; Fig. 3). The smallest average individual mass (2.95 ± 1.02 g) was at 1 m, and the largest average individual mass (7.33 ± 0.86 g) was at 2 m.

C. fluminea shells were found down to 6 m (Fig. 2). The distribution of shells with depth was not uniform (Kruskal–Wallis test, $P = 0.0002$) and differed from the distribution of live *C. fluminea* (Kolmogorov–Smirnov test, $P < 0.001$).

C. fluminea density differed significantly among substrate types (Kruskal–Wallis test, $P = 0.04$). Dead *C. fluminea* shells and course detritus had the highest density (43 ± 14 m^{-2}), and the lowest density (3.6 ± 3.6 m^{-2}) was found in silt (Table 2). Dead *C. fluminea* shells were not found uniformly among substrate types, and were most abundant in clay with stones (272 ± 56 m^{-2}; Kruskal–Wallis test, $P = 0.0008$).

Distribution of Benthic Animals

We found 38 taxa (species, genera or higher taxa), including 17 chironomids (identified to the species or genus level). The average density of benthic animals, excluding bivalves, over the entire reservoir was 901 ± 91 m^{-2} with a biomass of 2.76 ± 0.29 g m^{-2}. The most abundant insect larvae were the chironomid *Coelotanypus tricolor* (Lowe) (185 ± 25 m^{-2}, total number of samples = 96), *Chironomus* sp. (69 ± 17 m^{-2}), and the phantom midge *Chaoborus punctipennis* (Say) (137 ± 35 m^{-2}). Among oligochaetes, *Branchiura sowerbyi* Beddard was dominant (71 ± 12 m^{-2}). Only a single species of amphipod, *Hyallela azteca* (Saussure), was found (24 ± 11 m^{-2}).

Corbicula fluminea dominated the benthic biomass in the littoral zone from 1–4 m in the lower part of the reservoir (transects 1–4) and was responsible for more than 97% of the total wet mass of the benthic community. At depths ≥ 6 m, *Chironomus* sp., *C. punctipennis*, and *B. sowerbyi* were responsible for 43%, 17%, and 26% of the total benthic biomass, respectively.

In the upper region of the reservoir (transects 5 and 6) the average density (1165 ± 216 m^{-2}) and average biomass (3.57 ± 0.54 g m^{-2}) of benthic animals were marginally higher (Kruskal–Wallis test, density: $P = 0.073$; biomass: $P = 0.061$) than in the lower part (excluding *C. fluminea* and unionids density 843 ± 104 m^{-2}, biomass 2.60 ± 0.35 g m^{-2}). However, because of the presence of *C. fluminea* in the lower region of the reservoir, the total macrobenthos (including *C. fluminea*) biomass (74.5 ± 18.9 g m^{-2}) was 20 times greater than in the upper part.

There were three species of unionids in the lake, *Pyganodon grandis* (Say), *Ligumia subrostrata* (Say), and *Toxolasma texasensis* (Lea). Two *P. grandis*, one *L. subrostrata*, and six *T. texasensis* were found on transects 2, 3, and 4 on depths of 1–4 m (Fig. 4). These unionids completely overlapped with the distribution of *C. fluminea* (Kolmogorov–Smirnov test, $P > 0.10$).

For transects 1–4, the maximum density and biomass of unionids was in clay (17.9 ± 8.3 m^{-2}, 97.5 ± 42.8 g · m^{-2}, total number of samples $n = 12$) and in course detritus with *C. fluminea* shells (10.8 ± 5.6 m^{-2}, 98.4 ± 71.1 g · m^{-2}, $n = 12$). The lowest density and biomass of unionids was in silt (3.6 ± 3.6 m^{-2}, 14.9 ± 14.9 g·m^{-2}, $n = 12$). There were no significant correlations between the *C. fluminea* density or the density of *C. fluminea* shells and any invertebrate taxon.

DISCUSSION

C. fluminea *Distribution*

The exotic plant *Hydrilla verticillata* covers approximately 45% of Lake Nacogdoches and is the dominant macrophyte species in this community (Prater 1991). Another exotic species, *C. fluminea*, dominated the benthic community of this reservoir. However, the spatial distribution of these two nuisance species did not overlap. During our study, neither live *C. fluminea* nor dead shells were found in the upper part of the reservoir, which is covered with *H. verticillata*. Prater (1991) sampled the benthos of Lake Nacogdoches monthly over 12 mo in 1989–1990 and never found *C. fluminea* in the *H. verticillata* region of the reservoir as well. Two factors may contribute to the absence of *C. fluminea* in the upper part of the reservoir. First, dense *H. verticillata* mats may deplete the oxygen in the water to levels below those critical for *C. fluminea* survival. We found a significant decrease in oxygen in

TABLE 2.

Abundance of live *Corbicula fluminea* and shells in various substrata in Lake Nacogdoches in 2001.

Substrate Type	Density, Ind. m^{-2}	Biomass, g · m^{-2}	*C. fluminea* Shells m^{-2}
C. fluminea shells and course detritus	43.0 ± 14.0 (12)	162.68 ± 56.01 (12)	71.7 ± 25.6 (12)
Clay and stones	19.1 ± 14.5 (9)	95.36 ± 63.75 (9)	272.3 ± 55.5 (9)
Clay	25.1 ± 8.3 (12)	149.93 ± 66.26 (12)	100.3 ± 21.4 (12)
Sand	14.3 ± 14.3 (3)	45.87 ± 45.87 (3)	28.7 ± 28.7 (3)
Silt	3.6 ± 3.6 (12)	17.77 ± 17.77 (12)	112.9 ± 28.6 (24)

The abundance of live *C. fluminea* was estimated from transects 1–4, from 1–4 m. The abundance of dead shells was estimated from transects 1–4 and 1–6 m. Average values ± standard errors of the mean are given. Sample size in parentheses.

the portions of the lake covered with *H. verticillata*, but this difference was relatively small. Second, bottom substrates in the upper part of the reservoir are predominantly silty clay, which may be unfavorable for *C. fluminea*.

C. fluminea was found in all four transects in the lower part of Lake Nacogdoches at depths up to 4 m, and *C. fluminea* dead shells were found up to 6 m depth. Deeper in the reservoir, *C. fluminea* was probably limited by low oxygen, especially during the summer, when the water column is stratified and the oxygen content deeper than 6 m never exceeds 1 mg L^{-1} (Taylor 1980). *C. fluminea* is known to be intolerant of even moderate hypoxia (McMahon 1991, McMahon & Bogan 2001), and low oxygen is considered to be one of the main sources of mortality for *C. fluminea* (Sieckel 1986). Although live *C. fluminea* were most dense at 1 m depth and had highest total biomass at 2 m, their dead shells were most abundant at 3 and 4 m (Fig. 2), which suggested that the depth of maximum *C. fluminea* abundance may vary with time or that dead shells were transported to deeper water by water motion.

C. fluminea density and biomass also varied among substrate types. Clams were most abundant in sediments formed by shells and course detritus and least abundant on silt. The mean population density and biomass of *C. fluminea* we found were very similar to those found 10 years earlier by Prater (1991). He found that clam density in 1989–1990 varied from 0 to 60 m^{-2} (average 24.4 ± 5.5 m^{-2}). This suggests that the population density of *C. fluminea* in Lake Nacogdoches is rather stable. *C. fluminea* can occur in dense aggregations, exceeding 2000 m^{-2} (Gardner et al. 1976, Phelps 1992). These densities are much higher densities than those that have been recorded in Lake Nacogdoches. However, these higher densities were reported for a limited period of time, shortly after initial invasion (Phelps 1994) or from a local spot in a water body (Eng 1979). For example, after the initial invasion in the Potomac River in 1977, *C. fluminea* reached a maximum density in 1986 (722 g m^{-2} wet weight, including shell) but then sharply declined, and in 1992 was at 24.8% of 1986 levels (Phelps 1994). In the sediment bars of the Delta-Mendota Canal, the maximum density of *C. fluminea* at one site was 131,200 m^{-2}; however, the average density was much smaller (Eng 1979). In another Texas lake, Lake Arlington, the mean density of *C. fluminea* in 1975 was very similar to the densities we found in Lake Nacogdoches (32.1 ± 16.5 m^{-2}, Aldridge & McMahon 1978).

Dominance in Benthos

C. fluminea appears to dominate the benthic community of water bodies it invades (McMahon 1983, Counts 1986, McMahon 1991, Poff et al. 1993, McMahon 1999). We found that in littoral zone of Lake Nacogdoches *C. fluminea* comprises more than 97% of the total wet mass of the macrobenthic community. We found no correlations between *C. fluminea* density and biomass and other nonmolluscan invertebrates.

Impact on Unionids

Whether *C. fluminea* and native bivalves compete is controversial (McMahon 1999, Strayer 1999). According to some authors, *C. fluminea* may out compete native unionids (Kraemer 1979, Belanger et al. 1985, Leef et al. 1990, Howells 1992). The competitive advantage of *C. fluminea* over native bivalves has been suggested because it has a much higher filtering rate than native species (Mattice 1979, Lauristen 1986). In addition, by being able to use both filter and pedal feeding, *C. fluminea* may have an

advantage over native bivalves that are only able to filter feed (Hakenkamp & Palmer 1999). However, most of the evidence for the competitive impacts of *C. fluminea* on native bivalves is based on an analysis of their spatial distributions, and much of these data are anecdotal and qualitative rather than quantitative (Strayer 1999). According to many authors (reviewed in Strayer 1999), *C. fluminea* and native bivalves have nonoverlapping spatial distributions, implying that *C. fluminea* can out compete other bivalves. However, we found that in Lake Nacogdoches unionids and *C. fluminea* are both abundant and occupied the same areas. The depth distribution of *C. fluminea* and unionids was completely overlapping. In addition, both unionids and *C. fluminea* were abundant in the same type of substrate (course detritus with *C. fluminea* shells and clay). The lowest numbers and biomass of both *C. fluminea* and unionids were in silt.

Several other authors have found that unionids and *C. fluminea* coexist (Clarke 1988, Beaver et al. 1991, Miller & Payne 1994). These data may suggest that the impact of *C. fluminea* on native unionids is not as strong as the impact of zebra mussels, which can cause mass mortality of unionids (reviewed in Karatayev et al. 1997).

Impacts of Corbicula fluminea *versus* Dreissena polymorpha

In Lake Nacogdoches *C. fluminea* was never found in areas over grown by *H. vertecillata*. In contrast, zebra mussels are often found at their highest densities on submerged macrophytes, which they use as sites for attachment (Lewandowski 1982, Lyakhnovich et al. 1994, Karatayev et al. 1998). During our study, *C. fluminea* was most abundant on shelly sediment. This sediment is also one of the best substrates for the zebra mussel (Lyakhnovich et al. 1994, Karatayev et al. 1998). Silt is the poorest substrate for both *C. fluminea* (Duarte & Diefenbach 1994) and *D. polymorpha* (Zhadin 1946, Draulans & Wouters 1988, Karatayev & Burlakova 1995) and often limits their distributions. Belanger et al. (1985) found in their field and laboratory studies that *C. fluminea* preferred the following sediments in decreasing order: fine sand, organically enriched fine sand, and coarse sand. *C. fluminea*, a burrowing animal, preferred fine sediments; however, the zebra mussel, which attaches to hard substrate, forms especially high densities on rocks (Lyakhnovich et al. 1994, Burlakova 1998). Low oxygen may be another important limiting factor for both *C. fluminea* (McMahon 1991, McMahon & Bogan 2001) and the zebra mussel (Mikheev 1961, Spiridonov 1972, Shkorbatov et al. 1994).

Both *C. fluminea* (McMahon 1983, Counts 1986, McMahon 1991, Poff et al. 1993, McMahon 1999) and *D. polymorpha* (Sokolova et al. 1980, Karatayev et al. 1994) dominate benthic communities and are responsible for more than 95% of the biomass in lakes where they occur. *C. fluminea* live in soft sediment, crawl through sediment with a foot, and feed both as a filter feeder from the water column (Cohen et al. 1984, Boltovskoy et al. 1995), and from the sediments as a pedal feeder (Reid et al. 1992, Hakenkamp et al. 2001) and thus may negatively impact burrowing detritivores (McMahon 1999). Zebra mussels, in contrast, can live only on the surface of the sediments, where they attach to hard substrates and each other with proteinacious byssal threads creating complex three-dimensional structures (Karatayev et al. 2002). *D. polymorpha* constantly filter the water for both feeding and respiration. Filtered particles are either consumed or bound in mucus, preventing immediate re-suspension. This zebra mussel

activity builds a direct connection between the planktonic portion of water body and the benthos (benthic-pelagic coupling) and greatly enhances the rates of deposition of both organic and inorganic material on the bottom. *D. polymorpha* provide food and shelter for many benthic invertebrates, which have increased density and biomass in zebra mussel beds. Simultaneously other species (mainly filter feeders) may decrease or disappear from the community (Karatayev & Burlakova 1992, Stewart et al. 1998, Stewart et al. 1999). This well-documented effect of zebra mussel on benthic communities contrasts with the unknown impact of *C. fluminea* on composition, structure and densities of native invertebrates. In a recent study Hakenkamp et al. (2001) found that when they experimentally increased *C. fluminea* density in the field, there was no apparent impact on the abundance or taxonomic composition of the meiofauna.

In some circumstances *C. fluminea* may compete with native bivalves for food or substrate. In contrast, the negative impact of *D. polymorpha* on native unionids is more diverse. Besides resource competition, zebra mussels also show direct interference competition through overgrowth of unionids. By attaching to unionids, zebra mussels can make it more difficult for them to burrow and move through the sediment. They can weight down

their host unionid, resulting in burial in very soft sediments, can increase drag and the likelihood of dislodgement by water motion for species living near shore, prevent opening valves for respiration, feeding and reproduction, or preventing the closing valves (reviewed in Karatayev et al. 1997, Burlakova et al. 2000).

Mass mortalities of unionids caused by *D. polymorpha* overgrowth are most common during the initial stages of colonization, when mussel populations are growing rapidly. After initial peaks in zebra mussel abundance, *D. polymorpha* can coexist with unionid bivalves (Nichols & Amberg 1999, Burlakova et al. 2000). Similarly, we hypothesize that the strength of competition between *C. fluminea* and native bivalves may depend on various factors including unionid species, *C. fluminea* density, and time since *C. fluminea* invasion.

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PATTERNS OF EMERGENCE AND SURVIVAL OF *CONCHOPHTHIRUS ACUMINATUS* (CILIOPHORA: CONCHOPHTHIRIDAE) FROM *DREISSENA POLYMORPHA* (BIVALVIA: DREISSENIDAE)

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ABSTRACT This is the first study to quantify the periodic emergence of a *Conchophthirus* sp. from its bivalve host. Emergence rates of *C. acuminatus* from *Dreissena polymorpha* over the entire 24-day experiment appeared to be directly correlated with infection intensity. The rate of ciliate emergence from individual mussels varied considerably throughout the experiment at both 14°C and 21°C. It was not uncommon to have a sampling period in which no emergence was observed immediately followed by a period of high emergence, e.g., at 14°C from 0 to 25 ciliates and at 21°C from 0 to 720 ciliates. The total mean number of ciliates that were observed to have emerged from each mussel during the 24-day experiment was significantly higher at 21°C (207 ciliates/mussel) than at 14°C (29 ciliates/mussel). Our experiments suggested that *C. acuminatus* have a short survival period outside their host. Although we observed a maximum survival period of 144 hr (6 days), most ciliates died within 48 h.

KEY WORDS: *Conchophthirus acuminatus*, ciliate, commensal, host, bivalve, *Dreissena polymorpha*, zebra mussel, mantle cavity

INTRODUCTION

The ciliate *Conchophthirus acuminatus* (Claparède & Lachmann) (Scuticociliatida: Conchophthiridae) is the most common of 34 endosymbionts associated with zebra mussels (*Dreissena polymorpha* (Pallas)) (Molloy et al. 1997). Although not known from North America, this ciliate is very common in European zebra mussel populations, including in Bulgaria (Raabe 1934), Denmark (Fenchel 1965), Hungary (Raabe 1950), Macedonia (Raabe 1966), Poland (Dobrzanska 1958), and Switzerland (Claparède & Lachmann 1858). Its widespread distribution was recently confirmed by its presence in all 21 zebra mussel populations surveyed in Belarus (Burlakova et al. 1998, Karatayev et al. 2000a). Among all zebra mussel protozoan symbionts, this ciliate typically has the highest prevalence (i.e., percentage of mussels with ciliates) and intensity of infection (i.e., number of ciliates per infected mussel) (Molloy et al. 1997, Burlakova et al. 1998, Karatayev et al. 2000a).

Conchophthirus acuminatus appears to be very specific to *Dreissena* and has never been reported from any other host. Raabe (1950) never observed it in unionid mussels, even though they were sometimes completely covered by *C. acuminatus*-infected zebra mussels. Although its feeding on the sperm cells of *D. polymorpha* has been documented (Laruelle et al. 1999), *C. acuminatus* is likely a commensal organism which ingests a variety of organic particles present on *Dreissena*'s mantle epithelial surfaces (Molloy et al. 1997). *C. acuminatus* is typically found on the epithelial surfaces of the mantle, gills, visceral mass, and labial palps, and within gill water tubes and suprabranchial cavities (Laruelle et al. 1999).

As with other *Conchophthirus* spp., *C. acuminatus* appears to have an obligate association with its bivalve host, with the only free-living phase of its life cycle occurring during its transfer to new hosts. The longer these ciliates can live in open water, the greater their success in reaching new hosts, particularly distant zebra mussel populations. An investigation of this free-living phase in the *C. acuminatus* life cycle was the focus of this study.

In a series of laboratory experiments, we quantified the frequency that these ciliates emerged from zebra mussels and measured their survival rate in open water. The results presented herein are part of an extensive investigation that we, as members of the International Research Consortium on Molluscan Symbionts (Molloy 2003), are conducting to characterize the systematics, biology, ecology, and distribution of *Dreissena*'s endosymbionts (Molloy et al. 1996, Molloy et al. 1997, Molloy et al. 2001, Burlakova et al. 1998, Laruelle et al. 1999, Karatayev et al. 2000a, Karatayev et al. 2000b, Karatayev et al. 2002, Laruelle et al. 2002, Fokin et al. 2003). This current study, in particular, will hopefully contribute to a better understanding of the emergence patterns and subsequent free-living phase of *C. acuminatus* and will thereby provide insights into the life cycle of a commensal—a type of symbiont which, relative to parasites and mutualists, has received little research attention.

MATERIALS AND METHODS

Laboratory experiments were conducted during 1998–2002 in the Republic of Belarus using zebra mussels collected at a ca. 1.5 m depth from the Dnieper–Bug Canal (52°06′N, 26°00′E) and the Svisloch River (53°55′N, 27°32′E).

Emergence of C. acuminatus from D. polymorpha

To determine the frequency of emergence of *C. acuminatus* from zebra mussels, an experiment was conducted in April 1998 in which 48 mussels from the Dnieper–Bug Canal were placed individually in 20-mL Petri dishes containing a suspension of the alga *Scenedesmus acuminatus* (Lagerheim) in 10 mL of unchlorinated tap water. For 24 days, half of these dishes were held at 14 (±1)°C and half at 21 (±1)°C. Mean mussel lengths in the 14°C and 21°C dishes were, respectively, 13.8 mm and 14.3 mm (Tables 1 and 2). Every 2 to 3 days, the water in each dish was transferred to a plankton counting chamber and fresh, unchlorinated tap water and algae were added to each dish. Water in the counting chamber was examined for *C. acuminatus* using a stereomicroscope (20×), with ciliates counted and discarded.

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TABLE 1.

Pattern of emergence of *C. acuminatus* from *D. polymorpha* at the 14 (±1)°C.

Mussel No.	Mussel Length (mm)	Number of Ciliates Collected Outside Their Host										Total During Experiment	Infection Intensity on Day 24
		Day 3	Day 5	Day 7	Day 10	Day 12	Day 14	Day 17	Day 19	Day 21	Day 24		
1	13.4	6	0	0	1	4	1	1	0	11	7	31	34
2	13.2	1	0	0	1	0	6	1	0	4	6	19	74
3	15.5	1	0	1	2	5	3	0	2	17	24	55	313
4	14.0	3	0	0	1	0	0	1	2	17	6	30	19
5	14.6	2	1	2	3	3	2	2	0	25	28	68	125
6	13.1	2	0	0	1	0	—	1	1	6	1	12	1
7	13.2	0	0	2	1	2	0	2	1	14	5	27	117
8	13.6	0	0	0	0	0	5	1	1	4	4	15	3
9	13.0	0	0	1	0	0	2	0	2	8	24	37	41
10	13.5	1	0	0	3	2	3	2	4	11	7	33	24
11	14.0	1	0	0	2	1	0	1	0	8	8	21	13
12	14.1	4	6	26	19	0	8	13	4	3	12	95	0
13	14.0	1	0	0	3	0	1	0	1	4	6	16	5
14	14.0	0	0	0	0	2	0	2	2	5	29	40	89
15	14.2	1	0	3	3	1	0	2	3	5	1	19	0
16	13.9	0	0	0	1	0	0	0	1	13	6	21	89
17	14.1	0	0	3	0	0	6	9	5	5	6	34	10
18	13.6	3	0	2	1	1	0	0	0	2	10	19	4
19	13.6	0	1	1	1	0	2	0	0	6	3	14	69
20	13.9	1	3	1	1	2	0	—	1	5	7	21	16
21	13.2	0	2	4	2	1	1	0	4	6	18	38	20
22	13.4	1	0	0	1	0	2	0	0	4	17	25	77
23	13.6	1	0	1	2	1	0	0	0	0	1	6	11
24	13.6	0	1	0	0	2	0	1	0	0	5	9	10
Mean	13.8	1.2	0.6	2.0	2.0	1.1	1.8	1.7	1.4	7.6	10.0	29.4	48.5
SE	0.02	0.06	0.06	0.22	0.16	0.06	0.10	0.13	0.06	0.25	0.36	0.83	2.85

To determine infection prevalence and intensity at the beginning of the experiment, we dissected 13 14-mm long mussels from the above-mentioned Dnieper–Bug Canal sample. Infection prevalence and intensity were also calculated at the end of the experiment by dissecting the 48 mussels used in the Petri dishes. During dissection, mussel mantle cavities were repeatedly flushed with unchlorinated tap water using a pipette to remove all ciliates from exposed epithelial surfaces. Because *C. acuminatus* were also present within gill water tubes and suprabranchial cavities, gills were lacerated with forceps and then flushed by pipette. The number of *C. acuminatus* in all rinse water was determined in a plankton counting chamber using a stereomicroscope (20×).

Survival of C. acuminatus *Outside* D. polymorpha

Three laboratory experiments were conducted to determine how long *C. acuminatus* survive outside their host in open water. In all experiments, *C. acuminatus* were transferred with a pipette into dishes containing water. Dishes were then covered with lids to prevent evaporation and half of them were held at 14 (±1)°C and the other half at 21 (±1)°C. Using a stereomicroscope (20×), dishes were inspected until all ciliates had died.

Experiment 1

In November 1998, mussels were collected from the Svisloch River and dissected. Ciliates were held in groups of 10 in each of

six 10-mL Petri dishes containing 2 mL of unchlorinated tap water and were inspected daily.

Experiment 2

In January 2000, 40 *C. acuminatus* obtained by dissection from zebra mussels collected in Dnieper–Bug Canal were held individually in 10-mL Petri dishes containing 3 mL of unchlorinated tap water. Mortality was scored at 6, 21, 70, and 90 h.

Experiment 3

In July 2002, 20 *C. acuminatus* obtained by dissection from zebra mussels collected from the Svisloch River were held at 14 (±1)°C and 23 (±1)°C in 40 individual 4-mL plastic dishes containing 2 mL of filtered (100-μm mesh net) Svisloch River water. Mortality was scored at 6, 24, 30, 48, and 54 h. During each dish inspection, 1 mL of water in each dish was replaced with fresh filtered water. Since ciliates may be more sensitive to environmental changes than their hosts (Beers 1959), we followed Beers' suggestion to collect mussels as needed and to use the ciliates at once. Therefore, in experiment 3 we repeated the same exact procedure three times, starting on three consecutive days using ciliates from freshly collected mussels.

Data Analysis

The Box–Cox procedure (Krebs 1999) indicated that the best transformation to achieve a normal distribution was $X' = (X + 1)^{0.22}$.

TABLE 2.

Dynamics of the emergence of *C. acuminatus* from *D. polymorpha* at the 21 (±1)°C

Mussel No.	Mussel Length (mm)	Number of Ciliates Collected Outside Their Host										Total During Experiment	Infection Intensity on Day 24
		Day 3	Day 5	Day 7	Day 10	Day 12	Day 14	Day 17	Day 19	Day 21	Day 24		
1	13.1	2	0	0	3	0	0	70	176	15	26	292	0
2	13.1	1	0	17	99	29	9	22	15	16	2	210	0
3	14.0	2	9	2	2	5	3	2	85	171	35	316	7
4	15.5	0	720	186	45	14	5	4	0	12	1	987	63
5	14.0	1	3	6	4	0	3	31	20	12	5	85	1
6	15.5	9	0	6	120	159	87	156	32	24	47	640	143
7	15.8	1	0	1	2	0	0	1	5	5	32	47	49
8	15.2	43	9	22	6	1	4	14	7	4	4	114	129
9	14.6	20	2	0	0	3	9	105	24	37	16	216	114
10	13.0	0	2	0	28	15	9	34	6	9	3	106	168
11	14.3	3	72	3	1	4	11	39	14	24	41	212	732
12	13.5	0	3	10	2	1	2	0	1	1	5	25	262
13	14.5	1	0	0	0	1	3	66	45	15	7	138	331
14	14.5	1	2	1	2	0	5	8	3	6	11	39	158
15	15.2	9	76	1	1	5	0	6	29	9	6	142	1035
16	13.9	5	0	2	0	4	13	4	4	1	1	34	0
17	15.8	2	55	1	3	6	33	61	150	34	61	406	93
18	14.0	0	7	0	1	2	15	200	29	52	6	312	175
19	14.1	1	0	0	0	0	3	3	3	6	11	27	264
20	13.4	0	1	4	7	11	7	26	8	11	3	78	39
21	13.7	0	5	0	0	3	0	1	6	5	5	25	21
22	13.2	0	3	1	0	2	3	9	39	25	133	215	82
23	13.6	5	1	3	11	10	6	20	72	12	0	140	27
24	15.4	0	8	0	19	24	14	81	10	4	3	163	351
Mean	14.3	4.4	40.8	11.1	14.8	12.5	10.2	40.1	32.6	21.3	19.3	207.0	176.8
SE	0.04	0.39	6.10	1.57	1.30	1.34	0.74	2.16	1.91	1.43	1.23	9.17	10.23

To compare transformed data, we used Welch's approximate *t* test (or *t* test if variances were homogeneous) in Statistica software (Windows Release 6.0, StatSoft, Inc.). Effects were considered statistically significant at $P < 0.05$.

RESULTS

Emergence of C. acuminatus *from* D. polymorpha

The rate of ciliate emergence from individual mussels varied considerably throughout the experiment at both 14°C and 21°C. It was not uncommon to have a sampling period in which no emergence was observed, immediately followed by a period of high emergence, e.g., at 14°C from 0 to 25 ciliates (Table 1: mussel 5, day 19 vs. day 21) and at 21°C from 0 to 720 ciliates (Table 2: mussel 4, day 3 vs. day 5).

At 14°C, typically ≤3 ciliates were observed outside a host mussel each sampling day, but this pattern was typically interrupted by periods of higher emergence, particularly toward the end of the experiment (Table 1). The mean number of ciliates that were observed outside of the 24 mussels at 14°C ranged from 0.6 to 10.0 ciliates/mussel (Table 1). During the first 19 days of the experiment, a mean of 1.5 ciliates was observed outside the 24 mussels at 14°C (Table 1). Dissection data indicated that infection intensity in the 14°C mussels during the experiment remained constant at about 48 ciliates/mussel (day 0 and day 24 intensities of, respectively 47.3 and 48.5 ciliates/mussel, Table 1). This indicated that during the first 19 days of the experiment on average ca. 3% (i.e..

1.5/49.5) of ciliates were outside their hosts on a sampling day. Emergence rates increased toward the end of the 14°C experiment with a mean emergence of 10.0 ciliates/mussel at the termination of the experiment on day 24 (Table 1). Because the 24 mussels dissected at the end of the 14°C experiment had a mean infection intensity of 48.5 ciliates/mussel (Table 1), this indicated that ca. 17% (i.e., 10.0/58.5) of all ciliates present within the 24 dishes were outside their hosts on day 24.

A similar irregular pattern of ciliate emergence was observed at 21°C (Table 2). Typically ≤15 ciliates were observed outside a host mussel at 21°C, but the majority of mussels also had at least one sampling period during which very high numbers (e.g., 72–720 ciliates) were observed to have emerged. The total mean number of ciliates that were observed to have emerged from each mussel during the 24-day experiment was significantly higher at 21°C than at 14°C (Welch's *t* test: $t = 6.35$, $P < 0.001$) and was, respectively, 207.0 and 29.4 ciliates/mussel (Tables 1 and 2). The higher number of emerged ciliates in the 21°C dishes was almost certainly related to the significantly higher infection intensity that had developed in mussels at this warmer temperature. Mean infection intensity in the 14°C mussels at the end of the 24-day experiment was 48.5 ciliates/mussel (Table 1) and was not significantly different (*t* test: $t = 0.06$, $P = 0.95$) from the infection intensity at the beginning of the experiment, i.e., 47.3 ciliates/mussel. In contrast, mean infection intensity in mussels held at 21°C increased to 176.8 ciliates/mussel by the end of the experiment (Table 2) and differed significantly from the initial infection

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intensity (Welch's t test: $t = 2.32$, $P = 0.026$) and the infection intensity in mussels held at 14°C (t test: $t = 2.43$, $P = 0.019$). In contrast to the 14°C data, emergence rates at 21°C were not higher toward the end of the experiment. At the termination of the 21°C experiment on day 24, a mean of 19.3 emerged ciliates were observed (Table 2). Because dissections revealed that these 24 mussels had a mean infection intensity of 176.8 ciliates/mussel (Table 2), this indicated that ca. 10% (19.3/196.1) of all the ciliates in the 24 dishes were outside their host on day 24.

Survival of C. acuminatus *outside* D. polymorpha

In experiment 1, *C. acuminatus* exhibited mortality during first 24 h, but 20% were still alive after 96 h at 21°C and after 144 h at 14°C (Fig. 1). During experiment 2, there was a shorter survival period, and all ciliates died by 21 h at 21°C and by 90 h at 14°C (Fig. 2). In experiment 3 ciliates began to die during first 6 h at both temperatures (Fig. 3), and as in previous experiments, ciliates tended to perish faster at higher temperature.

DISCUSSION

Emergence of C. acuminatus *from* D. polymorpha

This is the first study to quantify the periodic emergence of a *Conchophthirus* sp. from its bivalve host. Emergence rates of *C. acuminatus* over the entire 24-day experiment appeared to be correlated with infection intensity. Higher infection intensities led to a higher emergence of ciliates possibly because of higher ciliate reproduction at 21°C compared with 14°C. We hypothesize that these results can explain the seasonal change in zebra mussels infection intensity with *C. acuminatus* that we have observed in the field, i.e., higher intensity in summer and lower in winter (Karatayev et al. 2000b).

The data at both 14°C and 21°C suggested that *C. acuminatus* emergence from an individual zebra mussel does not occur at a constant periodic rate, but is rather an irregular pattern marked occasionally with sudden fluctuations. When data on ciliate emergence was pooled for the entire test group of 24 mussels at either temperature, however, the day-to-day fluctuations in emergence rates were considerably reduced. This suggested that in nature the total number of *C. acuminatus* emerging from a single mussel might fluctuate markedly from day to day, but at the same time the
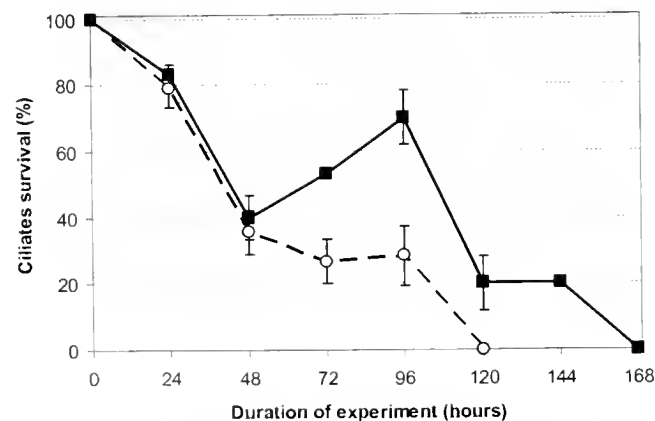
Figure 2. Experiment 2. Survival of *C. acuminatus* outside the host zebra mussel at 14 (±1)°C (solid line, filled squares) and at 21 (±1)°C (dashed line, open circles).

total number emerging from the entire zebra mussel populations would vary far less. Pooling data from all 24 dishes at each temperature provided rough estimates (e.g., 3%, 10%, 17%) of the total *C. acuminatus* outside their hosts, suggesting that a consid-

Figure 3. Experiment 3. Survival of *C. acuminatus* outside the host zebra mussel in three consecutive tests (A, B, and C) at 14 (±1)°C (solid line, filled squares) and at 23 (±1)°C (dashed line, open circles).
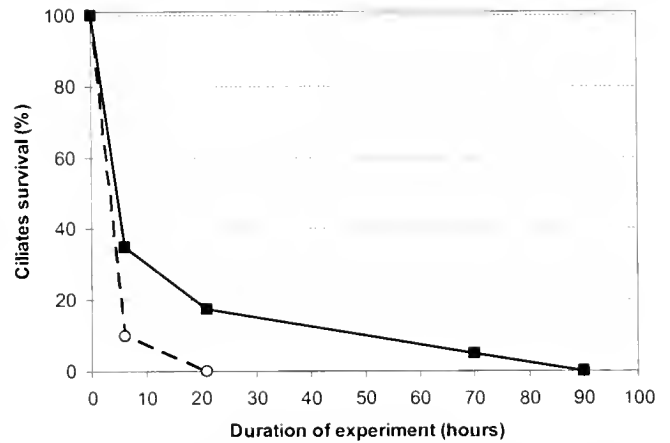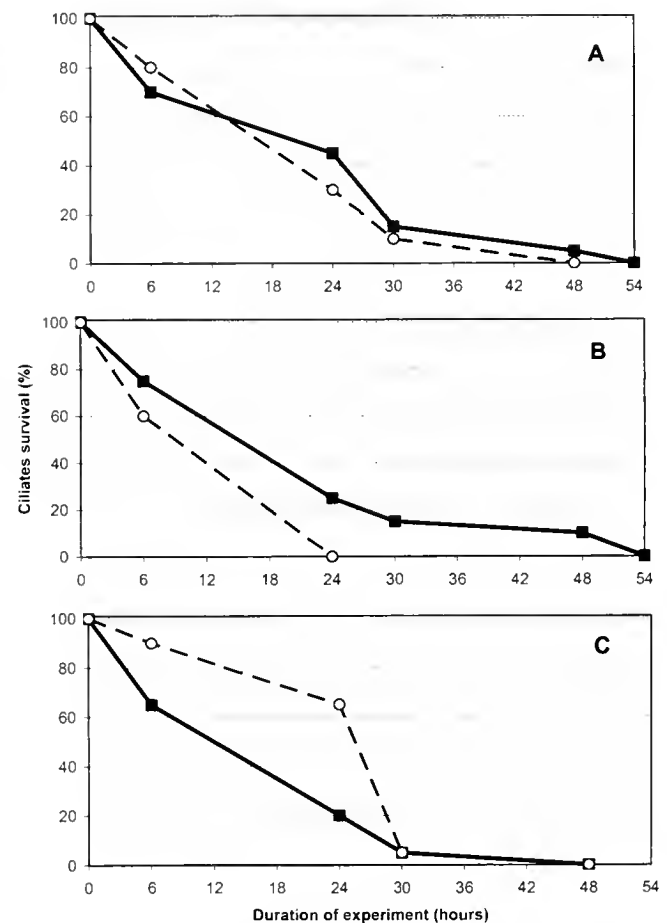
Figure 1. Experiment 1. Mean (±SE) survival of *C. acuminatus* outside its host zebra mussel at 14 (±1)°C (solid line, filled squares) and at 21 (±1)°C (dashed line, open circles).

erable portion of the *C. acuminatus* population might be in open water in search of new hosts. This, in addition to the commensal nature of this symbiont, is likely a key factor explaining why prevalence of this ciliate is typically near 100% in almost all European zebra mussel populations (Molloy et al. 1997, Karatayev et al. 2000a).

Fenchel (1965) observed that *Conchophthirus* spp. in nondreissenid bivalves quickly emerged in large numbers from their damaged or dying hosts. Burlakova et al. (1998) confirmed this same pattern in laboratory trials in which they recorded rapid and massive emergence of *C. acuminatus* from dying zebra mussels. Our present experiment supplements these latter studies by providing information on emergence patterns from live zebra mussels.

Because prevalence of *C. acuminatus* in zebra mussel hosts is frequently 100%, it was surprising, therefore, to observe in our experiment that some infected mussels (i.e., ciliates emerged from them during the experiment) were completely uninfected by the end of the experiment (Table 1, mussels 12 and 15; Table 2, mussels 1, 2, and 16). This suggests that *C. acuminatus* infection can be temporary. In nature, however, mussels are likely infected periodically by *C. acuminatus* from other infected zebra mussels, whereas in our experiment mussels were individually isolated, with transinfection prevented.

Hopefully this experiment has provided some insight into the frequency to which *C. acuminatus* emerge from their host zebra mussels. Future trials, however, may want to expand on its design as follows:

1. *More frequent observations.* We likely underestimated the numbers of ciliates that emerged. Since *C. acuminatus* is a relatively small organism (L × W ≅ 100 × 50 μm), it was extremely difficult to see dead/decomposing individuals using the stereomicroscope. Thus, our counts were almost exclusively based on observation of live ciliates exhibiting movement (i.e., swimming, cilia beating, etc.), and ciliates that emerged and died between the 2- to 3-day sampling periods were likely overlooked. A higher frequency of observations, possibly every 3 h, would be required to address this problem.

2. *Dishes with more than one mussel.* Host density may affect ciliate emergence rates, and this was not accounted for in our experimental design. The possibility exists *C. acuminatus* may be stimulated to emerge from their hosts when chemical cues indicate the presence of other nearby potential host zebra mussels, particularly uninfected juvenile mussels. Our study measured emergence only from isolated zebra mussels (i.e., one mussel per dish).

3. *Mussel siphoning observations.* Is ciliate emergence active (i.e., do they swim out of the mussel) and/or passive (i.e., ejected from the mussel)? Do ciliates emerge through the mussel's inhalant siphon and/or exhalant siphon? Do some ciliates reenter their hosts, and if so, through which siphon? Direct observation, including video recording, would be helpful to shed light on these questions.

Survival of C. acuminatus *Outside the Host*

Our experiments suggest that *C. acuminatus* have a short survival period outside their host. Although we observed a maximum survival period of 144 h (6 days), most ciliates died within 48 h. These results are similar to those of Beers (1959), who studied the survival of *Conchophthirus mytili* DeMorgan (syn. *Peniculistoma mytili* (DeMorgan)) inhabiting the mantle cavity of marine bivalve *Mytilus edulis* Linneaus. He found that a period of 84 h in open water was fatal for the ciliate at 14°C but ciliates died faster at 22°C (48 h) and 30°C (10 h). Fenchel (1965) found that 50% of *Peniculistoma mytili* survived outside their bivalve host for 100 h, *Ancistrum mytili* (Quennerstedt) for about 100 h, and *Ancistrocoma myae* (Kofoid and Busch), *Ancistrum caudatum* Fenchel, and *Thigmophrya saxicavae* Fenchel for ca. 50–100 h. Kidder (1934) studied *Conchophthirus* spp. from nondreissenid bivalves and found them to live not longer than 24 h.

Just because a ciliate is alive does not mean it is capable of reproduction—an essential requirement for establishing a population in a new host. Thus, future trials examining *C. acuminatus* survival should investigate the relationship between duration of time outside a host and the ability of such surviving ciliates to successfully reproduce following entry into a new zebra mussel host.

We set up our survival experiments with ciliates obtained by dissection of zebra mussels. Future trials may want to measure the survival of ciliates that have emerged naturally from their hosts. It is possible that this latter group ciliates may contain greater food reserves and thus may have great longevity in open water.

How does *C. acuminatus* maintain its infection in expanding zebra mussel populations? *Dreissena* spp. often spread to other waterbodies by the downstream dispersal of their planktonic larvae, sometimes being carried hundreds of kilometers from their origin (Stoeckel et al. 1997). These planktonic larvae lack a mantle cavity and are too small to contain *C. acuminatus*. Yet *C. acuminatus* is virtually ubiquitous in all freshwater European zebra mussel populations. Since zebra mussel larvae can stay suspended in downstream currents for more than a week (Hillbricht-Ilkowska & Stanczykowska 1969, Skalskaya 1976), it would appear from our experimental data that *C. acuminatus* would not be able to survive for as long a duration as the zebra mussel larvae that are at the leading edge of the dispersing population. Over time, however, *C. acuminatus* would likely establish itself throughout the entire expanded population by smaller incremental steps of dispersion.

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A NOVEL METHOD FOR LOCATING TAGGED INFAUNAL BIVALVES: SUBMERSIBLE PULSE TECHNOLOGY METAL DETECTORS

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ABSTRACT Hardclams, *Mercenaria mercenaria* (Linne, 1758), tagged with brass washers attached to the outer shell surface and replanted into their natural habitat, were located remotely through the use of a commercially available, fully submersible, pulse technology metal detector. The ability to remotely locate tagged, replanted clams can increase the speed and efficiency of field operations associated with studies of clam population dynamics. Also, this methodology can reduce localized disturbances to the habitat that routinely accompany extensive hand probing to relocate experimental clams in traditional tag and recapture based studies.

KEY WORDS: *Mercenaria*, tagging, infaunal, recapture, metal detector

INTRODUCTION

Studies on the population dynamics of infaunal bivalves routinely involve the mark and recapture of measured, marked individuals. Maximization of recovery rates of experimental clams returned to their natural habitats is essential to support robust analyses of various parameters related to population biology. Various studies have used fenced enclosures to retain experimental organisms within predescribed plots and minimize losses from the population under study. However, enclosures can alter various biotic and abiotic parameters of the microhabitat under study, induce localized erosion, call unwanted attention to the experimental plot from passers-by, and damage fragile habitats.

In cases where clam motility is considered to be minimal, enclosures can be deemed unnecessary and simple marking of the boundaries of the study plot followed by intensive hand probing of the substratum may be sufficient to recapture a statistically significant sample of marked individuals. However, complete reconnaissance of the study plot by hand probing can result in significant damage to the substratum and associated infaunal and epifaunal animals, and rooted or attached plants and algae. Also, even minimal lateral translocations of clams, by passive or active means, across the boundary of the study site can lead to loss of these organisms from the study population because of the impracticalities of hand probing extensive areas beyond the experimental plot limits. While some loss is expected through predation, erosive exhumation associated with stochastic high-energy events, etc., these losses could be significant in terms of previously unrecognized micro- to meta-displacements that have dispersal consequences when viewed cumulatively over time scales ranging from months to years (Prezant et al. 1990, Prezant et al. 1994).

To facilitate our ongoing studies of the population dynamics of *Mercenaria mercenaria* at St. Catherine's Island, Georgia, a typical barrier island ecosystem located near the apex of the Georgia Bight, a novel technique for locating marked, replanted individuals was developed. This new method for remote location of replanted infaunal clams has several distinct advantages to future studies of population dynamics of various clam species including maximizing recovery rates of tagged individuals from both within and outside of the study plot as well as limiting habitat disturbances during recovery operations.

MATERIALS AND METHODS

Inexpensive, common brass washers were obtained in several sizes ranging from approximately 10 to 30 mm in diameter from local building supply centers. Approximately 125 clams of varying sizes ranging from about 25 to 110 mm total length were hand collected from a variety of habitats on St. Catherine's Island, Georgia. The posterior portion of one or both valves was cleaned using a synthetic, abrasive scouring pad and fine sandpaper as necessary to prepare a clean surface for bonding of the washer. A variety of adhesives and glues were tried including two part epoxies and exterior grade construction adhesives.

Clams were held in *in vivo* positions (posterior end uppermost) by placing them into a shallow tray filled with beach sand. Adhesive was applied to the previously prepared and dried shell surface with care to not glue the two valves together. The washer was pressed into the adhesive, which was then allowed to dry or cure as per manufacturer's instructions. Some clams received two washers, one on each valve (see Fig. 1). In some cases, masking tape was used to hold the washer in place until the adhesive set up and the washer was secured firmly in place.

Clams were replanted in a variety of habitats including barrier beaches (quartz sand) and ebb-dominated point bars (richly organic detritus) and placed in *in vivo* orientation and substratum depths. A hand-held Fisher (Fisher Research Laboratory, Los Banos, CA) "Impulse" (fully marine submersible, pulse technology) metal detector was used to locate the tagged clams from 2 to 5 days after replanting. The metal detector was used according to the manufacturer's instructions with the search coil moved parallel to and just above the surface of the substratum in overlapping sweeping arcs.

RESULTS AND DISCUSSION

In a series of trials involving 15–25 brass washer-tagged *Mercenaria mercenaria*, nearly 100% of all clams were located using the submersible, pulse technology detector. Individual washers placed by hand could be located to depths of up to 20–25 cm, depending on substratum type and prevailing local conditions. In actual use as markers on clams, there was no discernible difference in the ability to locate clams with a single washer versus those with two washers under the conditions encountered in this study. Under
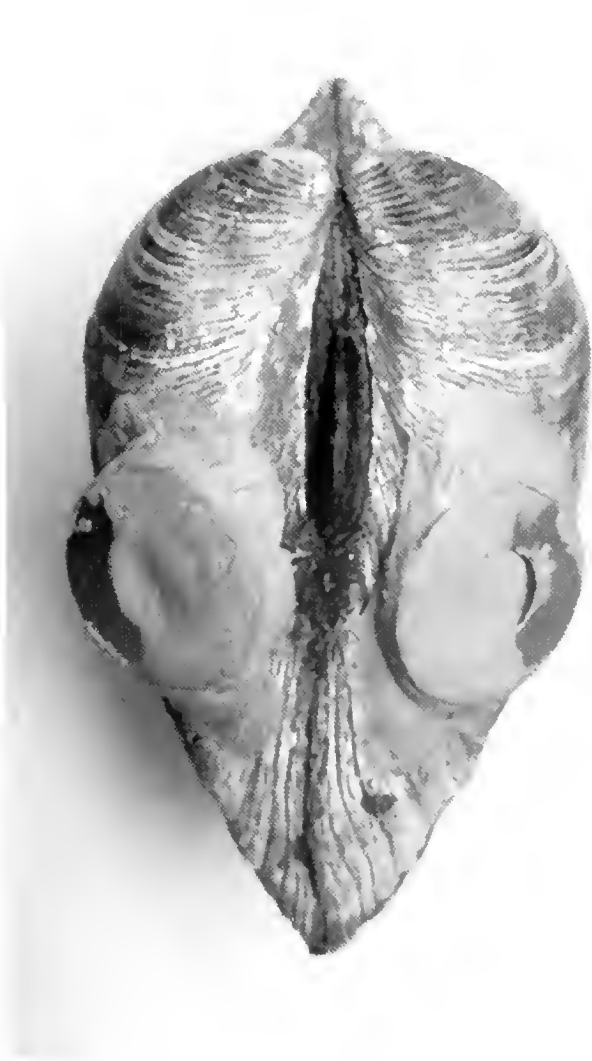
Figure 1. Posterior view of *Mercenaria mercenaria* with two brass washers (diameter = 22 mm) attached as targets for pulse technology metal detection.

other conditions (e.g., deeper burial) two washers should provide a greater potential target. The ability to ground balance the detector to eliminate the background signal from the naturally highly mineralized mud substrata, found commonly in the tidal tributary systems of St. Catherine's Island, was essential to discriminate the brass targets.

Because of the highly corrosive nature of marine sediments, many anthropogenic targets, especially those composed of ferrous compounds (nails, fishhooks, etc.) are rapidly oxidized and eliminated from the habitat. These trash items, if present, could result in false positive returns as they do in more traditional metal detecting scenarios. Pre-screening of clam relocation study sites for such unwanted targets with the detector could allow for study site optimization.

To maximize the discrimination capabilities of the detector, the brass washers were bonded to the exterior of the shell in an orientation that would cause them to be parallel to the surface of the substratum when the clam was in a normal living position. In doing so, the brass washer targets create low yet conspicuous surface irregularities on the shell. However, it has been our experience following direct observation of thousands of hardclams that con-

siderable epibiont growth, particularly oysters and barnacles, is known to occur on clams from native populations around the study sites at St. Catherine's Island, particularly those recovered from tidal creeks within well established salt marshes. Upon careful examination, even clams with heavy epibiont loads appear to be healthy and have growth rates similar to non-epibiont carrying clams (Walker & Tenore 1984, Walker 1985, Walker 1987). Therefore, it is highly unlikely that the presence of the brass washer has any direct deleterious effect on the health and viability of the tagged clam.

Detachment of targets from the valve surface occurred in a small percentage of the clams (<10%) resulting in retrieval of only the brass washer. Target loss can be minimized by careful surface preparation and adhesive choice to ensure solid bonding. While the two part (resin and hardener) products had excellent bonding characteristics, their use is more time consuming due to the need to mix small quantities at a time. The exterior grade construction adhesives, available in large tubes and extruded with the use of a standard caulking gun, were cheaper, easier to use, more time efficient, and had nearly the same efficacy as more expensive epoxy products. As shell surface characteristics can vary from habitat to habitat even within the same species, experimentation trials with different adhesives are recommended before large-scale deployment of tagged clams (Walker & Tenore 1984).

With the excellent sensitivity and discrimination capabilities of the submersible, impulse technology metal detector, tagged clams could be precisely located within a lateral distance of 5–20 cm depending on the size of the target (brass washer) and the depth of the target. Therefore, disturbance to the habitat by hand probing is minimized substantially as compared with hand probing of the entire study plot.

Detection of metal targets depends on a variety of factors, including target size and depth of burial. Burrowing depth of *Mercenaria mercenaria* is known to be positively correlated with clam size (Walker 1985, Walker 1987). Also, smaller clams are known to exhibit increased vertical motility (Walker 1985, Walker 1987) within the substratum. As such, small brass washers placed on small clams should have similar chance of detection as large washers placed on large clams. In theory, the impact of the washers on the clam, if any, would then be similar across the various size classes.

The use of pulse technology, submersible metal detectors for the location of tagged bivalves represents a simple and extremely cost effective methodology potentially applicable to a wide range of freshwater and marine clam species. Continuing improvements in metal detection technology should increase the practicality and efficacy of the use of metal detectors for studies of infaunal organisms. For example, metal detectors with target recognition capabilities could provide expanded opportunities to remotely recognize tagged clam cohorts by size without the need to recover individual clams.

While all detector use in our trials was performed at low tide with the substrata either fully exposed or covered by less than 15 cm of seawater, the fully submersible operation of this detector would allow for it to be used over the side of a small boat, preferably one with a fiberglass hull, or handheld by a person using snorkel or scuba gear.

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EFFECTS OF STARCH TYPE, MACROALGAL MEAL SOURCE, AND β-CAROTENE ON GONAD YIELD AND QUALITY OF THE GREEN SEA URCHIN, *STRONGYLOCENTROTUS DROEBACHIENSIS* (MÜLLER), FED PREPARED DIETS

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ABSTRACT Adult green sea urchins (*Strongylocentrotus droebachiensis*) were collected from the wild, placed in land-based tanks, and fed one of 12 prepared feeds or a control diet of kelp (*Laminaria longicruris* or *L. digitata*) for a period of 12 wk (April 8 to July 1, 1999). The prepared diets were formulated to examine three experimental factors: (1) starch type (corn, potato, or tapioca); (2) macroalgal meal source [kelp (*L. longicruris*) or rockweed (*Ascophyllum nodosum*) meal]; and (3) β-carotene concentration (0 or 200 mg kg^{-1} dry weight of feed). The experiment was a $3 \times 2 \times 2$ completely crossed design. A number of gonad attributes were quantified during the 12-wk experiment including percent yield, percent water, color, texture, firmness, and taste. Color was assessed subjectively by eye and objectively with a reflected-light, fiber-optic spectrophotometer to generate CIE L*, a*, and b* values. Results from sea urchins fed prepared feeds were compared and contrasted with those of wild specimens collected from the source population at weeks 0 and 12 of the experiment. After 12 wk, sea urchins fed prepared diets had significantly higher percent gonad yields (range of means: 19.2–24.3%) than sea urchins given kelp (mean ± SE: 14.5 ± 3.9%) or those collected from the wild at the end of the experiment (2.8 ± 0.5%). Percent gonad yield of sea urchins fed prepared feeds increased significantly over time, but was not significantly affected by starch type or macroalgal meal source. By the end of the experiment, feeds containing β-carotene had produced significantly lower percent gonad yield than feeds without the pigment. Increase in gonad yield was not a result of the addition of water because percent gonad water decreased significantly over time in sea urchins fed prepared diets. Gonad color of sea urchins fed prepared diets or kelp was, generally, pale yellow/orange to yellow-brown/orange-brown at the end of the experiment and did not differ significantly among any of the feeding treatments. Gonad color did improve significantly over time as evidenced by color ratings done by eye and spectrophotometric data. Gonad color was not significantly affected by starch type or macroalgal meal source, but was influenced by β-carotene concentration-feeds with pigment generally giving better gonad color than feeds without it. At the end of the experiment, sea urchins fed prepared diets had gonads that were typically smooth to very smooth with distinct gonad segment halves (texture rating), firm (firmness rating), and ranging from satisfactory to good in flavor (taste rating). Gonad texture and firmness of sea urchins fed prepared diets was as good or better than kelp-fed sea urchins or wild controls. Gonad taste, however, was significantly better in kelp-fed or wild individuals than in sea urchins given the prepared feeds. Gonad texture, firmness, and taste were generally uninfluenced by starch type, macroalgal meal source, β-carotene concentration, or time. Results indicate that gonad enhancement diets for sea urchins should incorporate pigment for optimum coloration, but that the type of starch or macroalgal meal used (at least of those tested) may be less critical for optimizing gonad quantity or quality.

KEY WORDS: β-carotene, gonad, macroalgal meal, prepared feed, roe quality, sea urchin, starch, *Strongylocentrotus droebachiensis*

INTRODUCTION

Sea urchins are harvested worldwide with the majority destined for the Japanese fresh fish markets. Japan is the world's largest consumer of sea urchin gonads, termed "roe" or "uni" in the commercial trade, importing approximately 6100 tonnes of sea urchin product worth USD million $251 in 1994 (Sonu 1995). Whereas market demand remains steady, recent years have seen declines in catch statistics in many of the world's major sea urchin fishing countries (Keesing & Hall 1998, Andrew et al. 2001). It is generally acknowledged that the only way that future market demand can be reliably met is through aquaculture production. Aquaculture production could involve spawning adult brood-stock and rearing larvae/juveniles through to market size or using enhancement techniques where commercial-size sea urchins of low gonad yield are captured from the wild, held in captivity, and fed natural or prepared feeds to increase their percent yield. The latter method, while still reliant on natural stock, could help wild-capture fishery sustainability by increasing gonad yield and quality and, hence, decreasing the necessary amount of stock required to generate the same level of income.

Whereas the major species of echinoids of economic interest for potential aquaculture development—*Diadema setosum, Echinus esculentus, Loxechinus albus, Lytechinus variegatus, Paracentrotus lividus, Psammechinus miliaris, Strongylocentrotus droebachiensis, S. franciscanus, S. intermedius, S. nudus, S. purpuratus, Tripneustes gratilla, T. ventricosus* (Lawrence & Bazhin 1998)—are predominantly macroalgal grazers (see review by Lawrence 1975), the use of macrophytes for feeding in large-scale enhancement or grow-out operations will be problematic due to the expense and logistics involved in the collection and storage of the massive quantities of algae required for such an undertaking. In addition, macrophytes can vary in nutritional quality with season and location and may contain a whole suite of fouling organisms. The evolution of a successful sea urchin culture industry will undoubtedly require the development of suitable, low-cost prepared diets that are easily stored and nutritionally reproducible.

A number of scientific studies have shown that enhancement of gonad yield in captive sea urchins can be readily achieved by feeding them prepared diets (Lawrence et al. 1992, Lawrence et al. 1997, de Jong-Westman et al. 1995a, Klinger et al. 1997, McBride et al. 1997, McBride et al. 1999, Barker et al. 1998, Fernandez &

Boudouresque 1998, Fernandez & Boudouresque 2000, Goebel & Barker 1998, Walker & Lesser 1998, Spirlet et al. 2000, Olave et al. 2001, Pearce et al. 2002a, Pearce et al. 2002b, Pearce et al. 2002c, Robinson et al. 2002). Surprisingly few investigations, however, have focused on how prepared diets affect gonad qualities such as color, texture, firmness, or taste (see Motnikar et al. 1997, Goebel & Barker 1998, Watts et al. 1998, McLaughlin & Kelly 2001, Pearce et al. 2002a, Pearce et al. 2002b, Pearce et al. 2002c, Robinson et al. 2002)—factors at least as important as gonad yield in the commercial roe industry.

Diets that have been developed specifically for gonad enhancement generally contain five major components: (1) feed binders (e.g., agar, gelatin, ligno-sulfonate, sodium alginate, starch); (2) protein sources (e.g., macroalgae, corn meal, fish meal, soybean meal, wheat meal); (3) lipid sources (e.g. corn oil, canola oil, fish oil); (4) vitamins and minerals; and (5) pigments (e.g., β-carotene, lutein, zeaxanthin, capsanthin). Whereas corn, potato, and wheat starches have been used individually as binders or fillers in a number of experimental diets (Lawrence et al. 1989, Klinger et al. 1994, de Jong-Westman et al. 1995a, de Jong-Westman et al. 1995b, Motnikar et al. 1997, Robinson & Colborne 1997, Barker et al. 1998, Goebel & Barker 1998, McBride et al. 1998, Pearce et al. 2002a, Pearce et al. 2002b, Pearce et al. 2002c, Robinson et al. 2002), no study has examined the potential effect of various starch types in prepared feeds on gonad quantity or quality in sea urchins. Starches may, indeed, be able to affect gonad quality. Previously, it was shown that prepared diets bound solely with unmodified corn starch produced significantly better-colored gonads than diets made with other binders such as gelatin, guar gum, or sodium alginate (Pearce et al. 2002a). The authors hypothesized that corn starch may have led to greater production of storage glycogen and, thus, a lighter or whiter gonad background and a brighter gonad color (Pearce et al. 2002a).

Several studies utilizing prepared diets for feeding sea urchins have incorporated kelp plants or kelp meal in the diets (Levin & Naidenko 1987, Klinger et al. 1997, Lawrence et al. 1997, McBride et al. 1998, Watts et al. 1998, Hávardsson et al. 1999, Pantazis et al. 2000, McLaughlin & Kelly 2001, Olave et al. 2001). Considerably fewer studies have used rockweed meal, derived from *Ascophyllum nodosum*, in prepared feeds (Pearce et al. 2002a, Pearce et al. 2002b, Pearce et al. 2002c). Of the common species of macroalgae available to *S. droebachiensis* in its natural environment, kelps (such as *Alaria, Laminaria, Macrocystis, Nereocystis*) are usually preferred, with fucoid algae (*Fucus* spp. and *A. nodosum*) generally eliciting intermediate to low preference (Vadas 1977, Larson et al. 1980, Himmelman 1984, Himmelman & Nédélec 1990). Green sea urchin populations in the Bay of Fundy may be an exception to this generalization, however, as they express a preference in feeding trials for *A. nodosum* (Mackay 1976), the most abundant alga present in the ecosystem. No study has tested the efficacy of kelp meal versus rockweed meal in prepared diets on the gonad yield or quality of sea urchins.

The development of suitable color in gonads of sea urchins fed prepared feeds can be problematic if appropriate levels of certain dietary pigment sources are not included in the diet (Barker et al. 1998, Grosjean et al. 1998, Watts et al. 1998, Pearce et al. 2002a, Pearce et al. 2002b). de Jong-Westman et al. (1995a) found that inclusion of β-carotene (60 mg kg^{-1} dry weight of feed) in prepared feeds significantly increased gonad growth in *S. droebachiensis* as opposed to feeds without the pigment, but did not examine the gonad quality of sea urchins given feeds containing

β-carotene. Research by Robinson et al. (2002) with *S. droebachiensis* has shown that β-carotene is an effective pigment source for producing the bright yellow/orange coloration sought after by the commercial roe market and that the most effective concentration is 200–250 mg kg^{-1} dry weight of feed. No studies have examined the effect of pigmentation source or concentration on gonad quality factors, such as texture, firmness, or taste. The objective of this study was to conduct a multi-factor experiment to examine the combined effects of (1) starch type (corn, potato, or tapioca); (2) macroalgal meal source (kelp or rockweed meal); and (3) β-carotene concentration (0 or 200 mg kg^{-1} dry weight of feed) on the gonad yield and quality of adult green sea urchins, *S. droebachiensis*, held in a land-based culture facility.

MATERIALS AND METHODS

Sea Urchin Collection and Maintenance

Adult green sea urchins, *S. droebachiensis*, were collected by SCUBA divers on March 24–27, 1999 off Bancroft Point, Grand Manan Island, Bay of Fundy, Canada (44°43'N, 66°44'W) on a rocky, cobble bottom at a depth of ~10 m (high tide). Mean test diameter and wet weight of a sub-sample of these sea urchins were 61.2 ± 4.7 mm and 100.5 ± 20.4 g, respectively (mean ± SD, n = 30). Sea urchins were placed in plastic tote boxes with ambient seawater and transported to the laboratory within 3 h of collection. They were then put in white plastic tanks (L × W × H: 50 × 50 × 28 cm) that were supplied with flow-through, ambient seawater at a flow rate of ~5 l min^{-1}. These tanks were equipped with double standpipes (ID of outer pipe: 35 mm; ID of inner pipe: 18 mm) and were designed so that seawater entering into the tanks at the top exited at the bottom. Initial stocking density was 100 urchins tank^{-1} or ~168 kg m^{-3} of water volume or ~14 kg m^{-2} of tank surface area. Sea urchins were starved for 12–15 d prior to experimentation to standardize relative hunger levels. Any individuals that died during that period were removed and replaced.

Seawater temperature was automatically recorded in a header tank every 15 min during the experiment by a temperature data logger. The temperature gradually increased during the experimental period (min: 3.6°C, max: 13.6°C, mean ± SD: 7.4 ± 2.0°C, n = 7839). Lighting for the experiment was provided by overhead fluorescent lights (34 W Sylvania "Cool White") set to a constant photoperiod of 15.5 h light and 8.5 h dark (i.e., photoperiod corresponding to early July in the Bay of Fundy).

Diet Preparation

Ingredients used in diets are shown in Table 1. Kelp meal was produced by collecting fronds of *Laminaria longicruris*, drying it (either solar drying or in a convection dryer at ~20°C), and grinding it into small flakes using a hammer mill. Rockweed meal, derived from *Ascophyllum nodosum*, was produced by Tidal Organics Inc. (Lower East Pubnico, Nova Scotia, Canada) and purchased from Shur-Gain, Maple Leaf Foods Inc. (Truro, Nova Scotia, Canada). Dulse powder, *Palmaria palmata*, was produced by and purchased from Roland's Sea Vegetables (Grand Manan, New Brunswick, Canada). Corn starch, corn oil, and molasses were purchased from a local supermarket. Potato starch was produced by World Flower (Germany) and purchased from East Coast Scale Company Ltd. (Dartmouth, Nova Scotia, Canada). Tapioca starch was produced by the National Starch and Chemical Company (Bridgewater, NJ) and purchased from Kennedy Distribution

TABLE 1.

Ingredients used in diets

Ingredient	Dry Weight (%)
Starch (corn, potato, or tapioca)	24.0 or 14.0
Macroalgal meal (kelp[a] or rockweed[b])	22.8
Rovimix β-carotene 0.2%[c]	0.0 or 10.0
Soybean meal	27.9
Dulse powder[d]	10.0
Molasses	5.0
Gelatin (pork)	5.0
Canola oil	2.0
Dicalcium phosphate	1.8
Lecithin	1.0
Ethoxyquin	0.2
Vitamin pre-mix[e]	0.1
Mineral pre-mix[f]	0.1
Vitamin C (Stay C)	0.1
TOTAL	100.0

[a] *Laminaria longicruris*

[b] *Ascophyllum nodosum*

[c] Contains 98% wheat middlings, 1.8% starch-coated matrix of gelatin and carbohydrates, and 0.2% β-carotene. Obtained from Shur-Gain, Maple Leaf Foods Inc.

[d] *Palmaria palmata*

[e] Contains ground wheat, vitamin E, vitamin C (Stay C), inositol, ethoxyquin, vitamin D_3, niacin, calcium pantothenate, vitamin K, soybean oil, vitamin B_{12}, biotin, riboflavin, pyridoxine, thiamine, vitamin A, and folic acid. Levels are proprietary information. Obtained from Shur-Gain, Maple Leaf Foods Inc.

[f] Contains ground wheat, manganese sulphate, iron sulphate, zinc sulphate, soybean oil, calcium iodate, selenium selenate, and copper chloride. Levels are proprietary information. Obtained from Shur-Gain, Maple Leaf Foods Inc.

Inc. (Moncton, New Brunswick, Canada). Pork gelatin, with a bloom factor of 175, was obtained from CSP Foods Inc. (Moncton, New Brunswick, Canada). This was found to be the best of a number of different binders tested in terms of maintaining pellet stability (Pearce et al. 2002a). Rovimix β-carotene 0.2% was produced by Hoffmann-La Roche Ltd. (Cambridge, Ontario, Canada) and purchased from Shur-Gain. This product contained 0.2% β-carotene, 1.8% starch-coated matrix of gelatin and carbohydrates, and 98% wheat middlings. Feeds with β-carotene added had a concomitant reduction in starch level while all other dietary ingredients remained at the same percentage (Table 1). All other ingredients were purchased from Shur-Gain.

A small Hobart mixer/grinder (Hobart Corporation, Troy, OH) was used to mix the dry ingredients with hot freshwater (~100°C) and to extrude a moist pellet (diameter: 5/16" or 7.9 mm). These pellets were then air dried at ~20°C in a forced-air drying oven and later stored at 3–5°C in covered plastic boxes until used in the experiment. Twelve different prepared diets were formulated to examine three experimental factors: (1) starch type (corn, potato, or tapioca); (2) macroalgal meal source [kelp (*Laminaria longicruris*) or rockweed (*Ascophyllum nodosum*) meal]; and (3) β-carotene concentration (0 or 200 mg kg^{-1} dry weight of feed). Each level of one factor was present in combination with each level of the other two factors in a totally crossed experimental design. For brevity and clarity, treatment names have been abbreviated using the starch type ("Corn" = corn starch, "Pot" = potato starch, "Tap" = tapioca starch), macroalgal meal source

("Kelp" = kelp meal, "Rock" = rockweed meal), and β-carotene concentration ("0" = 0 mg kg^{-1}, "200" = 200 mg kg^{-1}). For example, a feed containing corn starch, rockweed meal, and 200 mg kg^{-1} of β-carotene would be abbreviated "Corn Rock 200."

Experimental Protocols

The experiment was conducted for 12 wk (April 8 to July 1, 1999). In addition to the 12 prepared diet treatments (see "Diet Preparation"), there was a control treatment of kelp (fronds of *Laminaria longicruris* and/or *L. digitata*, predominantly the former). Three replicate tanks were established for each of the 13 treatments, each replicate having 100 sea urchins at the beginning of the experiment and being placed in a separate group in a completely randomized block design. Sea urchins were fed twice a week (generally Monday and Friday) at a rate of 3.0% body weight d^{-1} of kelp or 0.5% body weight d^{-1} of prepared feed (n.b., total food amounts took into consideration all days in between feedings including the day of feeding). Further research—examining gonad production of sea urchins fed the Corn Rock 0 diet at ration levels of 0.25, 0.50, and 1.00% body weight d^{-1} —has shown that gonad yield is maximized at 0.50% body weight d^{-1} (Pearce et al. 2002c). Sea urchins were hand fed and attempts made to ensure that all individuals had equal access to feed. Tanks were cleaned before feeding by removing the standpipes, allowing the tanks to drain, and washing the uneaten feed and fecal material out of the tanks with ambient seawater. While feeding rates were not measured directly, generally there was little uneaten food remaining in the tanks at the time of cleaning. Dead individuals were removed from the tanks as soon as they were observed, but not replaced.

A random sample of 30 sea urchins, chosen from extra individuals that were not part of the study, was taken at the beginning of the experiment to assess initial gonad yield, color, texture, and firmness. Yield and color were quantified every second week for the 12-wk duration of the experiment while texture and firmness were assessed on weeks 6 and 12 of the experiment. This was done by randomly sampling 10 sea urchins from each replicate tank. Gonad taste was assessed at weeks 6 and 12 of the experiment by randomly sampling three sea urchins from each replicate tank; each individual being sampled by two independent tasters, both unaware of treatment designation. Feeding rates were adjusted for sampled individuals but not dead ones, since percent mortality during the experiment was low [<10.3% per 12 wk in all treatments (see Results)]. Thirty sea urchins were randomly sampled from the wild source population at the end of the experiment for assessment of yield and quality.

Sampled sea urchins were vigorously shaken to remove excess external water and their test diameter and mass measured using digital calipers and a digital balance, respectively. Urchins were then cracked open, thoroughly drained of internal fluid, and re-weighed. Gonads were scooped out of the tests, rinsed in seawater, and gently shaken using forceps to remove as much water as possible, but not blotted dry. The gonads were then placed in pre-weighed aluminum pans, weighed, assessed for quality, dried to a constant weight in a 70°C oven for a minimum of 48 h, and then re-weighed. Color was assessed using 79 different paint card samples (Home Hardware, Beauti-Tone) that were later converted to a rating of 1–4 (see scale later). Gonad color was always assessed under standardized light conditions [i.e., 50 cm away from a single-point, artificial light source (20 W Sylvania Cool White fluorescent light) with no natural lighting]. At weeks 6 and 12 of

the experiment. gonad color was also subjectively rated without the use of the paint samples using the 10 randomly sampled individuals from each replicate tank. Gonad characteristics were quantified as follows:

Gonad Yield (%) = (wet gonad weight/whole urchin weight) × 100

Gonad Water (%) = [(wet gonad weight − dry gonad weight)/wet gonad weight] × 100

Gonad Color—Subjectively by Eye With or Without Paint Samples (Rating 1–4)

  1 = bright yellow or orange (equivalent to Grade A in commercial roe industry)
  2 = paler yellow or orange, mustard (Grade A or Grade B)
  3 = yellow-brown, orange-brown, red-brown, cream (Grade B or Grade C)
  4 = any other color (e.g., dark brown, grey) (Grade C)

Gonad Texture—Subjectively by Eye (Rating 1–4)

  1 = two distinct gonad segment halves, very smooth
  2 = two distinct gonad segment halves, smooth (distinction and smoothness <1)
  3 = distinction of gonad segment halves possible but <2, rough/granular
  4 = distinction of gonad segment halves not possible, rough/granular

Gonad Firmness—Subjectively by Eye (Rating 1–4)

  1 = very firm
  2 = firm
  3 = soft
  4 = very soft

Gonad Taste—Subjectively by Two Independent Tasters (Rating 1–6)

  1 = excellent (very sweet)
  2 = very good (very sweet, but <1)
  3 = good (sweet)
  4 = satisfactory (bland: not sweet, not bitter)
  5 = poor (bitter)
  6 = very poor (very bitter)

Gonad color was also objectively quantified at weeks 0, 6, and 12 using a reflected-light, fiber-optic spectrophotometer taking three replicate measurements of L^* (intensity or lightness), a^* (hue or redness), and b^* (chroma or yellowness) from each of 30 sea urchins at week 0 and three replicate measurements from each of five randomly sampled sea urchins from each replicate tank in weeks 6 and 12. For a full description of the spectrophotometric system see Robinson et al. (2002).

Statistical Analyses

For percent gonad yield, percent gonad water, gonad color done by eye, gonad texture, and gonad firmness of experimental animals, a mean was calculated using the 10 sea urchins sampled from each replicate tank (for the wild urchins sampled at weeks 0 and 12, thirty individuals were randomly placed in three groups of ten to obtain three mean values). This mean "tank" value was then used in subsequent statistical analyses ($n = 3$). For gonad taste, a mean value was calculated for each sea urchin using the two independent observations and this value was then used in the calculation of mean tank values. These tank values were then used in

statistical analyses ($n = 3$). For gonad color assessed with the spectrophotometer, mean values of L^*, a^*, and b^* were calculated for each sea urchin using the three measurements taken. These individual means were then used to calculate a tank mean from the five sampled sea urchins taken from each tank. Tank means were then used in subsequent statistical analyses ($n = 3$).

For the various gonad characteristics, two-way ANOVAs (completely randomized block design with tank as the blocking factor) were used to determine the significance of the differences among all treatments at the end of the experiment, including the kelp-fed sea urchins and wild samples taken at the beginning and end of the experiment. To examine more closely the combined effects of starch type, macroalgal meal source, and β-carotene concentration, four-way ANOVAs (starch type, macroalgal meal source, β-carotene concentration, and tank as a blocking factor) were conducted on the various gonad characteristics at the end of the experiment. To assess the effects of starch type, macroalgal meal source, and β-carotene concentration on percent gonad yield, percent gonad water, and gonad color over time, four-way repeated measures ANOVAs were used. In these analyses the blocking factor was left out so as not to produce a cumbersome five-way ANOVA. In multi-way ANOVAs with significant interaction terms, the effect of one main factor was examined within each level of the other main factors with two-way ANOVAs (treatment and block). Where significant P-values were generated in ANOVAs, Fisher's LSD post-hoc comparison tests were used to evaluate differences among pair-wise means ($P < 0.05$). Probability plots were used to confirm that data were normally distributed and Cochran's tests ($P < 0.01$) used to verify that variances were homogeneous.

RESULTS

Mortality

At the end of the 12-wk experiment, cumulative percent mortality in the various feeding treatments ranged from a low of 1.7 ± 0.7% (mean ± SE) for Corn Kelp 200 and Tap Kelp 200 to a high of 10.3 ± 5.9% for Pot Rock 0 with most treatments having a mean cumulative percent mortality under 5% (Fig. 1A). There were no significant differences in cumulative percent mortality among any of the treatments including the kelp control (2.3 ± 0.7%) (Table 2).

Gonad Yield

Mean percent gonad yield increased from 15.1% at the beginning of the experiment to at least 19.2% in all prepared diet treatments (Fig. 1B) with percent gonad yield increases per week ranging from a low of 0.3% for Tap Kelp 200 to a high of 0.8% for Tap Kelp 0. Mean percent gonad yields for the feeding treatments at the end of the 12-wk experimental period and the wild samples collected at the beginning and end of the experiment differed significantly (Table 2). All prepared feed treatments had significantly higher percent gonad yields (range: 19.2–24.3%) than the kelp control (14.5 ± 3.9%) or wild sample collected at the end of the experiment (2.8 ± 0.5%) (Fig. 1B). All prepared feed treatments except Tap Kelp 200 (19.2 ± 1.1%) and Tap Rock 200 (19.6 ± 2.1%) had significantly higher percent gonad yields than the wild sample taken at the beginning of the experiment (15.1 ± 1.0%) (Fig. 1B). There was only one significant pair-wise comparison among the prepared feeds at the end of the experiment: Tap Kelp 0 (24.3 ± 1.3%) had a higher percent gonad yield than Tap Kelp
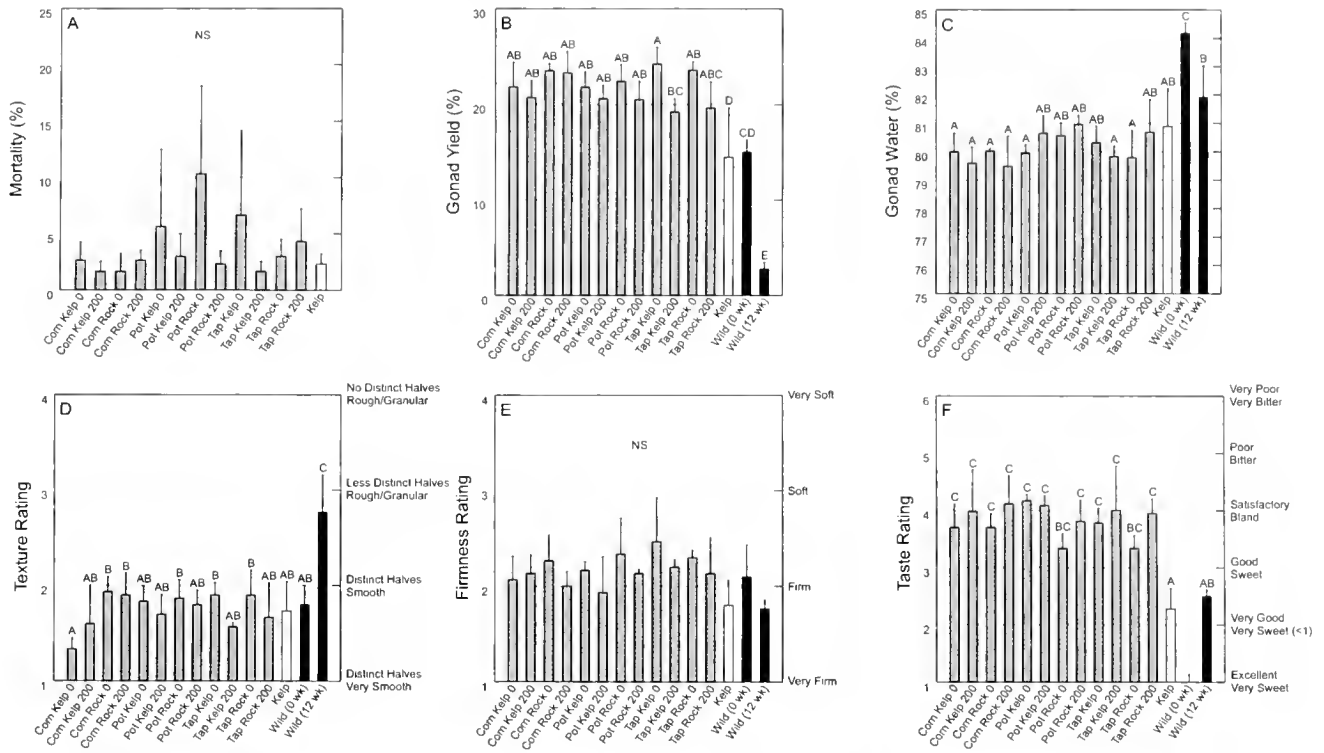
Figure 1. Mean cumulative percent mortality (A), mean percent gonad yield (B), mean percent gonad water (C), mean gonad texture rating (D), mean gonad firmness rating (E), and mean gonad taste rating (F) for all experimental treatments and wild controls at the end of the experiment. See text for full explanation of gonad quality ratings. Error bars are SE and $n = 3$. Letters above bars indicate the results of Fisher's LSD multiple comparison post-hoc tests showing significant pair-wise differences among experimental treatments and wild controls within each graph. "NS" denotes no significant differences among treatment means.

200 (Fig. 1B). The kelp control and wild sample collected at the beginning of the experiment had significantly greater percent gonad yield than the wild sample taken at the end of the experiment (Fig. 1B).

A 4-way ANOVA examining the effects of starch type, mac-roalgal meal source, β-carotene concentration, and block on percent gonad yield at the end of the experiment revealed a significant effect of β-carotene concentration, but no other significant main or interaction effects (Table 3). Feeds with 200 mg kg^{-1} of β-carotene produced significantly lower percent gonad yield than those with-

TABLE 2.

Results of separate 2-way ANOVAs on cumulative percent mortality, percent gonad yield, percent gonad water, gonad color rating, L*, a*, b*, gonad texture rating, gonad firmness rating, and gonad taste rating at the end of the 12-wk experiment. Sources of variation are treatment, block, and error.

Source	SS	DF	F-Ratio	P-Value	SS	DF	F-Ratio	P-Value	SS	DF	F-Ratio	P-Value
		Mortality				Yield (%)				Water (%)		
Treatment	228.97	12	0.94	>0.5	1253.83	14	11.37	<0.001	58.14	14	4.04	<0.001
Block	161.08	2	3.98	<0.05	15.93	2	1.01	>0.1	0.56	2	0.27	>0.5
Error	486.26	24			220.65	28			28.81	28		
	Color Rating (with paint samples)				Color Rating (without paint samples)				L* (brightness)			
Treatment	1.58	14	0.77	>0.5	1.13	14	1.35	>0.1	419.60	14	1.54	>0.1
Block	0.41	2	1.40	>0.1	0.29	2	2.42	>0.1	8.46	2	0.22	>0.5
Error	4.10	28			1.68	28			543.46	28		
	a* (redness)				b* (yellowness)				Texture Rating			
Treatment	85.56	14	1.63	>0.1	122.22	14	3.71	<0.005	3.98	14	2.89	<0.01
Block	3.72	2	0.50	>0.5	5.36	2	1.14	>0.1	0.30	2	1.51	>0.1
Error	104.74	28			65.89	28			2.76	28		
	Firmness Rating				Taste Rating							
Treatment	1.53	14	0.89	>0.5	13.26	13	3.65	<0.005				
Block	0.14	2	0.56	>0.5	0.19	2	0.33	>0.5				
Error	3.43	28			7.27	26						

TABLE 3.

Results of separate 4-way ANOVAs on percent gonad yield, percent gonad water, gonad color rating, L*, a*, b*, gonad texture rating, gonad firmness rating, and gonad taste rating at the end of the 12-wk experiment. Sources of variation are starch type (S), macroalgal meal source (M), β-carotene concentration (B), block, and error.

Source	SS	DF	F-Ratio	P-Value	SS	DF	F-Ratio	P-Value	SS	DF	F-Ratio	P-Value
		Yield (%)				Water (%)				Color Rating (with paint samples)		
S	6.17	2	0.52	>0.5	3.40	2	2.13	>0.1	0.02	2	0.10	>0.5
M	5.18	1	0.88	>0.1	0.34	1	0.42	>0.5	0.32	1	3.77	>0.05
B	44.98	1	7.64	<0.05	0.07	1	0.09	>0.5	0.19	1	2.20	>0.1
S × M	8.84	2	0.75	>0.1	0.38	2	0.24	>0.5	0.01	2	0.01	>0.5
S × B	24.18	2	2.05	>0.1	1.60	2	1.00	>0.1	0.12	2	0.71	>0.5
M × B	0.41	1	0.07	>0.5	0.22	1	0.28	>0.5	0.01	1	0.01	>0.5
S × M × B	1.44	2	0.12	>0.5	1.32	2	0.83	>0.1	0.05	2	0.30	>0.5
Block	7.72	2	0.66	>0.5	1.05	2	0.66	>0.5	0.61	2	3.58	<0.05
Error	129.53	22			17.53	22			1.88	22		
		Color Rating (without paint samples)				L* (brightness)				a* (redness)		
S	0.08	2	0.81	>0.1	8.41	2	0.23	>0.5	1.87	2	0.26	>0.5
M	0.20	1	4.08	>0.05	51.39	1	2.86	>0.1	1.22	1	0.33	>0.5
B	0.23	1	4.70	<0.05	45.70	1	2.54	>0.1	17.78	1	4.87	<0.05
S × M	0.01	2	0.07	>0.5	100.08	2	2.78	>0.05	12.86	2	1.76	>0.1
S × B	0.02	2	0.16	>0.5	27.82	2	0.77	>0.1	6.73	2	0.92	>0.1
M × B	0.03	1	0.68	>0.1	15.87	1	0.88	>0.1	12.91	1	3.53	>0.05
S × M × B	0.01	2	0.02	>0.5	33.70	2	0.94	>0.1	2.43	2	0.33	>0.5
Block	0.18	2	1.81	>0.1	2.34	2	0.07	>0.5	0.78	2	0.11	>0.5
Error	1.09	22			396.04	22			80.41	22		
		b* (yellowness)				Texture Rating				Firmness Rating		
S	13.58	2	3.20	>0.05	0.07	2	0.39	>0.5	0.17	2	0.68	>0.5
M	1.83	1	0.86	>0.1	0.32	1	3.53	>0.05	0.01	1	0.08	>0.5
B	4.93	1	2.32	>0.1	0.07	1	0.78	>0.1	0.28	1	2.21	>0.1
S × M	40.25	2	9.49	<0.001	0.31	2	1.69	>0.1	0.14	2	0.53	>0.5
S × B	18.14	2	4.28	<0.05	0.24	2	1.32	>0.1	0.03	2	0.11	>0.5
M × B	24.73	1	11.66	<0.005	0.01	1	0.05	>0.5	0.01	1	0.08	>0.5
S × M × B	1.54	2	0.36	>0.5	0.07	2	0.41	>0.5	0.08	2	0.32	>0.5
Block	1.50	2	0.36	>0.5	0.06	2	0.33	>0.5	0.09	2	0.37	>0.5
Error	46.65	22			2.00	22			2.83	22		
		Taste Rating										
S	0.07	2	0.12	>0.5								
M	0.54	1	1.84	>0.1								
B	0.92	1	3.13	>0.05								
S × M	0.59	2	1.00	>0.1								
S × B	0.08	2	0.13	>0.5								
M × B	0.29	1	1.00	>0.1								
S × M × B	0.07	2	0.11	>0.5								
Block	0.50	2	0.85	>0.1								
Error	6.46	22										

out the pigment (Fig. 2A). A 4-way repeated ANOVA examining the effects of time, starch type, macroalgal meal source, and β-carotene concentration on percent gonad yield showed a significant effect of time, but no other significant main or interaction effects (Table 4). Percent gonad yield increased over time with all pair-wise comparisons among weeks being significantly different except for comparisons between weeks 0 and 4, weeks 0 and 6, and weeks 4 and 6 (Fig. 2B).

Gonad Water

Mean percent gonad water decreased during the 12-wk experiment from 84.2 ± 0.3% at the beginning of the experiment to less than 81.0% in all feeding treatments at the end of 12 wk (Fig. 1C). A 2-way ANOVA analyzing the effects of treatment and block on percent gonad water at the end of the experiment showed a sig-

nificant effect of treatment (Table 2). All prepared feed treatments (range: 79.5–81.0%), as well as the kelp control (80.9 ± 1.0%) and wild samples taken at the end of the experiment (81.9 ± 0.8%), had significantly lower percent gonad water than the initial sample (84.2 ± 0.3%) (Fig. 1C). There were no significant pair-wise differences in percent gonad water among any of the prepared feeds or kelp control (Fig. 1C).

A 4-way ANOVA examining the effects of starch type, macroalgal meal source, β-carotene concentration, and block on percent gonad water at the end of the experiment revealed no significant main or interaction effects (Table 3). A 4-way repeated ANOVA examining the effects of time, starch type, macroalgal meal source, and β-carotene concentration on percent gonad water showed significant starch type and time main effects and a significant starch type × β-carotene concentration interaction (Table 4). For feeds with 0 mg kg⁻¹ of β-carotene, those containing potato
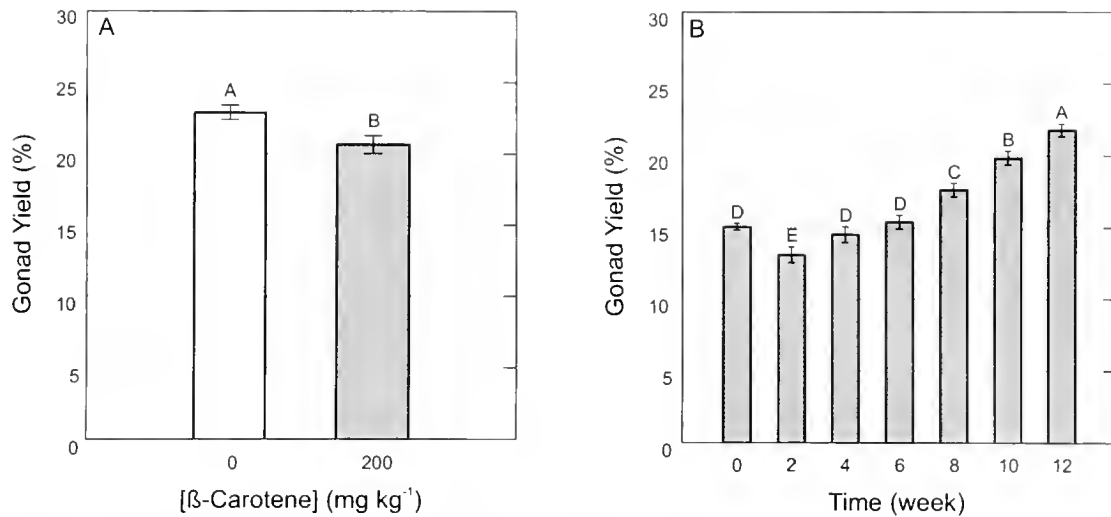
Figure 2. (A) Mean percent gonad yield at week 12 in feeding treatments with and without β-carotene. Error bars are SE and n = 18. Letters above bars indicate the results of an ANOVA showing significant difference between treatment means. (B) Mean percent gonad yield of all feeding treatments at each sampling interval. Error bars are SE and n = 36. Letters above bars indicate the results of a Fisher's LSD multiple comparison post-hoc test showing significant pair-wise differences among weeks.

starch produced gonads with significantly higher percent water than those containing tapioca starch (Fig. 3A). There were no significant differences, however, among starch types for feeds containing 200 mg kg^{-1} of β-carotene (Fig. 3A). There were no significant differences between the two β-carotene concentrations at any of the starch type levels. Percent gonad water decreased over time with all pair-wise comparisons among weeks being significantly different except for comparisons between weeks 0 and 2, weeks 0 and 4, and weeks 2 and 4 (Fig. 3B).

Gonad Color

At the end of the experiment, mean color ratings of sea urchin gonads from prepared diet treatments varied between 2.5 ± 0.3 (Tap Kelp 200) and 3.0 ± 0.1 (Corn Rock 0) for ratings done with paint samples and between 2.3 ± 0.2 (Corn Kelp 200) and 2.8 ± 0.1 (Tap Rock 0) for ratings done without paint samples (Fig. 4A, B). There were no significant differences at the end of the experiment among any of the prepared feeds, kelp control, or wild controls in either color rating data set (Table 2, Fig. 4A, B).

Four-way ANOVAs examining the effects of starch type, macroalgal meal source, β-carotene concentration, and block on gonad color ratings at the end of the experiment revealed a significant effect of β-carotene concentration for ratings done without paint samples, but not for ratings done with paint samples (Table 3). In both data sets, feeds with β-carotene produced better gonad color than those without the pigment (Fig. 5A). There were no other significant main or interaction effects in either color rating data set (Table 3).

A 4-way repeated ANOVA examining the effects of time, starch type, macroalgal meal source, and β-carotene concentration on gonad color ratings done with paint samples showed a significant effect of time, but no other significant main or interaction effects (Table 4). Gonad color improved over time; sea urchins sampled in weeks 6, 8, 10, 12 had significantly better gonad color than those sampled in weeks 0 and 4 while those measured in weeks 10 and 12 had significantly better gonad color than those measured in weeks 0, 2, and 4 (Fig. 5B). Time also significantly

affected gonad color ratings done without paint samples, although the effect of time was dependent on the interaction with macroalgal meal source (Table 4). For sea urchins fed kelp meal diets, gonad color ratings significantly improved at each subsequent sampling date; week 12 was significantly better than week 6, which was significantly better than week 0 (Fig. 5C). For sea urchins fed rockweed meal diets, gonad color ratings in weeks 6 and 12 were significantly improved from the beginning of the experiment, but there was no significant difference between weeks 6 and 12 (Fig. 5C). There was no significant difference in gonad color ratings between kelp and rockweed meal diets at weeks 0 or 6, but feeds containing kelp meal produced significantly better gonad color ratings than feeds containing rockweed meal by week 12 (Fig. 5D).

At the end of the experiment, mean values of L* of sea urchin gonads from prepared diet treatments varied between 45.4 ± 2.3 for Corn Rock 0 and 55.1 ± 0.4 for Corn Kelp 0 (Fig. 4C). There were no significant differences at the end of the experiment among any of the prepared feeds, kelp control (52.0 ± 1.3), or wild controls (0 wk: 49.5 ± 2.8, 12 wk: 44.3 ± 4.0) (Table 2, Fig. 4C). There were no significant main or interaction effects in the 4-way ANOVA conducted on week 12 L* data (Table 3) and only the effect of time was significant in the 4-way repeated ANOVA (Table 4). The mean value of L* was higher in week 12 than in weeks 0 or 6 of the experiment, but only the comparison between weeks 6 and 12 was significantly different (Fig. 6).

Mean values of a* (hue or redness) of sea urchin gonads from prepared diet treatments in week 12 varied between 19.2 ± 0.7 for Corn Kelp 0 and 23.0 ± 1.5 for Corn Kelp 200 (Fig. 4D). There were no significant differences at the end of the experiment among any of the prepared feeds, kelp control (20.3 ± 0.9), or wild controls (0 wk: 18.9 ± 1.3, 12 wk: 23.1 ± 1.4) (Table 2, Fig. 4D). There was a significant effect of β-carotene concentration on a* values at the end of the experiment, but no other significant main or interaction effects (Table 3). Feeds with 200 mg kg^{-1} of β-carotene produced significantly higher a* values than feeds with 0 mg kg^{-1} of pigment (Fig. 7A). Only the effect of time was significant in the 4-way repeated ANOVA (Table 4). Values of a* were

significantly higher in week 12 than in weeks 0 or 6 of the experiment (Fig. 7B).

At the end of the experiment, mean values of b* (chroma or yellowness) of sea urchin gonads from prepared diet treatments varied between 16.2 ± 0.2 for Corn Kelp 0 and 21.5 ± 1.4 for Corn Rock 0 (Fig. 4E). Mean values of b* for the feeding treatments at the end of the 12-wk experimental period and the wild samples collected at the beginning and end of the experiment differed sig-

TABLE 4.

Results of separate 4-way repeated ANOVAs on percent gonad yield, percent gonad water, gonad color rating, L*, a*, b*, gonad texture rating, gonad firmness rating, and gonad taste rating. Sources of variation are starch type (S), macroalgal meal source (M), β-carotene concentration (B), time (T), and error.

Source	SS	DF	F-Ratio	P-Value	SS	DF	F-Ratio	P-Value	SS	DF	F-Ratio	P-Value
	Yield (%)				Water (%)				Color Rating (with paint samples)			
S	55.48	2	0.83	>0.1	8.29	2	3.99	<0.05	0.02	2	0.06	>0.5
M	23.30	1	0.70	>0.1	0.10	1	0.09	>0.5	0.14	1	0.83	>0.1
B	126.68	1	3.80	>0.05	1.72	1	1.66	>0.1	0.35	1	2.16	>0.1
S × M	20.99	2	0.32	>0.5	1.69	2	0.81	>0.1	0.21	2	0.65	>0.5
S × B	40.66	2	0.61	>0.5	8.12	2	3.91	<0.05	0.07	2	0.21	>0.5
M × B	7.78	1	0.23	>0.5	0.09	1	0.09	>0.5	0.06	1	0.35	>0.5
S × M × B	25.93	2	0.39	>0.5	1.24	2	0.60	>0.5	0.03	2	0.10	>0.5
Error	799.39	24			24.95	24			3.91	24		
T	2121.78	6	98.88	<0.001	577.15	6	171.46	<0.001	6.25	6	9.59	<0.001
T × S	55.36	12	1.29	>0.1	10.26	12	1.52	>0.1	0.62	12	0.48	>0.5
T × M	19.80	6	0.92	>0.1	2.99	6	0.89	>0.5	0.88	6	1.35	>0.1
T × B	34.00	6	1.59	>0.1	1.24	6	0.37	>0.5	0.22	6	0.34	>0.5
T × S × M	34.39	12	0.80	>0.5	5.12	12	0.76	>0.5	0.67	12	0.52	>0.5
T × S × B	34.50	12	0.80	>0.5	10.06	12	1.50	>0.1	0.59	12	0.46	>0.5
T × M × B	22.60	6	1.05	>0.1	2.53	6	0.75	>0.5	0.23	6	0.36	>0.5
T × S × M × B	60.27	12	1.40	>0.1	7.80	12	1.16	>0.1	0.98	12	0.75	>0.5
Error	515.00	144			80.79	144			15.62	144		
	Color Rating (without paint samples)				L* (brightness)				a* (redness)			
S	0.10	2	0.59	>0.5	5.11	2	0.22	>0.5	6.43	2	0.88	>0.1
M	0.01	1	0.01	>0.5	0.63	1	0.05	>0.5	0.08	1	0.02	>0.5
B	0.11	1	1.25	>0.1	13.91	1	1.17	>0.1	10.73	1	2.94	>0.05
S × M	0.01	2	0.02	>0.5	9.69	2	0.41	>0.5	4.98	2	0.68	>0.5
S × B	0.03	2	0.18	>0.5	28.36	2	1.19	>0.1	1.12	2	0.15	>0.5
M × B	0.01	1	0.11	>0.5	21.54	1	1.81	>0.1	6.23	1	1.71	>0.1
S × M × B	0.04	2	0.23	>0.5	7.22	2	0.30	>0.5	2.05	2	0.28	>0.5
Error	2.06	24			285.29	24			87.53	24		
T	1.81	2	19.07	<0.001	145.14	2	3.70	<0.05	129.05	2	13.80	<0.001
T × S	0.09	4	0.47	>0.5	24.40	4	0.31	>0.5	24.17	4	1.29	>0.1
T × M	0.44	2	4.58	<0.05	84.40	2	2.15	>0.1	3.68	2	0.39	>0.5
T × B	0.13	2	1.40	>0.1	31.88	2	0.81	>0.1	9.18	2	0.98	>0.1
T × S × M	0.01	4	0.02	>0.5	114.50	4	1.46	>0.1	8.27	4	0.44	>0.5
T × S × B	0.04	4	0.22	>0.5	32.00	4	0.41	>0.5	11.61	4	0.62	>0.5
T × M × B	0.03	2	0.26	>0.5	10.77	2	0.28	>0.5	7.21	2	0.77	>0.1
T × S × M × B	0.06	4	0.29	>0.5	48.34	4	0.62	>0.5	2.40	4	0.13	>0.5
Error	2.28	48			940.94	48			224.40	48		
	b* (yellowness)				Texture Rating				Firmness Rating			
S	9.42	2	1.43	>0.1	0.18	2	0.96	>0.1	0.12	2	0.51	>0.5
M	3.20	1	0.97	>0.1	0.14	1	1.51	>0.1	0.01	1	0.11	>0.5
B	0.41	1	0.12	>0.5	0.03	1	0.29	>0.5	0.23	1	1.94	>0.1
S × M	19.25	2	2.93	>0.05	0.18	2	0.96	>0.1	0.06	2	0.26	>0.5
S × B	49.47	2	7.53	<0.005	0.13	2	0.67	>0.5	0.03	2	0.14	>0.5
M × B	0.50	1	0.15	>0.5	0.01	1	0.03	>0.5	0.01	1	0.01	>0.5
S × M × B	1.48	2	0.23	>0.5	0.06	2	0.31	>0.5	0.02	2	0.10	>0.5
Error	78.89	24			2.24	24			2.86	24		
T	878.57	2	116.57	<0.001	0.31	2	1.87	>0.1	1.61	2	5.66	<0.01
T × S	53.62	4	3.56	<0.05	0.13	4	0.39	>0.5	0.09	4	0.15	>0.5
T × M	18.44	2	2.45	>0.05	0.19	2	1.12	>0.1	0.01	2	0.02	>0.5
T × B	5.76	2	0.77	>0.1	0.05	2	0.27	>0.5	0.14	2	0.50	>0.5
T × S × M	36.46	4	2.42	>0.05	0.28	4	0.85	>0.1	0.57	4	1.01	>0.1
T × S × B	34.72	4	2.30	>0.05	0.43	4	1.28	>0.1	0.04	4	0.07	>0.5
T × M × B	62.60	2	8.31	<0.001	0.01	2	0.01	>0.5	0.03	2	0.10	>0.5
T × S × M × B	0.81	4	0.05	>0.5	0.30	4	0.90	>0.1	0.07	4	0.12	>0.5
Error	180.88	48			4.00	48			6.82	48		

Source	SS	DF	F-Ratio	P-Value
			Taste Rating	
S	0.05	2	0.04	>0.5
M	1.23	1	1.95	>0.1
B	0.01	1	0.01	>0.5
S × M	0.24	2	0.19	>0.5
S × B	0.16	2	0.12	>0.5
M × B	0.07	1	0.11	>0.5
S × M × B	1.10	2	0.87	>0.1
Error	15.14	24		
T	0.11	1	0.29	>0.5
T × S	0.07	2	0.09	>0.5
T × M	0.01	1	0.01	>0.5
T × B	2.08	1	5.75	<0.05
T × S × M	2.28	2	3.14	>0.05
T × S × B	0.02	2	0.03	>0.5
T × M × B	1.06	1	2.94	>0.1
T × S × M × B	1.69	2	2.34	>0.1
Error	8.70	24		

nificantly (Table 2). Gonads from wild sea urchins generally had lower b* values than those from sea urchins fed prepared feeds or kelp, although only Corn Rock 0, Corn Rock 200 (19.5 ± 0.5), and Tap Kelp 200 (20.9 ± 0.4) had significantly higher b* values than wild samples (Fig. 4E). In the 4-way repeated ANOVA, there was one significant main effect (time) and three significant interactions (starch type × β-carotene concentration, time × starch type, and time × macroalgal meal source × β-carotene concentration) (Table 4). Comparing the two concentrations of β-carotene at each inter-action level revealed three out of 12 (week 0 data not tested) pair-wise comparisons to be significant; feeds with 0 mg kg^{-1} β-carotene had significantly higher b* values than feeds with 200 mg kg^{-1} β-carotene for Corn Kelp and Pot Kelp at week 6 whereas the reverse was true for Tap Kelp at week 12 (Fig. 8). Comparing the three weeks at each interaction level revealed significant dif-

ferences for all treatments except Corn Kelp 0, Pot Kelp 0, Pot Rock 200, Tap Kelp 0, and Tap Rock 200; weeks 0 and 12 gen-erally had significantly higher b* values than week 6 (Fig. 8).

Gonad Texture, Firmness, and Taste

At the end of the experiment, mean gonad texture ratings of sea urchins fed prepared diets varied between 1.3 ± 0.1 for Corn Kelp 0 and 1.9 ± 0.1 for Corn Rock 0, Pot Rock 0, and Tap Kelp 0 (Fig. 1D). Mean texture ratings for the feeding treatments at the end of the 12-wk experimental period and the wild samples collected at the beginning and end of the experiment differed significantly (Table 2). All prepared feeds, kelp control (1.7 ± 0.2), and wild 0-wk control (1.8 ± 0.2) had significantly lower texture ratings than the wild 12-wk control (2.8 ± 0.3) (Fig. 1D). Among the prepared diets, Corn Kelp 0 had significantly better gonad texture than Corn Rock 0, Corn Rock 200 (1.9 ± 0.2), Pot Rock 0, Tap Kelp 0, and Tap Rock 0 (1.9 ± 0.2); there were no other signifi-cant pair-wise differences among prepared feeds (Fig. 1D). There were no significant main or interaction effects on gonad texture in either the 4-way ANOVA (Table 3) or 4-way repeated ANOVA (Table 4).

Mean gonad firmness ratings of sea urchins fed prepared diets varied between 1.9 ± 0.3 for Pot Kelp 200 and 2.5 ± 0.3 for Tap Kelp 0 (Fig. 1E). There were no significant differences among any of the prepared feeds, kelp control (1.8 ± 0.2), wild 0-wk control (2.1 ± 0.3), or wild 12-wk control (1.8 ± 0.1) (Table 2, Fig. 1E). A 4-way ANOVA examining the effects of starch type, macroalgal meal source, β-carotene concentration, and block on gonad firm-ness ratings at the end of the experiment revealed no significant main or interaction effects (Table 3). Time was the only significant effect in the 4-way repeated ANOVA (Table 4). Gonads were significantly firmer at week 6 than at weeks 0 or 12, but there was no significant difference in gonad firmness between weeks 0 and 12 (Fig. 9).

Mean gonad taste ratings of sea urchins fed prepared diets varied between 3.3 ± 0.2 for Pot Rock 0 and Tap Rock 0 and 4.2 ± 0.1 for Pot Kelp 0 (Fig. 1F). A 2-way ANOVA revealed sig-
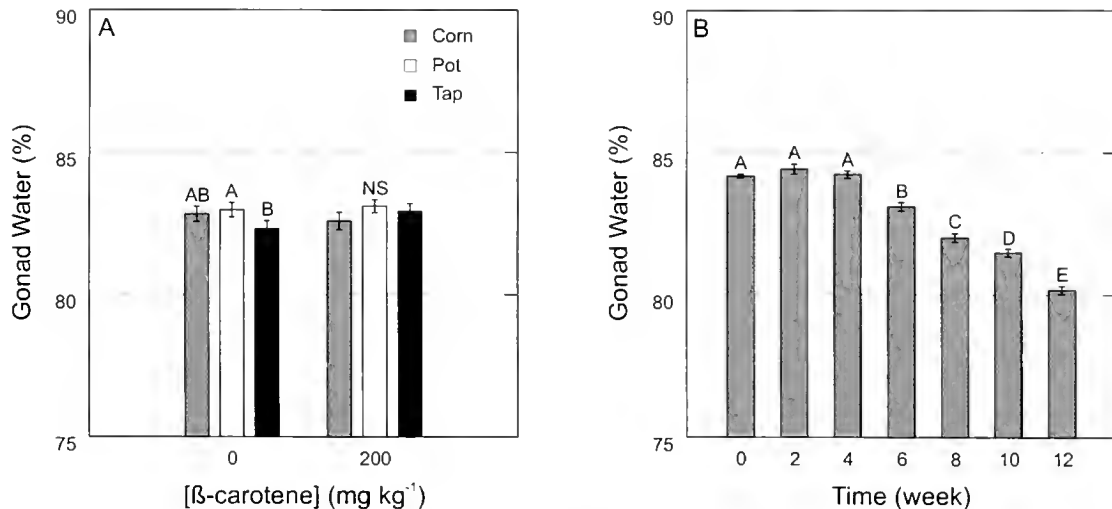

Figure 3. (A) Mean percent gonad water over the 12-wk experiment in feeding treatments with and without β-carotene with the three different starches. Error bars are SE and *n* = 42. Letters above bars indicate the results of Fisher's LSD multiple comparison post-hoc tests showing significant pair-wise differences among starch types at each β-carotene level. "NS" denotes no significant difference among treatment means. (B) Mean percent gonad water of all feeding treatments at each sampling interval. Error bars are SE and *n* = 36. Letters above bars indicate the results of a Fisher's LSD multiple comparison post-hoc test showing significant pair-wise differences among weeks.
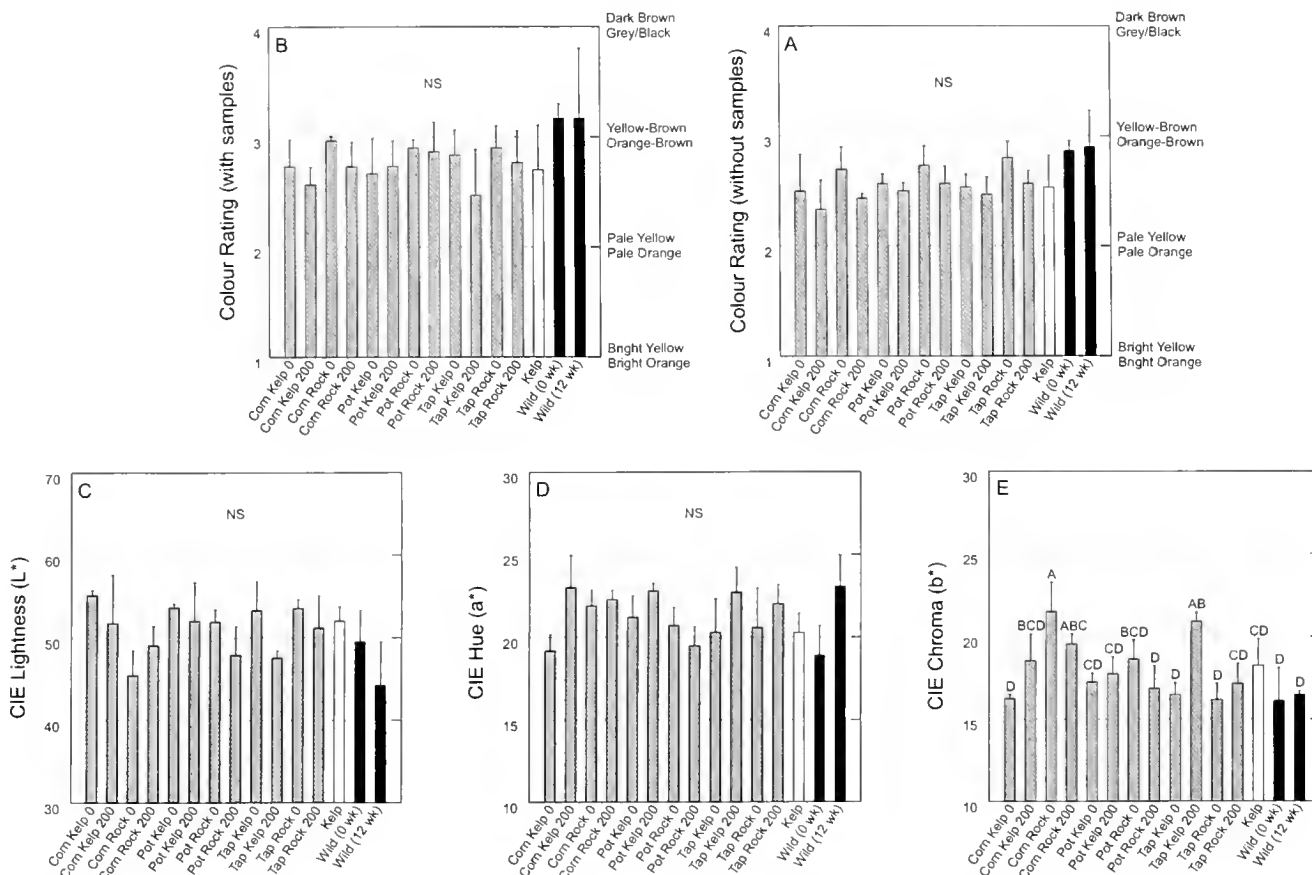
Figure 4. Mean color rating with paint samples (A), mean color rating without paint samples (B), mean CIE lightness (C), mean CIE hue (D), and mean CIE chroma (E) for all experimental treatments and wild controls at the end of the experiment. See text for full explanation of gonad quality ratings. Error bars are SE and *n* = 3. Letters above bars indicate the results of Fisher's LSD multiple comparison post-hoc tests showing significant pair-wise differences among experimental treatments and wild controls within each graph. "NS" denotes no significant differences among treatment means.

nificant differences among treatment means at the end of the experiment (Table 2). All prepared feeds produced significantly worse tasting gonads than the kelp control (2.3 ± 0.3) while the wild sample collected at the end of the experiment (2.5 ± 0.1) had significantly better tasting gonads than all prepared feeds except Pot Rock 0 (3.3 ± 0.2) and Tap Rock 0 (3.3 ± 0.2) (Fig. 1F). There were no significant pair-wise differences among any of the prepared feeds in terms of gonad taste ratings (Fig. 1F). A 4-way ANOVA examining the effects of starch type, macroalgal meal source, β-carotene concentration, and block on gonad taste ratings at the end of the experiment revealed no significant main or interaction effects (Table 3). A four-way repeated ANOVA on gonad taste ratings over time revealed no significant main effects and only one significant interaction—time × β-carotene concentration (Table 4). There was no significant difference between weeks 6 and 12 in gonad taste of sea urchins fed prepared feeds without β-carotene, but gonad taste was significantly better at week 6 than at week 12 for sea urchins given feeds with β-carotene (Fig. 10A). There was no significant difference in gonad taste of sea urchins fed feeds with or without pigment at either week 6 or week 12 (Fig. 10B).

DISCUSSION

The experiment was begun during the natural spawning season of the green sea urchin. Gonad yields of individuals sampled at the beginning of the experiment were high (15.1 ± 1.0%), but natural populations had completely spawned out by the end of the experiment in July (gonad yield = 2.8 ± 0.5%). In contrast, experimental sea urchins maintained in the laboratory under ambient temperature and fixed photoperiod and fed prepared diets did not undergo complete spawning. Partial spawning occurred in some individuals early in the experiment as evidenced by a slight drop in gonad yield during the second week of the experiment. This was also observed macroscopically as a number of individuals were leaking gametes early in the experiment. After the second week, however, there was a steady increase in gonad yield at each subsequent sampling period. This increase in gonad yield was not due to increasing water, however, since percent gonad water decreased during the experiment. These results are interesting from a commercial culture perspective since it shows that gonads of sea urchins fed prepared feeds can be enhanced even during periods of the year when natural populations are spawning. While sea urchins given prepared feeds showed an increase in percent gonad yields over the 12-wk experimental period, individuals fed kelp actually showed a decrease, albeit not significant, over the same time interval. Previous experiments have also shown that kelp can be an inferior feed in relation to prepared diets in terms of optimizing gonad yield (Pearce et al. 2002a, Pearce et al. 2002b, Pearce et al. 2002c). While kelp was inferior to prepared feeds in terms of increasing gonad yield, it did produce significantly better tasting gonads than the prepared diets.
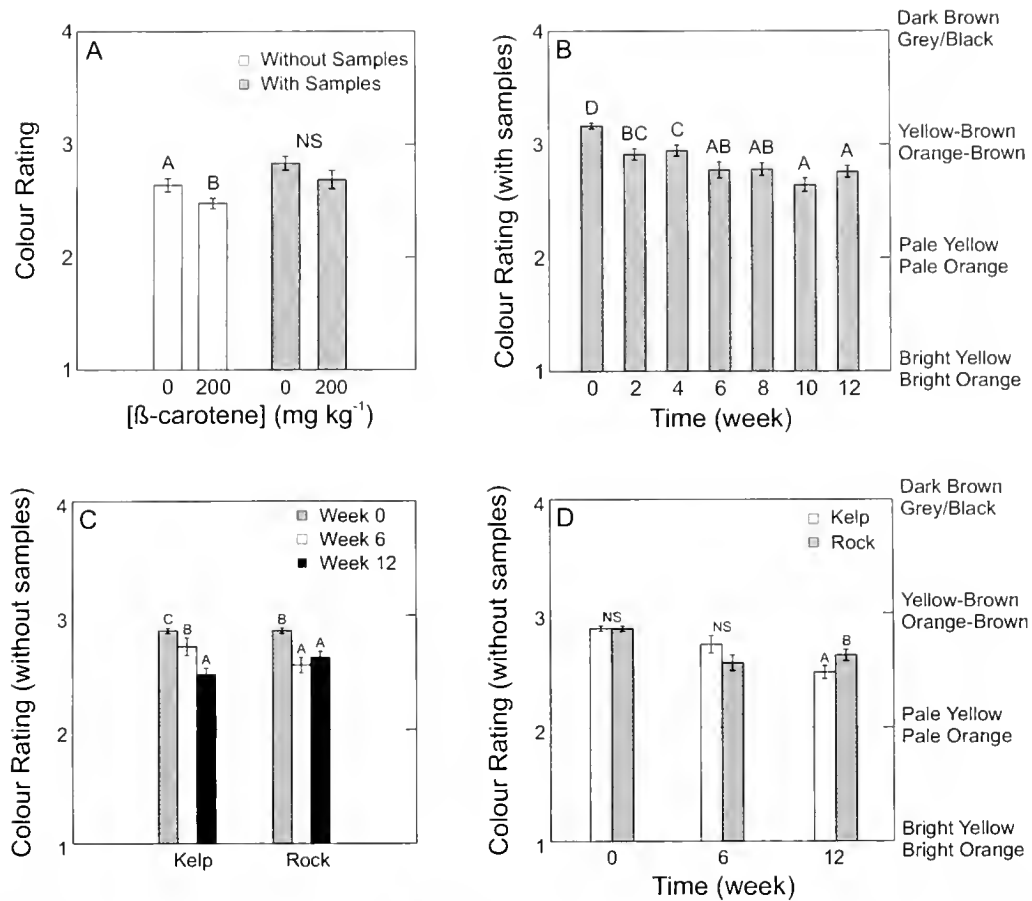
Figure 5. (A) Mean color rating (with and without paint samples) at week 12 in feeding treatments with and without β-carotene. Error bars are SE and *n* = 18. Letters above bars indicate the results of ANOVAs showing significant difference between treatment means. "NS" denotes no significant difference between treatment means. (B) Mean color rating (with paint samples) of all feeding treatments at each sampling interval. Error bars are SE and *n* = 36. Letters above bars indicate the results of a Fisher's LSD multiple comparison post-hoc test showing significant pair-wise differences among weeks. (C) Mean color rating (without paint samples) in feeding treatments with kelp or rockweed meal at the three sampling intervals. Error bars are SE and *n* = 18. Letters above bars indicate the results of Fisher's LSD multiple comparison post-hoc tests showing significant pair-wise differences among weeks at each level of macroalgal meal source. (D) Mean color rating (without paint samples) at the three sampling intervals for the kelp or rockweed meal treatments. Error bars are SE and *n* = 18. Letters above bars indicate the results of ANOVAs showing significant difference between treatment means. "NS" denotes no significant difference between treatment means.

Whereas all prepared feed treatments experienced gonad growth, the rate of increase (range: 0.3–0.8% wk^{-1}) was somewhat slower than published rates of increase in previous studies that have used prepared feeds to enhance *S. droebachiensis* (Klinger et al. (1997): 1.4% wk^{-1}; Motnikar et al. (1997): 1.2–2.6% wk^{-1}; Hávardsson et al. (1999): 0.7–0.8% wk^{-1}; Pearce et al. (2002a): 1.2–1.4% wk^{-1}; Pearce et al. (2002b): 0.9–1.3% wk^{-1}; Robinson et al. (2002): 1.6–2.2% wk^{-1}). Differences in rate of gonad increase may be attributed to variations in dietary components, especially protein concentration (de Jong-Westman et al. 1995a) and/or protein source ratio (Pearce et al. 2002b). Time of year may also affect rate of increase in percent gonad yield. In this study, while experimental sea urchins maintained in the laboratory did not spawn completely out, gonad yield dropped slightly in the second week of the experiment suggesting partial spawning in some individuals. Partial spawning was also evidenced by the presence of gonads leaking gametes in many individuals early in the experiment. This partial spawning event decreased the overall rate of gonad yield increase.

Gonad yield was unaffected by starch type or macroalgal meal source. This result was not surprising given that: (1) protein ap-
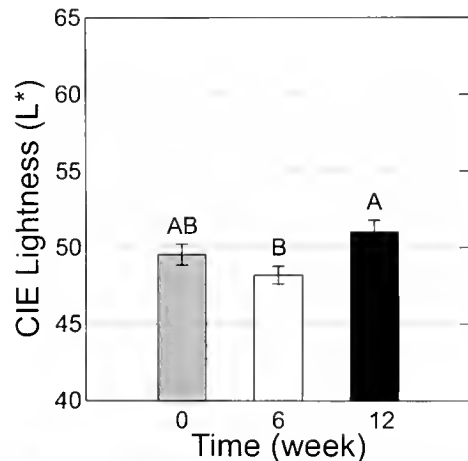

Figure 6. Mean CIE lightness (L*) of all feeding treatments at each sampling interval. Error bars are SE and *n* = 36. Letters above bars indicate the results of a Fisher's LSD multiple comparison post-hoc test showing significant pair-wise differences among weeks.
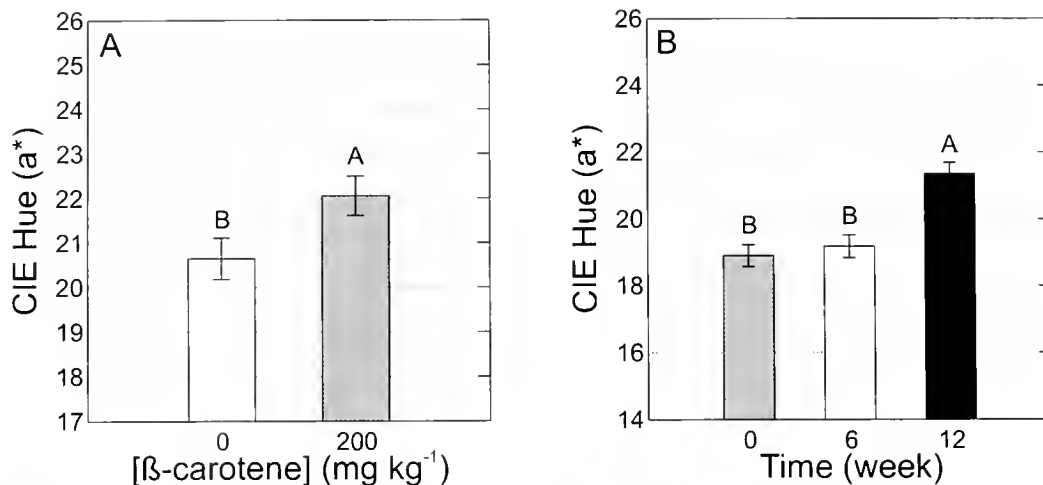
Figure 7. (A) Mean CIE hue or redness (a*) at week 12 in feeding treatments with and without β-carotene. Error bars are SE and *n* = 18. Letters above bars indicate the results of an ANOVA showing significant difference between treatment means. (B) Mean CIE hue or redness (a*) of all feeding treatments at each sampling interval. Error bars are SE and *n* = 36. Letters above bars indicate the results of a Fisher's LSD multiple comparison post-hoc test showing significant pair-wise differences among weeks.

pears to be the dietary component that may be predominantly responsible for gonad growth (de Jong-Westman et al. 1995a) and (2) the three starches contain minimum quantities of protein (<0.3%, manufacturer's specifications) while the two macroalgal meals have similar protein concentrations. The rockweed meal used in the experiment had a protein concentration of ~6% (Jeff Whitman, Intervest Trading, pers. comm.). Protein concentration of kelp can vary substantially with year, season, species, geographic location, and plant part, but Black (1950) reported that fronds of *L. saccharina*, collected between April and July of two successive years, had a range of 5.5–13.3% crude protein (% dry weight), depending on month of collection.

It is unclear why feeds with β-carotene (or more specifically Rovimix) supported significantly lower gonad growth than feeds without the pigment. Robinson et al. (2002) had similar results in

one of their experiments, but this finding is contradictory to the results of de Jong-Westman et al. (1995a) who reported that the inclusion of β-carotene in prepared diets significantly increased gonad growth in green sea urchins. More research on prepared feeds with β-carotene is required to fully understand the mechanisms at work.

The gonad color of sea urchins fed prepared diets improved over time. This was shown subjectively with both color rating schemes and objectively with L*, a*, and b* readings. These results indicate that formulated diets can be used to significantly improve sea urchin gonad color. Feeds containing β-carotene produced significantly better gonad color by the end of the experiment than feeds without the pigment. Similarly, Robinson et al. (2002) reported that they could significantly improve gonad color of *S. droebachiensis* by incorporating a spray-dried form of microalga
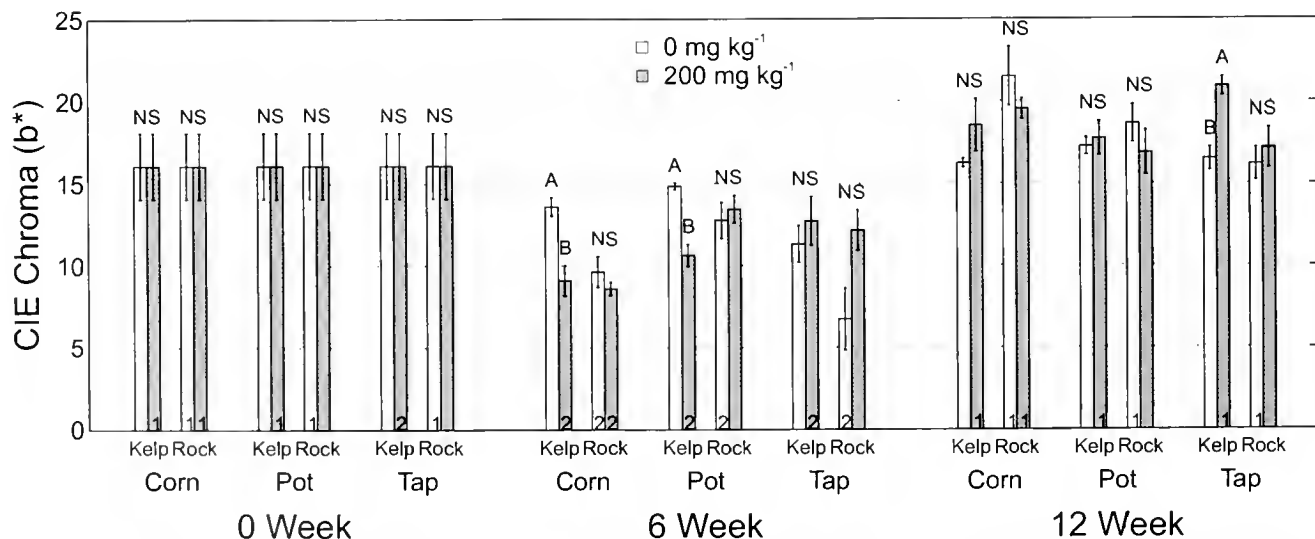

Figure 8. Mean CIE chroma or yellowness (b*) for each feeding treatment at each sampling interval. Error bars are SE and *n* = 3. Letters above bars indicate the results of ANOVAs showing significant pair-wise differences among β-carotene levels at each time/starch/macroalga level. Numbers at bottom of bars indicate the results of Fisher's LSD multiple comparison post-hoc tests showing significant pair-wise differences among time intervals within each starch/macroalga/β-carotene level.
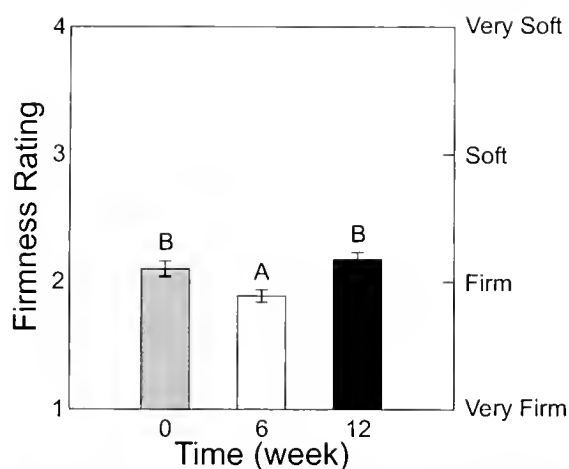
Figure 9. Mean firmness rating of all feeding treatments at each sampling interval. Error bars are SE and $n = 36$. Letters above bars indicate the results of a Fisher's LSD multiple comparison post-hoc test showing significant pair-wise differences among weeks.

(*Dunaliella salina*), rich in β-carotene, into prepared diets. They tested prepared feeds containing a range of β-carotene concentration (50, 100, 250, 500 mg kg^{-1} dry weight of feed) and found that 250 mg kg^{-1} was the most effective level at producing suitable gonad color (Robinson et al. 2002). Similarly, McLaughlin and Kelly (2001) reported that prepared diets containing the microalga *Phaeodactylum tricornutum* (having as its major carotenoid pigments fucoxanthin and diadinoxanthin and small amounts of β-carotene) significantly improved gonad color of the sea urchin *Psammechinus miliaris* over control diets without microalgae added. In contrast, Goebel and Barker (1998) reported that prepared feeds containing synthetic β-carotene did not significantly affect gonad color of the sea urchin *Evechinus chloroticus*. They used a much higher concentration (i.e., 60 parts per thousand) than recommended by Robinson et al. (2002) for optimizing color enhancement. Prepared diets containing astaxanthin or canthaxanthin—higher oxidation-state carotenoid pigments—do not significantly improve gonad color of *P. miliaris* (Cook et al. 1998, Kelly et al. 1998) or *S. droebachiensis* (Hávardsson et al. 1999, Pearce et al. 2002a). This suggests that sea urchins cannot use higher oxi-

dation state carotenoids, such as astaxanthin, as metabolic precursors for β-carotene or echinenone. β-carotene is a precursor of echinenone (Griffiths & Perrott 1976, Tsushima & Matsuno 1990, Tsushima et al. 1993), both pigments being major common carotenoids found in the gonads of a number of echinoid species including *S. droebachiensis* (Griffiths & Perrott 1976, Matsuno & Tsushima 2001).

Gonad yield, color, texture, firmness, and taste were not significantly affected by varying the starch type or macroalgal meal source indicating that gonad quantity and quality are independent of these two factors, at least with the starch and algal sources tested. This suggests that economic considerations or product availability, rather than biological factors, may influence the choice of starch or macroalgal meal sources for inclusion in sea urchin feeds. On the east coast of Canada for instance, rockweed meal is readily available commercially and considerably cheaper than kelp meal (non-bulk pricing: $0.80 CAD kg^{-1} and $30.80 CAD kg^{-1}, respectively). Algal meal made from *Ascophyllum nodosum* is often sold and packaged erroneously as "kelp meal". End-users of kelp meal should confirm with producers or suppliers to ensure they are receiving a product made from kelp.
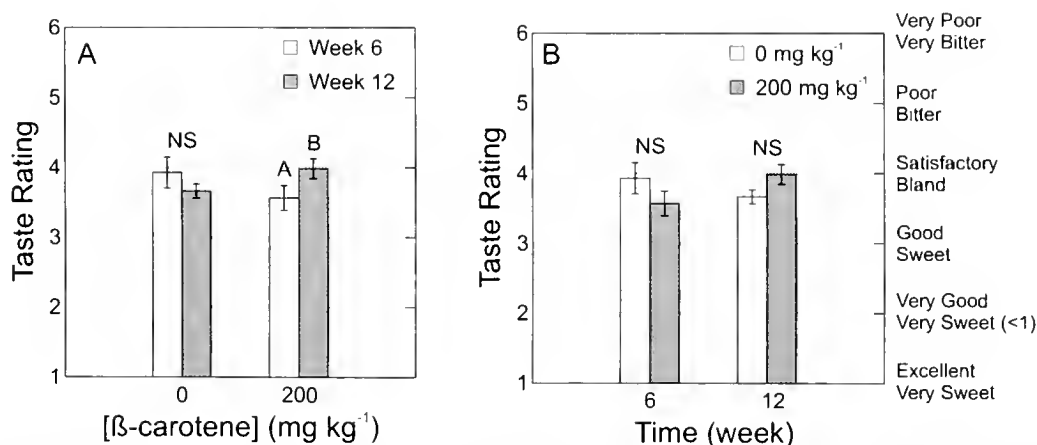
Figure 10. (A, B) Mean gonad taste rating of feeds with and without pigment (0 and 200 mg kg^{-1} β-carotene) at weeks 6 and 12. Error bars are SE and $n = 18$. Letters above bars indicate the results of ANOVAs showing significant pair-wise differences between pigment concentrations. "NS" denotes no significant difference between treatment means.

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PRODUCTION OF RED SWAMP CRAWFISH (*PROCAMBARUS CLARKII*) IN EARTHEN PONDS WITHOUT PLANTED FORAGE: ESTABLISHMENT, MAINTENANCE AND HARVEST OF POPULATIONS

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ABSTRACT Two separate studies were conducted in successive years to evaluate the effect of initial stocking densities and restocking versus natural recruitment during successive years on the production of red swamp crawfish (crayfish), *Procambarus clarkii*, in earthen ponds without planted forage. In the first study, permanently flooded ponds (mean depth = 1.05 m) that contained no crawfish populations were stocked with adult populations of red swamp crawfish (assumed male:female ratio of 1:1) at initial biomass densities of 168.8, 225; and 281.8 kg/ha. Production was compared with unstocked ponds with recruitment populations arising from animals that remained after harvest of the previous season (initial stocking density of 225 kg/ha). Crawfish populations were fed a commercially available 25% crude protein crawfish feed, twice daily and trap harvested. The mean yield and individual harvest weight of natural recruitment ponds (1589 kg/ha) was not significantly different from that of ponds that were initially stocked at the increasing densities (1623, 1755, and 1816 kg/ha, respectively). In the second study, restocking (112 kg/ha) versus natural recruitment and crawfish versus catfish feed were evaluated. Crawfish were harvested by both trap and seine. The mean yield and harvest weight of crawfish harvested from natural recruitment ponds (2374 kg/ha, 19.1g) was not significantly different from that of crawfish harvested from ponds that were restocked with broodstock (2160 kg/ha, 18.7g). Mean individual weight of harvested crawfish decreased from 28.5 g in October to 18.4 g in July. There were no significant differences relative to the different feeds used. The results suggest that after initial stocking, good management practices should result in sufficient recruitment to obviate restocking while still achieving consistent annual yields.

KEY WORDS: crawfish aquaculture in earthen ponds, *Pracambarus clarkii*, management

INTRODUCTION

Louisiana provides 90% of the US supply of crawfish (crayfish), approximately 47,240 mt per year (mean of 1991–1995) from a combination of forage-based culture fisheries and capture fisheries of *P. clarkii*, the red swamp crawfish (Huner 1997). Traditional culture of red swamp crawfish is based upon a flood and drain management of shallow ponds in association with a planted forage that is the basis for stimulation of the detrital food chain (Huner et al. 1994). The practice of double-cropping crawfish with a forage-feed, such as rice, may not always be possible or preferred in states outside of Louisiana. As commonly practiced in Louisiana, double-cropping with a forage limits realization of the true potential of crawfish growth and production because this management practice actually limits the time when ponds can be flooded during the growing and harvest season. In addition, the presence of forage restricts the mode of harvest to trap (Huner et al. 1994). In addition to Louisiana, commercial culture of the red swamp crawfish, *Procambarus clarkii* is conducted in Mississippi, Texas, Delaware, North Carolina, Arizona, and many other states in the United States (Huner et al. 1994).

Two of the major management problems associated with crawfish culture in systems with planted forage are depletion of sufficient amounts of food prior to the end of the growing and harvest season, and chronically low levels of dissolved oxygen. Production ponds are actually shallow flooded fields where rice is planted. In these systems, crawfish consume food derived directly or indirectly from the decomposition of the forage during the fall. However, by spring, this food resource is often depleted and a large population of resident crawfish that has yet to be harvested is left without sufficient food resources. As a result, growth during the

rising spring temperatures cannot be fully realized (Avault & Brunson 1990). Warm-water temperatures combined with the characteristic decomposition of plant material in this system are conducive to low levels of dissolved oxygen. Levels of dissolved oxygen less than 3 ppm are considered unsuitable for crawfish (Huner et al. 1994). Frequent incidence of low levels of dissolved oxygen induces stress in the crawfish, and feed efficiencies correspondingly decrease (Avault & Brunson 1990). Under these adverse conditions crawfish may even seek to respire atmospheric oxygen, and even emigrate from the pond.

A management approach that eliminates the use of planted forage in crawfish farming is attractive because permanently flooded, deeper ponds permit better control of the quality of water and food within the production system (D'Abramo & Niquette 1991, McClain & Romaire 1995). Permanently flooded, deeper ponds (D'Abramo & Niquette 1991) will result in water temperatures that are cooler and not so susceptible to wide daily fluctuation. Transition from an extensive to semi-intensive production system offers resident crawfish populations a potentially more suitable environment, as described by McClaine and Romaire (1995).

In an effort to move toward semi-intensive management and more reliable production from year to year in crawfish farming, D'Abramo and Niquette (1991) evaluated the feasibility of seine harvest and feeding of formulated pelleted diets as alternatives to exclusive trap harvest and the use of planted or volunteer forage as a food resource. Studies were designed to determine the best management practices for the production of red swamp crawfish in earthen ponds without planted forage. They observed that two different initial stocking rates of broodstock did not appear to affect mean harvest weight, suggesting a high degree of plasticity and unpredictability associated with production systems based on natural recruitment.

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MATERIALS AND METHODS

Common Pond Management Practices

Levels of dissolved oxygen and temperature for all experimental ponds were recorded daily for the entire harvest season to guide the implementation of different management practices. When the levels of dissolved oxygen were anticipated to decrease below 5 mg/L, pond water was aerated using a 0.5 hp aerator (Air-O-Lator Corporation, Kansas City, MO). Emergency aeration was provided by PTO driven paddlewheels when levels of dissolved oxygen decreased below 3 mg/L. Every third day during May through August, pH was recorded during the late afternoon. If pH values exceeded 9.2, all ponds received an application of cracked corn (56.3 kg/ha) to increase the production of CO_2 and thereby lower pH.

Study 1

Before stocking, 12 earthen ponds ranging in water surface area from 0.040 to 0.053 ha were treated with a chemical insecticide (Ambush, ICI Agricultural Products) at a concentration of 50 ppb. After 24 h the ponds were drained, filled half way, and again drained to remove any residual insecticide. This procedure effectively eliminated any resident crawfish from the designated experimental ponds. Ponds were then filled (mean depth of 1.05 m) and initially stocked with adults (assumed male:female ratio of 1:1) at biomass densities of 168.75, 225, and 281.75 kg/ha into ponds. The latter stocking densities exceeded those recommended for conventional forage based ponds (56-84 kg/ha) in an attempt to determine whether feeding could increase survival of young and total annual harvest. An additional treatment consisted of four ponds that already contained populations composed of animals remaining from the harvest of the previous season (initial first year stocking density of 225 kg/ha) and natural recruitment.

Crawfish populations were fed a commercially available 25% crude protein crawfish feed (Arcadiana Choice "25") twice daily. Feeding rates (Table 1) were based on an estimated pond biomass and water temperature. Total feed provided per treatment was in proportion to the initial stocking biomass.

Crawfish were harvested using pyramid style traps constructed of 1.91-cm diameter hexagon mesh with 61-cm extended necks

and three funnel openings between 3.8 and 4.4 cm. Traps were baited with approximately 150 g of a commercially available bait (Acadiana Choice - Medium), on Monday and Wednesday and harvested on Tuesday, Wednesday, Thursday, and Monday. Crawfish were harvested a total of 97 days during periods of November 21, 1995 to December 7, 1995 and March 3, 1996 to August 1, 1996. On one day during each week, up to 50 individuals harvested from each pond were randomly selected and individual weight and sex were recorded. Males were classified as sexually active (form I) or sexually inactive (form II) (Hobbs 1974).

All ponds were harvested once per month from May to August with a 1.91-cm mesh, knotted nylon seine, 1.5 m in height, that was modified through the attachment of a heavy nylon mud line to the existing lead line. Fifty individual crawfish were randomly selected from each seine harvest of each pond, and individual weight and sex were recorded.

Study 2

The relative value of an annual management practice of restocking (112 kg/ha) versus natural recruitment was investigated. In addition, two feeds, a 25% crude protein formulated crawfish feed (Acadiana Choice 25) and a sinking pelleted catfish feed (28% crude protein), were evaluated. Eight ponds, previously stocked in the first study, were randomly assigned to each of the two treatments, four ponds per treatment. An additional eight ponds from the previous year were restocked with crawfish (112 kg/ha) at an assumed male:female ratio of 1:1. Four ponds were randomly assigned to each feed thereby providing a 2 × 2 factorial arrangement of treatments. The annual rate of application of the commercially manufactured pelleted feeds was 3,700 kg/ha, proportionally distributed over eight months based upon pond water temperature and estimated resident biomass (Table 1).

Harvest was by trap and seine. Harvest by trap occurred on 99 days extending over 9 mo, from October 15, 1996 through December 13 and from March 5 to August 1, 1997. All ponds were seine harvested once in May, June, and August. The same traps, trap density, and seine described in study 1 were used. Trap harvest occurred on Monday, Tuesday, Thursday, and Friday. After harvest of unbaited traps on Mondays and Thursdays, traps were baited with approximately 150 g of a commercially bait (Acadiana Choice - Jumbo) and harvested on the following Tuesday and Friday, respectively. Compared with study 1, this management protocol used one less day of baiting and modified the two 72 h unbaited soak periods of study 1 to one 48 h and one 72 h. Data were collated to examine the relationship of yield to water temperature based upon whether the traps were baited.

Once weekly, up to 50 crawfish from each pond representing the different treatments were randomly selected. In addition, 50 individual crawfish were randomly selected from each pond that was seine harvested. In both instances, individual weight, sex, and reproductive form of the males (Hobbs 1974) were recorded. These data were collected to determine the composition of the crawfish population at different times of the year and identify any possible bias in harvest method relative to sex or male reproductive condition.

Statistical Methods

For study 1, an analysis of variance (ANOVA) using SAS (SAS Institute 1988) was used to determine whether significant differences in mean individual weight, total production, and survival

TABLE 1.

Monthly feeding rates by percent of total and kg/ha/yr.

Month	Percent of Total	
	Study 1	Study 2
January	0	0
February	0	0
March	15	8
April	15	15
May	25	20
June	20	20
July	5	8
August	0	0
September	0	0
October	5	11
November	10	10
December	5	8
Total feed fed (kg/ha/year)	*	3700

* 2400 = low density; 3200 = mid density; 4000 = high density.

existed among treatments. For study 2, a two-way analysis of variance (ANOVA), using the general linear model procedure (PROC GLM) of SAS (SAS Institute 1988), was used to determine whether significant differences in mean individual harvest weight, total production (kg/ha), and survival (%) existed between treatments and whether a significant interaction between factors (feed and stocking procedure) existed. An ANOVA was also conducted to determine whether the proportions of each sex and male developmental stage harvested were significantly different for the treatments in each study. All differences were considered significant at $P \leq 0.05$. The relationship between trap yields and water temperature for traps with and without bait was examined via linear regression and the best fitting lines were then calculated.

RESULTS

Study 1

The mean yield of natural recruitment ponds (1589 kg/ha) was not significantly different from that of ponds that were initially stocked at densities of 168.75, 225, and 281.75 kg/ha (1623, 1755, and 1816 kg/ha, respectively). Mean individual weight of crawfish harvested from natural recruitment ponds (17.3) was also not significantly different from that of ponds that were initially stocked at densities of 168.75, 225, and 281.75 kg/ha (17.9, 19.1, and 16.9 g, respectively).

Yields from seine harvests, individual harvest weights, M/F ratios, and % of form 1 males are presented in Table 2. Seine harvest comprised 13–35% of the total wet weight harvest per month. The mean percent of form 1 males that were trap harvested increased from 4% in April to 96% in August and declined to 12% by December. When seine harvest was conducted, the proportion of form 1 males was significantly lower than that obtained by trap harvest for the same week (Fig. 1).

Study 2

The interaction between quality of feed and stocking procedures was not significant ($P = 0.45$). The mean yield of natural recruitment ponds (2374 kg/ha) was not significantly different
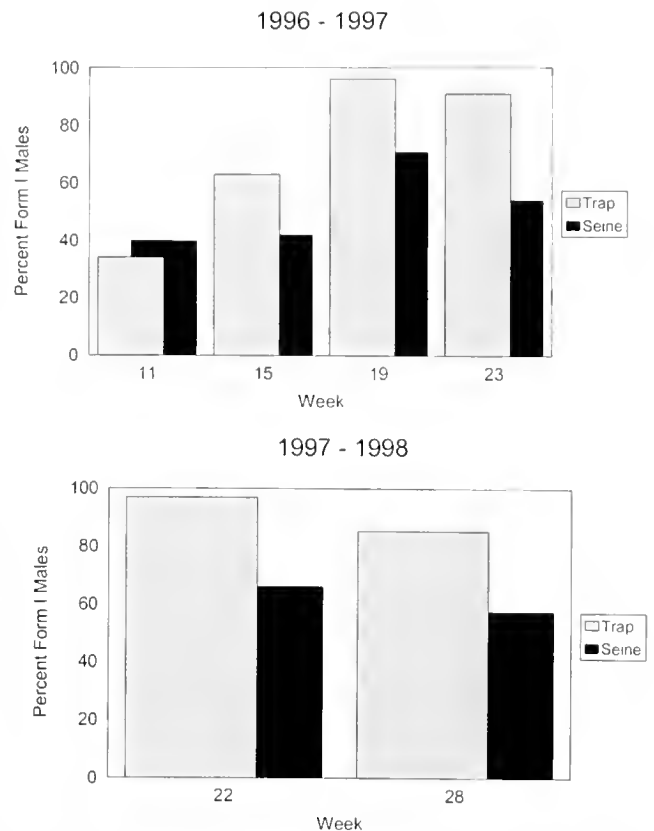

Figure 1. The relative percentages of form 1 males harvested by trap and seine during the same week.

from that of ponds that were restocked with broodstock (2160 kg/ha). Mean individual weight of crawfish harvested from natural recruitment ponds (19.1 g) was not significantly different from that of restocked ponds (18.7 g) and decreased in all ponds from 28.5 g in October to 18.4 g in July. There were no significant differences in production based upon feeds used.

Yields, individual weights, M/F ratios, and % of form 1 males from seine harvests are presented in Table 3. Seine harvest comprised 10–40% of the total wet weight harvest per month. The mean percent of form 1 males in all ponds increased from 5% in March to 95% in August. The proportion of form II males in seine harvests indicates that they were not harvested in the same proportion by trap (Fig. 1).

For crawfish harvested by trap, the relationship between mean harvest/trap/day and temperature, with and without the use of formulated bait, is presented (Fig. 2). The best fitting lines derived from harvest data obtained from baited and unbaited traps were determined. The intersection of the best fitting lines for unbaited and baited traps is 15C. Above this water temperature, the use of formulated bait becomes increasingly more effective than the use of no bait as the divergence of lines indicates. Soak time for baited traps was approximately 24 h, whereas the soak time for un-baited traps was either 48 or 72 h.

DISCUSSION

Populations of crawfish initially stocked into earthen ponds without planted forage appear to be self-sustaining for successive annual harvests when sufficient food is provided, good water quality is maintained, and the harvest practices outlined in studies 1

TABLE 2.

Mean yields from individual seine harvests relative to stocking density treatments (Study 1).

1995–1996	May 8	June 5	July 1	July 30
Kg/ha				
High	40.2	101.9	136.4	71.5
Medium	76.5	108.4	165.6	55.0
Low	58.8	71.9	126.6	73.8
Average wt. (g)				
High	12.0	13.7	14.8	18.8
Medium	13.0	14.6	15.7	18.3
Low	13.5	15.0	16.4	19.7
M/F ratio				
High	1.08	0.91	1.04	0.91
Medium	1.05	0.8	1.02	0.85
Low	1.15	1.46	0.98	0.94
Form 1 male (%)				
High	39	42	72	47
Medium	39	37	71	64
Low	42	47	69	51

TABLE 3.

Mean yields, male:female ratios, and percent form 1 males from individual seine harvests relative to treatments representing different management practices (Study 2).

1996–1997	May 6	June 20	Aug 1
Kg/ha			
Nat. Rec./crawfish	82	126.2	84
Nat. Rec./catfish	27.8	85.8	54.7
Restock/crawfish	118.2	144.1	96.2
Restock/catfish	41.2	93.6	105.8
Average wt. (g)			
Nat. Rec./crawfish	15.6	15.5	17.3
Nat. Rec./catfish	13.5	16.1	14.8
Restock/crawfish	15.3	15.1	16
Restock/catfish	14.4	15.3	16.3
M/F ratio			
Nat. Rec./crawfish		1.11	0.99
Nat. Rec./catfish		0.88	0.69
Restock/crawfish		1.0	0.9
Restock/catfish		0.97	.77
Form 1 males (%)			
Nat. Rec./crawfish		48.9	68.4
Nat. Rec./catfish		68.6	66.4
Restock/crawfish		55.7	63.5
Restock/catfish		57.4	66.6

and 2 are conducted. Increases of 33.3% and 66.7% in stocking biomass did not affect total annual production, even with corresponding increases in feeding rates. This suggests that a carrying capacity controlled by a variety of abiotic and biotic factors is reached and maintained as crawfish are harvested, i.e. removed from the pond. When ponds are well managed, restocking is unnecessary unless an annual harvest is unexpectedly low, suggesting that insufficient broodstock would be present for satisfactory recruitment for the following year.

Active harvest by seine revealed that information about population characteristics collected through passive trap harvest is not accurate. Data obtained from siene harvest show that the proportion of form II males in the pond population during May, June, and July is higher than that suggested by data obtained from trap harvest. Form II males are not caught by trap in the same proportion as they are present in the male population. This observation may be a manifestation of a behavioral hierarchy that exists among different males due to differential attraction to bait as a source of food. Another behavioral-based explanation is that form II males are more likely to leave the trap after the bait has dissolved as suggested by Romaire (1995).

At water temperatures below 15°C, use of the commercial formulated bait in traps was not effective in this study. Above 15°C, baited traps are more effective, daily harvest exceeding that of unbaited traps by approximately 0.01 kg/trap for every degree Celsius increase. As a result, a difference of 0.06 kg harvested/trap/day is realized by 20°C and 0.13 kg harvested/trap/day by 30°C. When bait is used, the higher daily yields/trap begin to be consistently realized at a water temperature of ≥17C and collectively contribute to an overall increase in annual yield. The temperature-dependent effectiveness of the bait used in this study may be related to its ingredient composition. Effective baits for trap harvest at temperatures <17 C may be achieved through modification of the ingredient composition. Further research needs to be conducted to evaluate different harvesting efforts, strategies, and formulated baits within specific temperature ranges.

The lack of significance of many of the management practices designed to improve production and evaluated in our studies may simply be due to the lack of sufficient control. Production from year to year is based upon a level of recruitment that cannot be totally controlled. However, a management strategy that does not incorporate an annual drain down followed by the planting of forage seems to offer a greater chance for consistent production from year to year. Nonetheless, the draining of conventional ponds for the planting of forage does offer two indirect benefits, control,
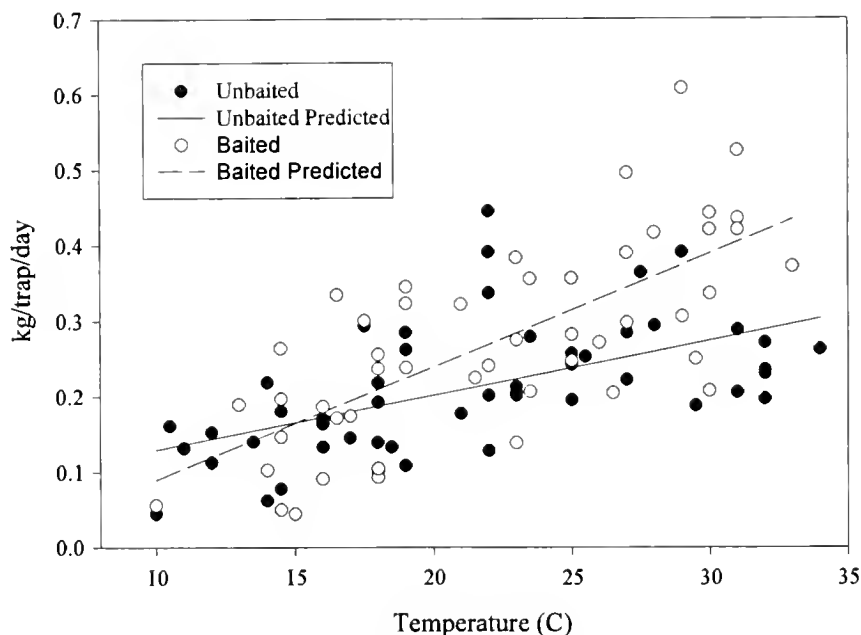

Figure 2. Linear regression displaying predicted mean yields (kg/trap/day) for harvest at different water temperatures with ($n = 50$) and without ($n = 51$) the use of formulated bait.

if needed, of predaceous fish that unintentionally become establish in the ponds and the oxidation of anaerobic sediments that may have resulted. Under the management practices described in this investigation, an annual production of approximately 1700 to 1800 kg/ha can be expected. Higher yields could be realized with the implementation of different harvest strategies. However, these approaches need to be weighed against the labor required. Practices that are designed to increase production may be inherently limited because of density-dependent factors, including cannibalism, for which management has little control. Some increase in production could be realized by methods to reduce the incidence of cannibalism. In addition, changes in the distribution of production over an annual cycle may be possible, but the increases may not be realized because only a particular range of production can be realized given the restrictions imposed by the nature of the management system. The quality of food provided can have an effect but once a certain level of quality of feed is attained, similar levels of production will be achieved, because the feeds primarily serve as an indirect source of nutrients. Most of the food provided appears to stimulate secondary productivity within a pond similar to the detrital food chain that is stimulated by the decomposition of planted forage in traditional ponds. The level of secondary productivity is limited and thereby controls the production of crawfish that can be realized. Certainly, routine removal by harvest does contribute to higher production. The red swamp crawfish is a species that has many characteristics that make it appealing as an aquaculture spe-

cies. However, some management practices are probably not successful simply because of the restrictions imposed by the nature of the production system. In addition, the innate level of variation among production ponds may preclude identification of significant treatment-related differences that would only be observed with a inordinate number of replicates. Any management practices that do succeed must still remain within the confines of realizing a positive net return. Those management practices that are ultimately identified as being most efficient and cost-effective must be transferred to larger (at least 0.5 ha) ponds to verify applicability.

ACKNOWLEDGMENTS

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PRODUCTION OF RED SWAMP CRAWFISH *(PROCAMBARUS CLARKII)* IN EARTHEN PONDS WITHOUT PLANTED FORAGE: EVALUATION OF TRAP AND SEINE HARVEST STRATEGIES

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ABSTRACT   The effect of trap density and trap versus seine harvest on the production of red swamp crawfish were evaluated in earthen ponds without planted forage. Crawfish were harvested from traps at densities of either 81 or 121/ha, 4×/week, from October through July of the following year. In another treatment crawfish were harvested from traps at a density of 121/ha when water temperatures were >19°C and seine harvested when water temperatures were between 15 and 19°C. Mean annual production ranged from 2173 to 2606 kg/ha, and mean harvest weight ranged from 16.6 to 17.8 g. Total production and catch per unit effort for seine and trap harvests at water temperatures between 15 and 19°C were not significantly different. Mean individual weight of seine harvested crawfish was significantly less (11.4 g) than that of trap-harvested crawfish (21.6 and 17.6 g). In a second study, the effects of different harvesting strategies and two formulated feeds were evaluated. Crawfish were fed either a 32% crude protein, extruded, slow-sinking formulated diet or a 32% crude protein, pelleted sinking diet, and harvested from traps either 3× (81 traps/ha) or 2× (121 traps/ha) per week. Trap harvest at 81 traps/ha, 3×/week and 121 traps/ha, 2×/week produced 2447 and 1884 kg/ha, and a mean individual harvest weight of 18.7 and 19.8 g, respectively. A significantly lower individual weight (16.2 g) was associated with the pelleted sinking feed relative to the extruded, slow-sinking feed. However, mean total production was not significantly different between treatments. Over 90% of the annual yield was harvested from April through October when water temperatures were >19°C.

KEY WORDS: crawfish aquaculture in earthen ponds, *Procambarus clarkii*, seine and trap harvest

INTRODUCTION

The goal of harvest strategies is to enhance efficiency of the labor expended while maximizing production. In traditional forage-based farming of the red swamp crawfish (crayfish), labor accounts for 50–70% of the total direct operational expenses and is primarily attributed to harvesting. Therefore, efficient harvest based upon conditions of when, how, how often and at what level of effort to harvest, is critical to the economic success of crawfish farming systems. The amount of labor is affected by trap density, number of harvest days/week, number of trap sets/day (the number of times a trap is prepared, with or without bait, to harvest crawfish daily), and current market price of crawfish (Romaire 1995).

The size of harvested crawfish also must be considered because quality of the product relative to market demand should not be compromised in exchange for a reduction in labor. In forage-based ponds for culture of crawfish, when soak time increases, larger but fewer crawfish are harvested from traps (Romaire 1995). Therefore, a harvesting strategy also must consider the number of crawfish that are harvested at one set. Inverting traps to prevent access when bait is not provided might result in an increase in mean size at harvest and yield because crawfish would be allowed to feed, reproduce, and molt in the pond for longer periods of time during the harvest season.

A decrease in catch per unit effort for trap harvest is encountered commonly for consecutive harvest days in production ponds with (McClain et al. 1998) and without (D'Abramo & Ohs 2003) planted forage. Proper management of the harvest schedule could lead to a more consistent yield with a corresponding decrease in labor. Trap density is also a component of an optimal harvest strategy, whereby a reduction in cost per unit effort can be realized.

Water temperature is also a major factor that influences the efficiency of harvest. Temperature-related differences in harvest strategy can also optimize trapping effort. Significant increases in catch per unit effort (CPUE) were achieved with the use of commercially manufactured baits when water temperature is equal to or exceeds 19 C (D'Abramo & Ohs 2003). A similar relationship between temperature and CPUE using formulated bait was observed in production ponds with planted forage (Romaire 1995). Efficient, cost-effective harvest at water temperatures less than 19°C requires a different approach that may include bait, soak time, or method of harvest.

A preliminary investigation of the utility of seine harvest of crawfish in production ponds without planted forage was conducted by D'Abramo and Niquette (1991). However, consistent yields were not achieved and mean individual weight of harvested crawfish declined because as the number of harvested crawfish accumulated in the seine, the ability of small crawfish to escape through the mesh decreased. D'Abramo and Ohs (2003) used periodic seine harvesting at pond water temperatures >19°C in an attempt to reduce biomass density and density-dependent growth reduction, with the intent to increase total annual yield. However, seine harvest may be most effective at water temperatures <19°C when the effectiveness of a passive trap harvest in conjunction with a formulated diet decreases.

In the absence of planted forage, formulated diets are needed to serve as both a direct and an indirect source of protein and other nutrients for the growth of crawfish. Uneaten food serves as both an inorganic and organic fertilizer of the pond, thereby contributing to an increase in natural food of the crawfish through stimulation of the detrital food chain. Diets that are more water stable will presumably increase the possibility of crawfish using the feed as a direct source of nutrients. An extruded feed is generally more water stable than a pelleted feed due to the heating process used in manufacture (De Silva & Anderson 1995). Buoyancy may also play an important role because a slow-sinking pellet contains air pockets that contribute to a lower rate of sinking and greater potential for distribution throughout the pond. This study evaluated trap density (81/ha versus 121/ha), and the effectiveness of seine versus trap harvest when pond water temperatures ranged between 15 and 19 C.

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MATERIALS AND METHODS

Study 1

Twelve earthen ponds with established crawfish populations from initial stocking either 1 or 2 y previously and, ranging from 0.045 to 0.069 ha in surface area, were used in this study. The duration of the study period was September through July of the following year. There were three treatments, four ponds/treatment. For the first treatment, eight seine harvests were conducted between October 31 and March 17 when water temperatures were between 15 and 19°C. When water temperatures consistently exceeded 19°C, crawfish were then trap harvested exclusively at a trap density of 81/ha. For the second treatment, crawfish were harvested exclusively by trap at a density of 121/ha for the entire study period. For the third treatment, the harvest schedule was the same as the second treatment except trap density was 81 traps/ha. An additional three seine harvests were performed when water temperatures were >19°C, once each in May, June, and July. Pyramid traps used in this study were constructed with 1.91-cm wire mesh (Gulf Coast Wire Products, Kaplan, LA). The traps had three funnel entryways, elongated necks that extended above the water surface, and polyvinyl chloride-retaining rings at the top. Traps were harvested four days per week (Monday, Tuesday, Thursday, and Friday) at temperatures >19°C. Harvests on Tuesday and Friday occurred after baiting on the previous days (24 h soak). Harvests on Thursday and Monday occurred with no bait after 48 and 72 h soak times, respectively. Traps were baited with approximately 150 g of a commercially available bait (Gros Rouge, Cargill, Minneapolis, MN). Four ponds were randomly assigned to each treatment stocked previously (1 or 2 y). No harvest was conducted when pond water temperatures were below 15°C. Crawfish were fed a 28% protein extruded, slow-sinking formulated feed for nine months (Table 1).

Traps were harvested a total of 105 days extending over 11 mo, from September 16 through July 31. The seine used for harvest was nylon, 1.5 m in height, and consisting of 1.9 cm mesh and was modified through the attachment of a heavy nylon mud line to the existing lead line. Once per week, after harvest, up to 50 crawfish from each pond were randomly selected and individual weight and sex were recorded. From each seine harvest, fifty individual crawfish were also randomly selected and individual weight and sex were recorded.

Levels of dissolved oxygen and water temperature for each of the experimental ponds were recorded daily for the entire year. If dissolved oxygen was anticipated to decline below 5 mg/L, surface aeration was provided by a 0.5-hp Aquarian aerolators (Air-O-Later Corp., Kansas City, MO). Additionally, tractor powered paddlewheels were used when the concentration of dissolved oxygen was anticipated to decline below 3 mg/L. From May to August, pH was measured every third day from water samples collected from each pond. In June, all ponds were treated with gypsum at approximately 182 kg/ha to control the sporadic and rapid increase in pH. The value of different harvesting methods and strategies was compared through calculation of CPUE. The calculations were based upon the assumptions that one worker (laborer) with a boat can harvest 150–300 crawfish traps/h (Romaire 1995), and a crew of three laborers, with the proper equipment, can seine harvest a 1 ha pond in 1 h. Seine harvest requires the removal of traps. However, no additional investment of labor is necessary if the traps are removed at the same time they are last harvested. The different labor investments required for the different strategies of harvest during water temperatures between 15 and 19°C, were standardized by assuming a 1 ha production pond. CPUE (kg/ha/laborer/h) was calculated by dividing the total harvest (kg/ha) for the entire period when water temperatures between 15 and 19°C by the number of harvest days, and then dividing by the number of hours required to complete harvest.

Study 2

Twelve earthen ponds were used in the evaluation of the effects of an extruded feed and an increased trap density. There were three treatments, four replicates (ponds) per treatment. Nine ponds had been in continuous production for either 2 or 3 y as part of previous investigations. The remaining three ponds were stocked with a 1:1 ratio of males to females at 112.5 kg/ha during July 1998. One of these ponds was randomly assigned to each of the three treatments. The management practices represented by the first treatment were the feeding of a 32% crude protein, pelleted, formulated diet, and a trap density of 81/ha. Traps were harvested 3 days/week, 2 consecutive days, followed by 24 h of soak. Traps were then inverted one day, baited the following day, and then harvested after a 24 h soak time. The second treatment was the same as the first treatment except a 32% crude protein, extruded, slow-sinking, formulated diet was fed. The final treatment consisted of the feeding of extruded, slow-sinking formulated diet, a trap density of 121/ha, and 2 consecutive harvest days/week with a 24 h soak time. When traps did not contain bait, they were inverted.

Harvest was conducted with the pyramid traps described in study 1. Trapping with bait occurred at water temperatures >19°C using a 100-g piece of formulated bait (Gros Rouge, Cargill, Minneapolis, MN). When water temperatures were between 15 and 19°C, traps were not baited and remained soaked. Under these conditions, a higher mean harvest weight would be expected because smaller crawfish would have more time to exit out of the trap. Dissolved oxygen concentrations and pH were measured and managed as described in study 1.

Some management constraints were imposed on trap harvest to maximize return on trapping effort. If the weekly harvest yielded

TABLE 1.

Monthly feeding rates (percent of total) and total amount of feed fed annually for studies 1 and 2.

Month	Percent of Total	
	Study 1	Study 2
January	0	7.25
February	0	6.5
March	10	7.0
April	17	11.5
May	15	14.25
June	14	12.5
July	10	7.0
August	0	4.0
September	8	7.75
October	10	8.0
November	10	7.0
December	6	7.25
Total feed fed (kg/ha/y)	4400	5635

<15 kg/ha/treatment, or mean individual harvest weight of the crawfish was <15 g/treatment/week, trap harvest was suspended for the next week. Also, if the average water temperature for all ponds decreased to <15°C, then harvest was suspended and resumed when water temperatures returned to 15°C.

From September 8 until November 3, crawfish in all ponds, representing all three treatments were fed a 32% crude protein, sinking, formulated feed manufactured by pelletization (Producers Feed Company, Isola, MS). Thereafter, the diets that were part of the previously described three treatments were fed. The results of the proximate analysis of each of the two different feeds used as part of the investigation are presented in Table 2.

CPUE (kg/ha/laborer/h) was calculated as described for the first study. To evaluate the differences between trap densities, a theoretical 1 ha pond was used and it was also assumed that 150 traps can be harvested per hour by one laborer with a boat. Total weight harvested (kg/ha) was determined for each pond day when trap harvest was conducted. Yields were either combined or separated to reflect harvest yields at water temperatures of either >19C or between 15 and 19°C. The cumulative harvest weights were divided by the number of harvest days, and then divided by the number of laborer hours required for trap harvest.

Statistical Analysis

A one-way analysis of variance using the general linear model of SAS (Statistical Analysis System, version 8.1, Cary, NC) was used to determine whether differences existed among treatments for mean yields (kg/ha and number/ha), mean individual weights and mean CPUE overall and relative to harvest temperature of the pond water. Significant differences were identified at the $P \leq 0.05$ level.

RESULTS

Study 1

The mean total production (kg/ha), and mean individual harvest weight (g) of crawfish harvested from ponds with a trap density of 81/ha were not significantly different from those ponds with a trap density of 121/ha (Table 3).

Total yield (kg/ha) at water temperatures between 15 and 19°C was not significantly different among treatments (Table 4). However, the mean individual weight (g) of the crawfish harvested by seine (11.4 g) was significantly less than those harvested from traps at densities of 81/ha (21.6 g) and 121/ha (17.6 g).

Seine harvest required a greater amount of labor than trap harvest and contributed to the lowest CPUE (10.1 kg/ha/laborer hour).

TABLE 2.

Results of the proximate analysis for extruded, slow-sinking, and pelleted diets fed to crawfish in ponds without planted forage.

Component (% Dry Weight)	Extruded Slow-Sinking Diet	Pelleted Diet
Ash	8.4	7.1
Crude protein	37.8	39.1
Crude lipid (acid hydrolysis)	5.4	4.3
Crude fiber	6.7	6.2
Nitrogen-free extract (carbohydrate), by difference	41.7	43.3

TABLE 3.

Mean annual production (kg/ha) ± SE and mean individual weight (g) ± SE of harvested crawlish (Study 1).

Treatment	Total Production (kg/ha)	Mean Individual Weight (g)
81 traps/ha	2606 ± 400	17.8 ± 0.4
121 traps/ha, trap <19°C	2318 ± 211	16.7 ± 0.6
121 traps/ha, seine <19°C	2173 ± 239	16.6 ± 1.0

CPUE was highest for trap harvest at 121 traps/ha (13.9 kg/ha/ laborer hour) during water temperatures between 15 and 19 C (Table 4).

Each of the three seine harvests conducted during the summer months when water temperatures exceeded 19 C in ponds that contained 81 traps/ha yielded between 50 and 100 kg/ha. Greater yield was achieved from a cumulative four day trap harvest than one seine harvest during the same week two of three times. The individual harvest weight of seine harvested crawfish was less than that of trap harvested crawfish collected during the first seine harvest in May. Under an equal number of trap days, the yields for the two different trap densities were similar. Further evaluation of trap density and trapping effort is warranted.

Study 2

The mean total production, mean individual weight, and mean number/ha did not differ significantly among treatments (Table 5). A 33% decrease in the number of harvest days for ponds containing 121 traps/ha resulted in a 23% decrease in annual production (kg/ha) and a 27% decrease in total number of crawfish harvested per hectare. CPUE (kg/ha/laborer hour) for the trap harvest conducted twice per week was 58% greater than that conducted 3×/ week.

At water temperatures between 15 and 19°C mean total production (kg/ha), mean individual weight, and mean number/ha were not significantly different among treatments (Table 6). To evaluate the feasibility of trap harvest at water temperatures less than 19°C, CPUE was calculated and multiplied by a market price of $2.75 US/kg. Maximum return for one hour of labor to harvest 81 traps/ha three times a week would be 37.8% less than the return realized from harvest of 109 traps/ha twice a week.

TABLE 4.

Total production (kg/ha), CPUE (kg/ha/laborer hour), and mean individual weight (g) of crawfish harvested when water temperatures were between 15 and 19°C (trap harvest was conducted for 15 separate days and seine harvest occurred eight different times, study 1).

Treatment	Total Production (kg/ha)	CPUE (kg/ha/laborer hour*)	Mean Individual Weight (g)
81 traps/ha, trap	293	10.5	21.6
121 traps/ha, trap	284	13.9	17.6
121 traps/ha, seine	298	10.1	11.4

* Assumed labor required: one laborer can harvest 150 traps per hour, three laborers can seine harvest a 1 ha pond in 1 hour. Values are based upon the mean of each treatment.

TABLE 5.

Mean total annual production (kg/ha) ± SE and mean individual weight (g) ± SE of harvested crawfish (Study 2).

Treatment	Mean Total Production (kg/ha)	CPUE (kg/ha/laborer hour*)	Mean Individual Weight (g)	Number/ha
81 traps/ha 3×, ESS	2447 ± 169	13.2	18.7 ± 0.9	130,764 ± 3,781
81 traps/ha, 3×, PS	2294 ± 309	12.4	16.2 ± 0.5	141,233 ± 18,416
121 traps/ha. 2×, ESS	1884 ± 260	20.8	19.8 ± 1.5	95,928 ± 18.878

At trap densities of 81/ha and 121/ha, the total number of harvest days was 100 and 67 days, respectively. Either a extruded slow-sinking (ESS) or a pelleted sinking (PS) diet was fed.

* Assumed labor required: one laborer can harvest 150 traps her hour. Values are based upon the mean of each treatment.

For trap harvest when water temperature exceeds 19°C, mean total production (kg/ha), mean individual weight, and mean number/ha were not significantly different. However, with a mean increase of 500 kg/ha and 32,000 crawfish/ha, the potential economic impact is obvious (Table 7). A mean weight increase of 1 g with the decreased trapping effort was not statistically significant.

DISCUSSION

Large inherent variation of production parameters of ponds within the same treatment does present some problems in the identification of the relative value of different management strategies. This condition is characteristic of the system under investigation, that is, production from one year to the next cannot be directly controlled and is principally determined by recruitment success. Nonetheless, some recommendations can emerge and future areas of investigation can be defined.

Results indicate either an extruded, slow-sinking diet or a pelleted 32–35% crude protein, sinking diet can be fed, and selection should be determined by cost and availability. A sinking catfish diet that is not particularly water stable works as well as a formulated diet. These results suggest that stimulation of the detrital food chain may be the best way to serve the nutritional needs of the crawfish as long as a selective harvest schedule is sufficiently intense to remove a satisfactory amount of biomass through time. Further investigation into the use of other alternative feedstuffs is warranted because the cost of feed represents a large proportion of the total operational costs. The results of the two studies suggest that trap density is sufficient at 81/ha and that trap harvest is a better strategy when water temperature is <19°C. Although the catch per unit effort is greater at a density of 121/ha because of less labor for harvest, this apparent benefit must be weighed against the cost of additional traps and the higher production that can be achieved for the entire harvest season when traps are harvested three times/week. The comparatively poor performance of seine versus trap harvest is probably caused by a less-than-efficient design for harvest. A design specific to the harvest of pond raised crustaceans may result in an attractive option. Other potential approaches to enhance yield from seine harvest would be provision of food (bait) just prior to a scheduled harvest, and/or harvest soon after dusk when foraging activity is believed to be highest.

An alternative management strategy that needs investigation is a modification in the proportion of trap days per month when trap harvest is conducted at water temperatures >19°C. This procedure would consist of a decrease in trapping effort from March through May, followed by a corresponding increase in effort from June through October. The ultimate goal of this management strategy would be maintenance of equivalent annual production but with the amount of production being proportionately greater when traditional capture and culture fisheries can no longer provide the product. Those management practices that are ultimately identified as being most efficient and cost-effective must be transferred to larger (at least 0.5 ha) ponds to verify applicability.

ACKNOWLEDGMENTS

TABLE 6.

Total production (kg/ha), CPUE (kg/ha/laborer hour), and mean individual weight (g) of crawfish harvested when water temperatures were between 15 and 19°C (Study 2).

Treatment	Total Production (kg/ha)	CPUE (kg/ha/man hour*)	Mean Individual Weight (g)	Number/ha
81 traps/ha, 3×, ESS	200	4.5	20.7	9679
81 traps/ha, 3×, PS	126	2.8	19.8	7721
121 traps/ha, 2×. ESS	136	6.2	20.7	7082

Trap harvest was conducted a total of 24 and 16 days at trap densities of 81/ha and 121/ha, respectively. Either an extruded slow-sinking (ESS) or a pelleted sinking (PS) diet was fed.

* Assumed labor required: one laborer can harvest 150 traps per hour.

Total annual production (kg/ha), CPUE (kg/ha/laborer hour), and mean individual weight (g) of crawfish harvested when water temperature was >19 C (Study 2).

Treatment	Total Production (kg/ha)	CPUE (kg/ha/man hour*)	Mean Individual Weight (g)	Number/ha
81 traps/ha, 3×, ESS	2246	16.0	18.6	121,085
81 traps/ha, 3×, PS	2168	15.4	16.0	133,512
121 traps/ha, 2×, ESS	1748	25.5	19.7	88,846

Trap harvest was conducted a total of 76 and 50 days at trap densities of 81/ha and 121/ha, respectively. Either an extruded slow-sinking (ESS) or a pelleted sinking (PS) diet was fed.

* Assumed labor required: one laborer can harvest 150 traps per hour.

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DISTRIBUTION, SHELTER FIDELITY, AND MOVEMENTS OF SUBADULT SPINY LOBSTERS (*PANULIRUS ARGUS*) IN AREAS WITH ARTIFICIAL SHELTERS (*CASITAS*)

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ABSTRACT In Bahía de la Ascensión, a large bay on the Caribbean coast of Mexico, artificial shelters (casitas) have been used in the fishery for spiny lobsters (*Panulirus argus*) for several decades. We selected two bay sites that differed in their ecological characteristics: site 1 was a protected inner-bay site, rich in benthic vegetation (settlement and postsettlement habitat) and site 2 was a more exposed, outer-bay site, closer to the coral reef tract, with less vegetation and more open hard bottoms. In each site, we explored the size distribution, population density, and patterns of aggregation of lobsters in casitas, as well as the site and shelter fidelity and the short-term movement ranges of individually tagged subadults (mean ± SD carapace length: 68.1 ± 10.9 mm). We expected that, owing to its lush vegetation, site 1 would have a higher density of lobsters of a smaller mean size than site 2, but that because of the occurrence of casitas in both sites, site and shelter fidelity and the movement ranges of subadult lobsters would be similar in both sites. As expected, site 1 had significantly more lobsters encompassing a wider size range, but with a smaller mean size, than site 2. Lobsters were also more aggregated beneath casitas in site 1 than in site 2. Subadult lobsters exhibited similar site fidelity and short-term movement ranges in both sites, but a marginally higher shelter fidelity in site 2. However, shelter fidelity in both sites was lower than expected based on studies conducted by other workers in areas with natural shelters only. Although not conclusive, our results suggest that, because casitas might all afford a similar shelter quality to lobsters, lobsters in areas with casitas exhibit lower shelter fidelity and wider movement ranges than lobsters in areas with natural shelters only.

KEY WORDS: *Panulirus argus*, artificial shelters, casitas, site fidelity, shelter fidelity, movements

INTRODUCTION

The spiny lobster *Panulirus argus* (Latreille, 1804), a major fishing resource throughout the Caribbean area, has several ontogenetic shifts in habitat and sociality during its benthic life. After a protracted, oceanic larval phase, the postlarvae of *P. argus* settle in shallow, vegetated habitats, where the ensuing algal-phase juveniles (6 to 15–20 mm carapace length, CL) remain widely dispersed, displaying asocial behavior. The postalgal juveniles (15–20 to approx. 45 mm CL) remain close to the settlement habitats but occupy crevice-type shelters and become socially gregarious. The subadults (45–80 mm CL) are more nomadic and may aggregate in large shelters but tend to migrate towards nearby coral reef tracts as they approach the adult phase (>80 mm CL). Adults dwell in caves and crevices in coral reefs and rocky bottoms on wide expanses of continental shelf and undergo massive, organized seasonal migrations (reviews in Herrnkind 1980, Butler & Herrnkind 1997).

Shelter availability plays an important role in the survival of spiny lobsters (Smith & Herrnkind 1992, Mintz et al. 1994, Briones-Fourzán & Lozano-Álvarez 2001) and much of the individual and social behavior of spiny lobsters revolves around the shelter (Childress & Herrnkind 1996, 2001). Spiny lobsters must balance their need to remain in a shelter to avoid predation with the opposite need of leaving that shelter to forage (Sih 1992, Vannini & Cannicci 1995) but have the ability to relocate known shelters (Herrnkind et al. 1975, Cobb 1981, Nevitt et al. 2000, Lozano-Álvarez et al. 2002). On the other hand, spiny lobsters prefer shelters that allow cohabitation (Spanier & Zimmer-Faust 1988, Eggleston et al. 1990, MacDiarmid 1994), and individuals of *P. argus* may use conspecifics as cues both to locate and to assess the quality of a shelter (Ratchford 1999, Nevitt et al. 2000, Childress & Herrnkind 2001).

Vegetated and hard-bottom habitats have a fractal structure, which decreases the amount of shelter for large animals compared with small animals (Morse et al. 1985, Caddy 1986). Paucity of shelter may affect the movements and residence time of spiny lobsters in different ways. In areas poor in shelter, juveniles may exhibit either high rates of nomadism (Herrnkind 1980), which increases their risk of predation, or restricted foraging movements, which precludes them from exploiting available food resources (review in Lipcius & Eggleston 2000) and may result in a poor nutritional condition (Briones-Fourzán et al. 2003). Also, shelter scarcity would increase shelter fidelity in spiny lobsters, i.e., the propensity of lobsters to return to a previously used shelter (Herrnkind et al. 1975, Ratchford 1999).

Casitas, or artificial shelters for spiny lobsters, have been empirically used for a number of decades in the fishery for *P. argus* in Bahía de la Ascensión, a large, shallow bay on the Caribbean coast of Mexico (Briones-Fourzán et al. 2000; Fig. 1). Casitas may increase lobster abundance and biomass in areas with limited natural shelter (Briones-Fourzán & Lozano-Álvarez 2001) by increasing protection from predators (Eggleston et al. 1990, Mintz et al. 1994). Casitas used in Bahía de la Ascensión are scaled to accommodate mostly subadults and adults (i.e., lobsters >45 mm CL), but because of their gregarious behavior lobsters that occupy casitas are 10–145 mm CL (Lozano-Álvarez et al. 1991). However, mean size of lobsters is generally larger in "outer-bay" sites (sites between the mouth of the bay and the reef tract, see Fig. 1) than in "inner-bay" sites (elsewhere in the bay; Eggleston et al. 1990, Lozano-Álvarez et al. 1991).

The area of the bay suitable for using casitas has been divided in parcels (called campos) allotted to the members of the local fishing cooperative. Fishers decide how many casitas and where to deploy them within their campos. Favored substrates are vegetated habitats and hard bottoms. Unvegetated soft bottoms are generally avoided because on these substrates casitas tend to sink or their sheltering space becomes obstructed by sediment (Briones-Fourzán et al. 2000). Therefore, although Camarena-Luhrs et al. (1996) estimated an average of 3.3 casitas ha⁻¹ in some bay areas,
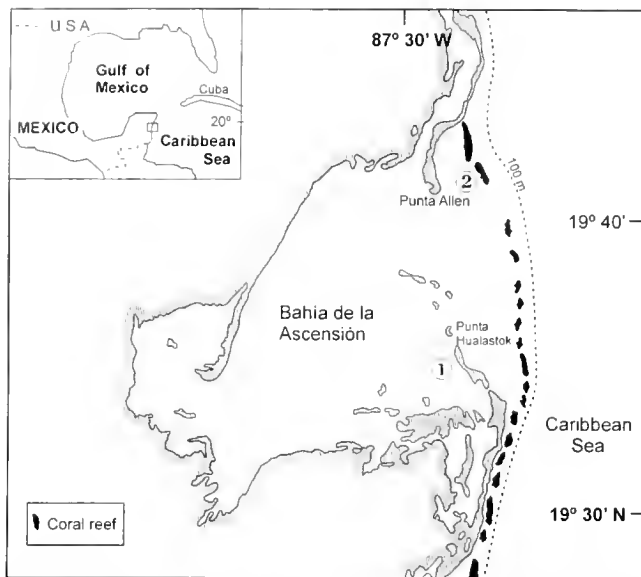
Figure 1. Location of the two study sites: an inner-bay site (site 1) and an outer-bay site (site 2) in Bahía de la Ascensión (Caribbean coast of Mexico). Black areas represent the coral reef tract.

the distribution of casitas throughout the fishing areas in the bay is highly heterogeneous.

Lozano-Álvarez (1995) hypothesized that, in addition to decreasing predation risk of lobsters, the occurrence of numerous casitas over large expanses could allow spiny lobsters to exploit food resources over more extensive areas, because after their nocturnal foraging excursions lobsters could retreat into any casita available in their vicinity. Moreover, lobsters foraging close to a casita may be attracted by chemical cues emanating from other lobsters already sheltered in that casita (Ratchford & Eggleston 1998, Nevitt et al. 2000). This hypothesis implies a low shelter fidelity among lobsters occurring in areas with casitas.

We explored the lobster density and the pattern of lobster aggregation in casitas in two sites in Bahía de la Ascensión that differed in their environmental characteristics: an inner-bay site (site 1) and an outer-bay site (site 2). Based on previous studies (e.g., Lozano-Álvarez et al. 1991, 1994), we expected 1) a smaller mean size and a higher abundance of lobsters in site 1 than in site 2, and 2) larger aggregations of lobsters in casitas in site 1 than in site 2. We also explored the site and shelter fidelity and the short-term movements among casitas of lobsters >45 mm CL (i.e., subadults and young adults). Despite the environmental differences between both sites, we hypothesized that, owing to the presence of casitas, (1) site and shelter fidelity of lobsters would be similar in both sites, and (2) short-term movements of these lobsters would be similar between both sites but greater than those reported for areas with natural shelters only.

MATERIALS AND METHODS

Study Sites

Site 1 was located west of Punta Hualastok, an inner-bay area highly protected from wave surge (Fig. 1). The water in this site was very calm and reddish in color as a result of the thick mangrove forests bordering the nearby coasts to the east and south of the site. Depth was 3–4 m. The bottom in site 1 was mostly fine

calcareous sand and mud, extensively covered with dense meadows of macrophytes that included mixed seagrass (*Thalassia testudinum* and *Syringodium filiforme*) and abundant macroalgae, such as *Laurencia intricata*, *Dictyota divaricata*, *Jania adhaerens*, *Caulerpa* sp., *Halimeda incrassata*, *H. monile*, *Batophora oerstedii*, and *Ripocephalus phoenix*.

Site 2 was located in an outer-bay zone, leeward of the coral reef tract (Fig. 1) and was more exposed to wave surge than site 1. Water in site 2 was generally very clear, and depth was 3–3.5 m. The bottom in site 2 was coarse calcareous sand with a few small coral heads and patches of exposed calcareous pavement. The bottom type changed gradually towards the coast, where a few patches of dense vegetation were interspersed with vast expanses of sparse vegetation and open sand. The macrophytes consisted of mixed seagrass with interspersed macroalgae, especially *Halimeda* spp., *Laurencia scoponia*, *Penicillus dumetosus*, *Udotea flavellum*, *U. conglutinata* and *U. spinulosa*.

Lobster Sampling

In Bahía de la Ascensión, casitas harbor more lobsters towards the end of the closed season (1 March–30 June), which is reflected in significantly higher catches during the first month (July) than during the rest of the fishing season (August–February) (Lozano-Álvarez et al. 1991). Therefore, to avoid bias in our results caused by fishing activities, our study was conducted in June 1990 and May through June 1991.

Two divers towed by a boat in a systematic pattern surveyed each of the two sites for casitas. When a casita was found, it was marked with an individually numbered buoy. Casitas were more widely dispersed in site 1 than in site 2. We marked 22 casitas in site 1 and 25 in site 2. The size and shape of the 47 casitas were similar (~1.8 m long × 1.2 m wide × 6–8 cm high) and all were constructed with the same materials (a palm-trunk frame and a ferrocement roof). We delimited the area enclosing the marked casitas in each site with additional buoys, measured the distance between adjacent buoys, and estimated the surface area of each site. This was approximately 25 ha in site 1 and 12 ha in site 2. The delimited areas were surveyed again, but no further casitas were found.

Divers censused the lobsters beneath the 22 casitas in site 1 on six occasions between 15 and 23 June 1990. On each of the first 4 days, all the lobsters sheltering beneath a randomly chosen casita were prodded into the cod-end of a seine net (Lozano-Álvarez et al. 1991, Lipcius et al. 1998). The cod-end was kept underwater at the side of the boat to maintain the lobsters submerged and protected from direct sunlight. Lobsters were then extracted from the net one at a time to determine their sex and to measure CL with calipers (±0.1 mm, between the rostral horns and the posterior margin of the cephalothorax). Subadults (individuals ≥45.0 mm CL) were then tagged and returned to their original casita. Tags consisted of a color-coded flag of adhesive tape held by a rubber band around the carapace between the fourth and fifth pair of pereiopods that allowed for identification of both the individual and the casita from where it was extracted. Over subsequent surveys, we recorded the data of resighted lobsters and of the casitas where they sheltered.

Lobsters beneath the 25 casitas in site 2 were censused on 15 occasions between May 7 and June 9, 1991. On eight dates between May 7 and 24, all the lobsters from one casita were measured and the subadults tagged. In addition to the color-coded tag,
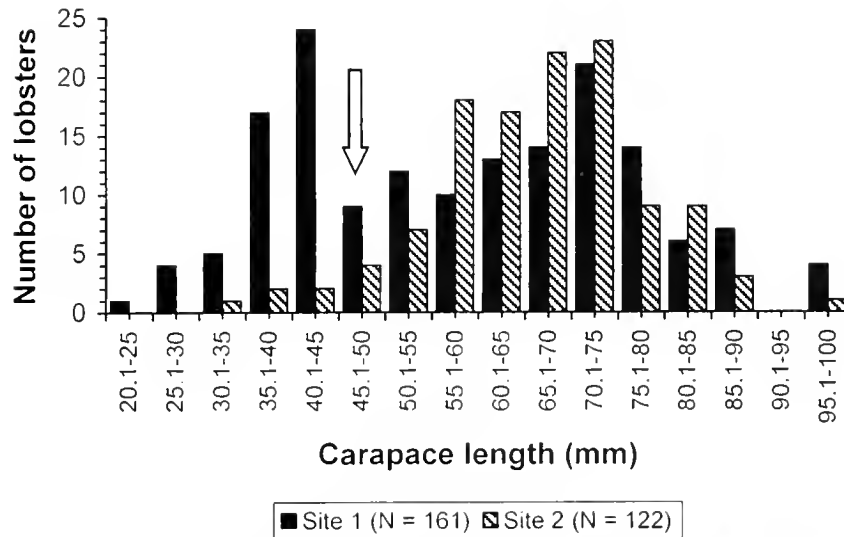
Figure 2. *Panulirus argus*. Size distribution of lobsters captured from beneath casitas in an inner-bay site (site 1) and an outer-bay site (site 2) in Bahía de la Ascensión. The arrow indicates the class size from which individuals were tagged.

which aided in the rapid identification of the original casita, we also applied to these lobsters individually numbered Australian "spaghetti" tags (Chittleborough 1974), modified for small lobsters (Lozano-Álvarez 1992, Negrete-Soto et al. 2002), on the dorsolateral muscle between the cephalothorax and abdomen. With these tags, Lozano-Álvarez (1992) estimated a tag-related mortality of ~5% after three months in individuals of *P. argus* over the same size range as in our study.

Lobster Density and Patterns of Aggregation in Casitas

In each site, lobster population size, losses (death + emigration) and immigration were estimated using the Fisher-Ford model (Fisher & Ford 1947), which relies on several tagging and recapture dates as well as on multiple recaptures of individuals. When capture-recapture data are scarce, 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, Negrete-Soto et al. 2002). The Fisher-Ford model assumes a constant survival rate (ϕ) but provides a method to test for this assumption (Begon 1979). Because the number and frequency of sampling dates varied between sites, we used only the data from censuses conducted over consecutive days (four dates in site 1 and six dates on site 2) to estimate lobster abundance. This would also increase the probability of a constant survival rate over such short periods. We then obtained the density of lobsters in each site by dividing the number of lobsters estimated by the model over the site area (Begon 1979). We also estimated the density of the portion of the lobster population sheltering in casitas in each site by dividing the daily number of lobsters censused beneath casitas over the site area. The propensity of lobsters to aggregate in casitas was analyzed in each site by plotting the number of lobsters in each casita vs. the number of casita surveys over the sampling period (Briones-Fourzán et al. 2000) and fitting the data to a random distribution.

Site and Shelter Fidelity Among Lobsters

The percent of tagged lobsters that were resighted at least once in each site was considered as a measure of site fidelity (Butler &

Herrnkind 1997). Because consecutive censuses were conducted 1 to 6 days apart, we used two measures of shelter fidelity 1) the percent of occasions a tagged lobster returned to the shelter it used the previous day (Ratchford 1999), and 2) the percent of occasions a tagged lobster returned to the shelter it occupied on the previous census date throughout the study periods. We compared site and shelter fidelity of lobsters between sites using contingency table analyses (Zar 1999).

Movements of Lobsters

Although lobsters may forage following complex, circuitous routes (Jernakoff 1987), we considered as the minimum daily distance moved by a tagged lobster the distance measured on a straight-line between casitas occupied by that lobster on consecutive days (Acosta & Butler 1997, Ratchford 1999). We used contingency table analyses (Zar 1999) to compare between sites the median daily distance moved by those lobsters that shifted casitas on the first post-tagging day and during the first post-tagging week (Jernakoff et al. 1987). We also measured the angle between casitas occupied by tagged lobsters on consecutive dates with an underwater compass, and analyzed the circular distribution of the angles with a Rayleigh test (Zar 1999) to determine whether lobsters showed directional or random movements. To assess the movements of lobsters over periods longer than those encompassed by our study, fishermen were requested to report the capture of tagged lobsters and their location of capture after the opening of the fishing season on July the first of each year.

RESULTS

Size Distribution of Lobsters

The number of lobsters extracted from four randomly chosen casitas in site 1 was 161, over a size range of 22.3–99.5 mm CL (mean ± SD: 58.7 ± 17.5 mm CL). In site 2, 122 lobsters were extracted from eight casitas. These lobsters were 34.8–96.9 mm CL (mean ± SD: 66.9 ± 10.9 mm CL) (Fig. 2). Mean size of lobsters was significantly different between sites (Student's *t*-test with log-transformed data to homogenize variances, t = 5.024,

df = 272, P < 0.0001). The difference was the result of the greater occurrence of small, postalgal juveniles (i.e., juveniles <45 mm CL) in site 1 (Fig. 2). Postalgal juveniles made up 31.7% of lobsters sampled in site 1 but only 4.5% of lobsters sampled in site 2. When postalgal juveniles were excluded from the comparison, the mean size of subadults was similar in both sites (site 1: 68.15 ± 12.4 mm CL; site 2: 68.25 ± 9.3 mm CL; t = 0.008, df = 215, P > 0.50). Sex ratio was around 1:1 in both sites.

Lobster Density and Patterns of Aggregation in Casitas

We tagged and returned to their original casitas 136 subadults and young adults (45.1–97.5 mm CL) in site 1, and 117 (45.1–96.9 mm CL) in site 2. Of these, 67 (49.3%) were resighted at least once in site 1 and 71 (60.7%) in site 2. Table 1 shows the statistics derived from the Fisher-Ford model for each site. As expected, population size, losses and immigrations were higher, but more variable, in site 1 than in site 2. Survival rate (ϕ) was also higher in site 1 (ϕ = 0.865) than in site 2 (ϕ = 0.745). Based on the estimates of population size, mean ± SD lobster density was estimated as 47.7 ± 9.7 lobsters ha^{-1} in site 1 and 25.8 ± 3.8 lobsters ha^{-1} in site 2 (Table 1). These mean densities were significantly different (t = 4.673, df = 6, P = 0.0034).

The daily number of lobsters beneath the 22 casitas in site 1 ranged from 295 to 467, yielding a density of 11.8–18.7 lobsters in casitas ha^{-1} (mean ± SD: 16.6 ± 2.7). In site 2, the daily number of lobsters in the 25 casitas fluctuated between 111 and 174, yielding a density of 9.3–14.5 lobsters in casitas ha^{-1} (mean ± SD: 13.1 ± 1.6), significantly different from that of site 1 (t = 3.653, df = 19, P = 0.0017). When considering only those dates included in the Fisher-Ford model, the mean number of lobsters beneath casitas accounted for 34.4% and 52.1%, respectively, of the mean number of lobsters estimated throughout sites 1 and 2.

The distribution of lobsters in casitas departed significantly from a random distribution in both sites (site 1: χ^2 = 156.865; P < 0.001; site 2: χ^2 = 40.493; P < 0.001). However, lobsters tended to be more aggregated in site 1 than in site 2 (Fig. 3). In site 1, 52% casitas harbored over 20 lobsters and the maximum number of lobsters sheltering beneath a casita was 60, whereas in site 2 these figures were, respectively, 3% and 40. In both sites, some casitas harbored no lobsters (Fig. 3), but casitas with no lobsters on a given date had lobsters on the following date and vice versa.

Site and Shelter Fidelity Among Lobsters

Table 2 summarizes the results on site and shelter fidelity of subadult lobsters in both sites. Some lobsters that were not resighted on the first few post-tagging days were seen again later, whereas others were never seen again. Site fidelity was higher, but not significantly different, in site 2 than in site 1. Mean shelter fidelity A (percent of occasions a tagged lobster returned to the same casita it used the day before) did not differ significantly between sites, whereas the difference in mean shelter fidelity B (percent of occasions a tagged lobster returned to the casita it used on the previous census date throughout the study period) was marginally significant. The P values may indicate that the power of the tests was low, but the overall results suggest that lobsters in site 2 exhibited slightly higher site and shelter fidelity than lobsters in site 1.

Movements of Lobsters

On the first post-tagging day, lobsters that shifted casitas moved 58–416 m overnight in site 1 (median distance = 165 m) and 25–290 m in site 2 (median = 108 m). The medians were not significantly different (χ^2 = 2.110; df = 1; P = 0.220). During the first post-tagging week the movements remained similar, both within (median of site 1: 133 m; of site 2: 110 m) and between sites (χ^2 = 1.100; df = 1, P = 0.431). Therefore, lobsters from both sites exhibited similar movement ranges during the first post-tagging week. Lobsters that used more than two casitas moved 155–400 m among casitas over the study periods. The movements of lobsters within site 1 (mean angle ± angular deviation: 154.6° ±

TABLE 1.

Panulirus argus: statistics of the Fisher-Ford model for spiny lobsters in (a) an inner-bay site (site 1) and (b) an outer-bay site (site 2) in Bahía de la Ascensión, México.

Sampling Date	Spiny Lobsters		Population Estimates			Density (Lobsters ha^{-1})
	Captured	Tagged	Size (N)	Losses	Emigration	
Inner-bay (site 1)						
15 June	295	37	—	—	—	—
16 June	410	46	1196	161	401	47.8
17 June	467	17	1435	194	−294	57.4
18 June	466	0	948	128	—	37.9
Mean ± SD			1196 ± 246			47.7 ± 9.7
Outer-bay (site 2)						
07 May	175	40	—	—	—	—
08 May	185	19	346	88	62	28.8
09 May	131	9	320	82	117	26.7
10 May	146	14	355	91	9	29.6
11 May	160	0	273	70	47	22.8
12 May	169	0	251	64	—	20.9
Mean ± SD			320 ± 45			25.8 ± 3.8

All lobsters were captured from beneath artificial shelters (casitas). Losses are deaths + emigration. Density of lobsters was estimated by dividing the population size over the surface area of each site (25 ha in site 1, 12 ha in site 2).
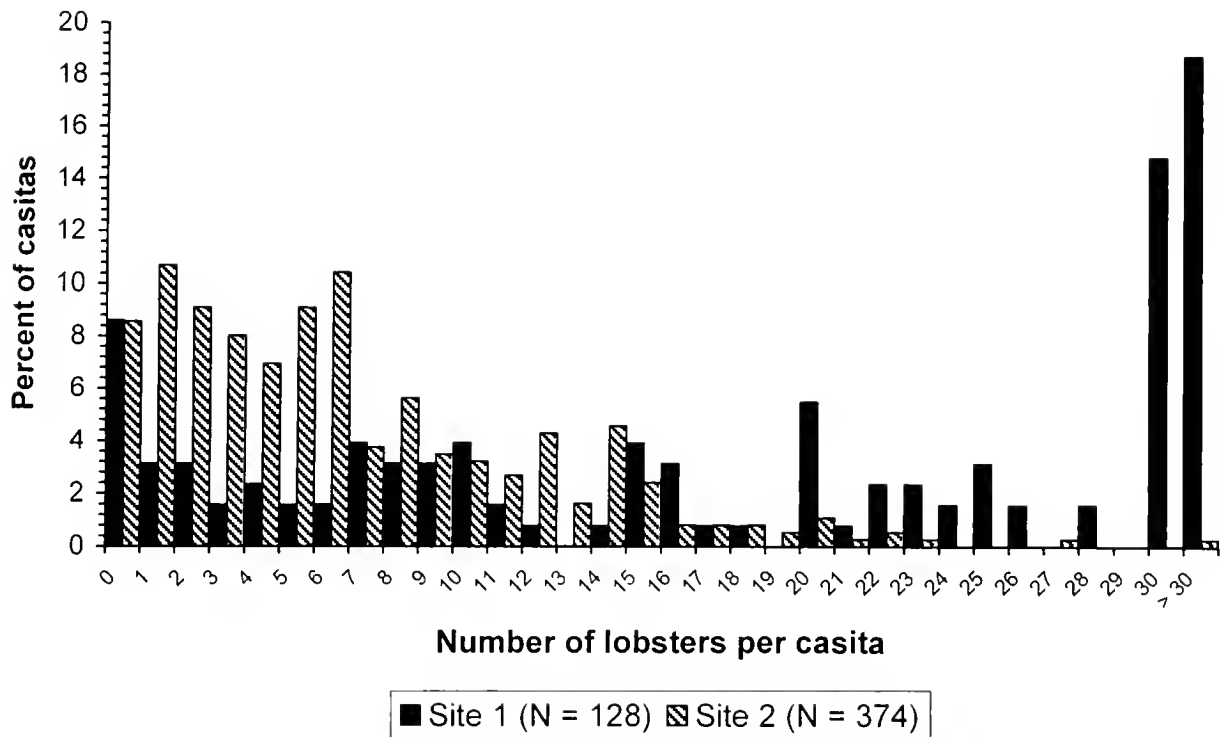
Figure 3. *Panulirus argus*. Distribution of lobsters beneath casitas in an inner-bay site (site 1) and an outer-bay site (site 2) in Bahía de la Ascensión. N is the number of casita surveys conducted throughout the study period in each site.

71.6°) were non-directional (Rayleigh's test: $z = 2.162$, $n = 45$, $P > 0.10$). In contrast, movements of lobsters within site 2 (mean angle ± angular deviation: 82.6° ± 69.1°) were not uniformly distributed around the circle ($z = 3.1$, $n = 42$, $P < 0.05$). These lobsters showed a tendency to move towards the coral reef, which lies at 80° from site 2 (V-test, $u = 2.493$, $n = 42$, $P < 0.01$).

Fishermen recaptured 33 lobsters tagged in site 1 during July 1990, 4–8 wk after being tagged. Of these, 17 remained within site 1, but 16 were recaptured 2,000–14,600 m away from this site. In contrast, fishermen recaptured 20 lobsters tagged in site 2 during July 1991 (8–13 wk after being tagged), of which 19 remained within site 2 and only one was caught outside this site (distance not recorded). Lobsters recaptured by fishermen (67.5–84.2 mm CL) had increased 4.3–20.2 mm in 6–13 wk.

DISCUSSION

As expected, the inner-bay site (site 1) had significantly more lobsters encompassing a wider size range, but with a smaller mean size, than the outer-bay site (site 2). Although we sampled site 2 one year later than site 1, our results are consistent with previous findings. In Bahía de la Ascensión, larger lobsters occur in many bay areas but are more common in the outer-bay, whereas smaller lobsters commonly occur at higher densities in more protected inner-bay areas, rich in settlement and post-settlement habitats (Eggleston et al. 1990; Lozano-Álvarez et al. 1991, 1994). Similar results have been obtained in shallow areas of northern Quintana Roo (Arce et al. 1997, Sosa-Cordero et al. 1998) and Belize (Acosta 1999).

TABLE 2.

Panulirus argus: comparisons of site and shelter fidelity of subadult spiny lobsters tagged in an inner-bay site (site 1) and an outer-bay site (site 2) in Bahía de la Ascensión, México.

Site	Number of Lobsters		Site Fidelity (%)	Shelter Fidelity (%)	
	Tagged	Resighted		A	B
Inner-bay (site 1)	136	67	49.3	18.4	31.6
Outer-bay (site 2)	117	71	60.7	30.3	47.4
χ^2 value			3.31	3.17	3.94
P value			0.069	0.074	0.047

All lobsters were captured from beneath artificial shelters (casitas). Site fidelity is the percent of tagged lobsters resighted at least once within the respective site. Two measures of shelter fidelity were considered: (A) the percent of occasions a tagged lobster returned to the same casita it used the day before, and (B) the percent of occasions a tagged lobster returned to the same casita it used on the previous census date. Census dates were 1 to 6 days apart. Degrees of freedom = 1 in all comparisons.

Density of lobsters beneath casitas was also higher and lobsters were more aggregated in site 1 than in site 2. Lobsters tend to aggregate more beneath large artificial shelters deployed over vegetated habitats, where juvenile density is higher, than over hard bottoms (Lozano-Álvarez et al. 1994, Mintz et al. 1994, Arce et al. 1997, Sosa-Cordero et al. 1998, Briones-Fourzán et al. 2000, Briones-Fourzán & Lozano-Álvarez 2001). This pattern of aggregation may indicate a "guide-effect," which is a consequence of conspecific attraction related to lobster density (Childress & Herrnkind 2001).

Short-term movements (and hence site fidelity) of lobsters could be affected by disturbance caused by capture and tagging (Herrnkind 1980). However, initial capture had only short-term effects, and tagging had no additional effect on the movement of individual *Jasus edwardsii* (MacDiarmid et al. 1991); capture and handling had no short-term effects on movements of individually tagged *P. cygnus* (Jernakoff et al. 1987), and disturbance of lobsters had no apparent effect on the selection of shelter by other lobsters (Ratchford 1999). Disturbance probably had little effect on our tagged lobsters because there were no significant differences in movement ranges and site fidelity between our study sites, and tag-related mortality was unlikely in either site. Therefore, lobsters that were not resighted may have been predated, moved beyond the boundaries of the sites, or occupied unsurveyed natural shelters throughout the sites.

We did not survey the potential natural shelters occurring in each of our sites; this would have been a formidable task given their large surface area. However, it has been shown that benthic vegetation, in addition to providing settlement habitats and feeding areas, may also provide shelter to juvenile *P. argus*. In Bahía de la Ascensión, Lipcius et al. (1998) plotted algal biomass vs. survival of tethered juvenile *P. argus* (30–75 mm CL) and obtained a hyperbolic habitat-survival function. Their results indicate that even a modest increase of algal biomass, which increases the architectural complexity of the habitat, significantly enhances the survival of juvenile *P. argus*. In Belize, greater numbers of juvenile *P. argus* moved into and from habitats surrounded by seagrass than those surrounded by rubble, which suggests that vegetated substrates may function as movement corridors for juvenile lobsters, facilitating their dispersal to areas containing new resources (Acosta 1999). This would explain the greater variations in population estimates of juveniles in our site 1 compared with site 2. Moreover, Acosta and Butler (1997) found that large juveniles of *P. argus* have similar survival when sheltering among mangrove prop roots and in coral crevices. Our inner-bay site, in addition to having more benthic vegetation, was close to thick mangrove forests; therefore, the higher survival rate estimated for lobsters in site 1 may reflect the additional protection provided by these vegetated substrates. Also, the lesser habitat complexity in site 2, where vegetation was scarce, could underlie the slightly higher shelter fidelity exhibited by lobsters in site 2 compared with site 1.

Herrnkind (1980) devised a conceptual model postulating that lobsters in areas of abundant food and shelter will tend to be residential, whereas lobsters in areas of scarce shelter and disperse food supply will tend to be more nomadic owing to intraspecific competition for shelter. But evidences for a relationship between site and shelter fidelity, lobster size, and shelter abundance remain equivocal (Herrnkind et al. 1975, Herrnkind 1980, MacDiarmid et al. 1991, Acosta & Butler 1997, Butler & Herrnkind 1997, Briones-Fourzán & Lozano-Álvarez 2001). Some studies report that smaller lobsters display stronger shelter fidelity than larger lob-

sters, whereas others report that subadults and young adults are more transient and nomadic (which implies a low shelter fidelity) than old adults. However, these evidences have been obtained in areas with natural shelter only. For example, in the case of *P. argus*, average shelter fidelity A of tagged subadult and young adult individuals was estimated at 38% (range: 15–88%) by Ratchford (1999), similar to the 42% reported for old adults by Herrnkind et al. (1975), and Acosta and Butler (1997) found average den residence times for postalgal *P. argus* of 2.0 to 4.38 days over five consecutive days (equivalent to a shelter fidelity A of 40–87%). Compared with these values, the average shelter fidelity A of our subadult *P. argus* (18.4% in site 1; range: 20–60%, and 30.3% in site 2; range: 20–75%) was rather low.

The occurrence of casitas could partially explain these results, as proposed by Lozano-Álvarez (1995), because casitas presumably reduce competition for shelter by allowing cohabitation of large numbers of individuals. However, based on laboratory experiments, Ratchford (1999) suggested that the longer a lobster resides in an area and becomes more familiar with the shelters in that area, the lower its shelter fidelity will appear. This could also explain the overall low shelter fidelity A of our lobsters as well as the marginal difference in shelter fidelity B among lobsters between our sites 1 and 2. The large number of postalgal juveniles cohabiting in casitas with subadults in the inner-bay site 1, rich in settlement and post-settlement habitats, suggests that these subadults had probably remained in that area since settlement. But this inner-bay area may cease to be an appropriate habitat once subadult lobsters reach a critical size. These subadults would then immigrate to other outer-bay habitats (Cruz et al. 1986, Lozano-Álvarez et al. 1991), thus explaining the distant locations where lobsters tagged in site 1 were recaptured by fishermen a few weeks later. In contrast, individuals beneath casitas in site 2 were mostly subadults, which had probably immigrated recently to this site from other, more vegetated areas. The proximity of the coral reef, the habitat preferred by subadults and adults, could also underlie the more directional movements of subadults towards this habitat in site 2.

Some species of spiny lobsters are highly mobile [e.g. *Panulirus cygnus* (Jernakoff 1987, Jernakoff et al. 1987) and *P. argus* (Herrnkind et al. 1975, Ratchford 1999)] and others are more sedentary [e.g. *Jasus edwardsii* (MacDiarmid et al. 1991) and *P. guttatus* (Negrete-Soto et al. 2002, Lozano-Álvarez et al. 2002)]. However, even in highly mobile species, the extent of the daily movement range appears to depend on the occurrence of suitable structured shelter. Previous studies estimating the daily movements of tagged postalgal juveniles and adults of *P. argus* have been conducted in areas with natural shelters only. Herrnkind et al. (1975) used sonic tags to individually track 27 large, adult *P. argus* (average size approx. 110 mm CL) in a coral reef habitat over five consecutive nights. These lobsters typically moved 30–90 m overnight and used three or four dens within 140 m, with a maximum den shift just under 500 m (Herrnkind 1980). In shallow coastal areas, postalgal juveniles (average size approx. 37 mm CL) moved 5.4 to 24.5 m overnight when shifting shelters (Acosta & Butler 1997), whereas lobsters 70.6–134.0 mm CL moved 10–185 m overnight when shifting shelters and up to 270 m among shelters over a period of four weeks (Ratchford 1999). Our subadult *P. argus* (mean size: 68 mm CL) moved 25–416 m overnight when shifting casitas, and 155–400 m among casitas over the study periods. These movements are greater than those reported by Acosta and Butler (1997) for postalgal juveniles and Ratchford

(1999) for subadults and adults in areas with natural shelters only, suggesting that the occurrence of casitas does increase the movement range of subadult *P. argus*.

Areas with few natural, appropriate shelters would favor behavior that allows lobsters to efficiently relocate previously used shelters (Ratchford 1999). Because of their physical properties, casitas allow cohabitation of many individuals over a wide size range and afford—at least in theory—a similar quality of shelter, although the latter may vary somewhat depending on the type of substrate around individual casitas (Meiners-Mandujano 2002). Therefore, areas with numerous casitas would allow lobsters to forage over greater areas by reducing their need to relocate a previously used casita. Moreover, a lobster could be attracted to any nearby casita at the end of its foraging excursion by cues emanating from lobsters already sheltered in that casita (Nevitt et al. 2000, Ratchford & Eggleston 2000, Childress & Herrnkind 2001). This would be reflected in low values of shelter fidelity A and wide short-term movement ranges, as suggested by our results.

These results are, however, inconclusive, because to fully test this hypothesis it would have been necessary to compare shelter fidelity and movement ranges of subadult lobsters in areas of the bay with and without casitas. This was unfeasible because an estimated 20,000 casitas occur throughout the lobster habitats in Bahía de la Ascensión (Briones-Fourzán et al. 2000). However, preliminary results of a controlled field experiment recently conducted in the reef lagoon at Puerto Morelos, Mexico indicate a significant increase in the daily movements of postalgal juvenile *P. argus* after the introduction of casitas scaled to their size (Meiners-Mandujano 2002, Lozano-Álvarez et al., unpublished data).

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TETRAPLOID INDUCTION BY HEAT SHOCKS IN CHINESE SHRIMP, *FENNEROPENAEUS CHINENSIS*

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ABSTRACT Tetraploid induction in the Chinese shrimp *Fenneropenaeus chinensis* was studied. Tetraploid larvae were successfully produced through mitosis I inhibition by heat shock in this species. Proper time window for tetraploid induction was optimized, and the highest induction level was more than 90% as measured by flow cytometry. At spawning temperature of 16°C, the best starting time for heat shocks was 98 to 110 min postfertilization. Tetraploid embryos had less viability compared with diploids. The highest tetraploid level detected at nauplius stage was 38%. Further work is needed to increase the viability of tetraploid larvae.

KEY WORDS: tetraploid, heat shocks, flow cytometry, *Fenneropenaeus chinensis*

INTRODUCTION

Chinese shrimp *Fenneropenaeus chinensis* is one of the most important aquaculture species in China. Because of its meat quality and cold resistance, the Chinese shrimp is one of the best species for shrimp culture. In recent years, the prevalence of virus disease has devastated shrimp culture worldwide. Genetic improvement is being used to enhance growth rate or disease resistance in culture fish and shellfish.

It was reported that triploid shellfish were useful for aquaculture because of their sterility, superior growth and improved meat quality, and increased disease resistance (Allen et al. 1989, Hand et al. 1998, Guo 1999). In fish, triploids were produced to improve growth (Flajshans et al. 1993, Pandian & Koteeswaran 1998), control reproduction, or reduce contamination for transgenic species (Devlin & Donaldson 1992, Pandian & Marian 1994). Since triploid induction was rarely 100% effective, the best way to produce triploids is using tetraploids to hybridize with diploids (Arai et al. 1993, Guo et al. 1996). Tetraploid production however, is challenging according to the reports to date because of the low viability of tetraploids. Until now, tetraploid production has been successful only in a few species of fish and shellfish (Guo & Allen 1994, Pandian & Koteeswaran 1998, Yang et al. 2000). Theoretically, tetraploid induction can be achieved by inhibiting mitosis of fertilized eggs. Through this method, production of tetraploids has been reported in a few fish species (Thorgaard et al. 1981), but there is no report on successful tetraploid production through inhibiting mitosis I in shellfish. Tetraploid embryos have been produced in the Pacific oyster by heat shock induced mitosis I inhibition, but the larvae did not survive beyond metamorphosis (Guo et al. 1994). Tetraploids could be produced by inhibiting the first polar body of the eggs from triploids (Guo & Allen 1994, Eudeline et al. 2000, He et al. 2000) or by inhibiting meiosis I of diploid zygotes (Yang et al. 2000, Zhang et al. 2000). Studies on chromosome manipulation for cultured shrimp have progressed slowly. Successful triploid production for shrimp was reported in a few species (Xiang et al. 1998, 2001; Li et al. 1999, 2002, 2003; Norris et al. 2001). Because of difficulties with artificial fertilization in shrimp, there is to date no way to induce triploids on large scale. The only way to induce triploids in shrimp is to treat fertilized eggs from one shrimp at a time. The need for tetraploids seems more

urgent in shrimp than in other species. To our knowledge, there has been only one report about tetraploid induction in shrimp (Xiang et al. 1993). In this study, tetraploid induction was performed and optimal treatment conditions for tetraploid induction were determined in Chinese shrimp.

MATERIALS AND METHODS

Source of Gravid Shrimp

Gravid shrimp were collected from the wild from the Yellow Sea or from an over-wintered population from a hatchery nearby Qingdao. The gravid shrimp were brought into the aquarium of our institute and put into 4 m³ tanks. Twenty individuals were put in each tank, where the seawater temperature was set at 12–13°C. At that time, ovaries of the gravid shrimp were at stage IV. The shrimp were kept at 12–13°C for 3–4 days to acclimate them to the conditions of our laboratory. Then water temperature was raised gradually (0.5°C/day) to the proper spawning temperature (16–18°C). Meanwhile, the normal light cycle for these tanks was reversed according to the method that was developed in our laboratory to make shrimp spawn at daytime (Xiang et al. 1993). Gravid shrimp were fed with polychates and fresh clam meat.

Collection of Fertilized Eggs

Shrimp with good ovary development that would spawn in 1 or 2 days were put into 300-L tanks with controlled temperature and light cycle. According to their behavioral changes, shrimp that would spawn immediately were taken out and put into 20-L containers. Usually the spawning process for gravid shrimp lasts about 10 min. After the spawning, they were placed in larger tanks to be cultured until re-maturation again. Spawned eggs were collected and concentrated for tetraploid treatment.

Treatment of Fertilized Eggs

Experiments were designed to compare tetraploid induction level under treatments of different starting time, different intensity, and different treatment duration. Heat-shocks were used to inhibit the first mitosis of fertilized eggs. Proper window for starting treatments was determined according to the tetraploid induction efficiency from a 3–4 min heat shock of 33–34°C applied at different starting times from 90–114 min. Tetraploid induction efficiency was determined at embryo stage using flow cytometry then induction efficiency under different treatment intensity, including heat-shock temperature and duration time, was compared. For each treatment, about 700–800 fertilized eggs taken out from the con-
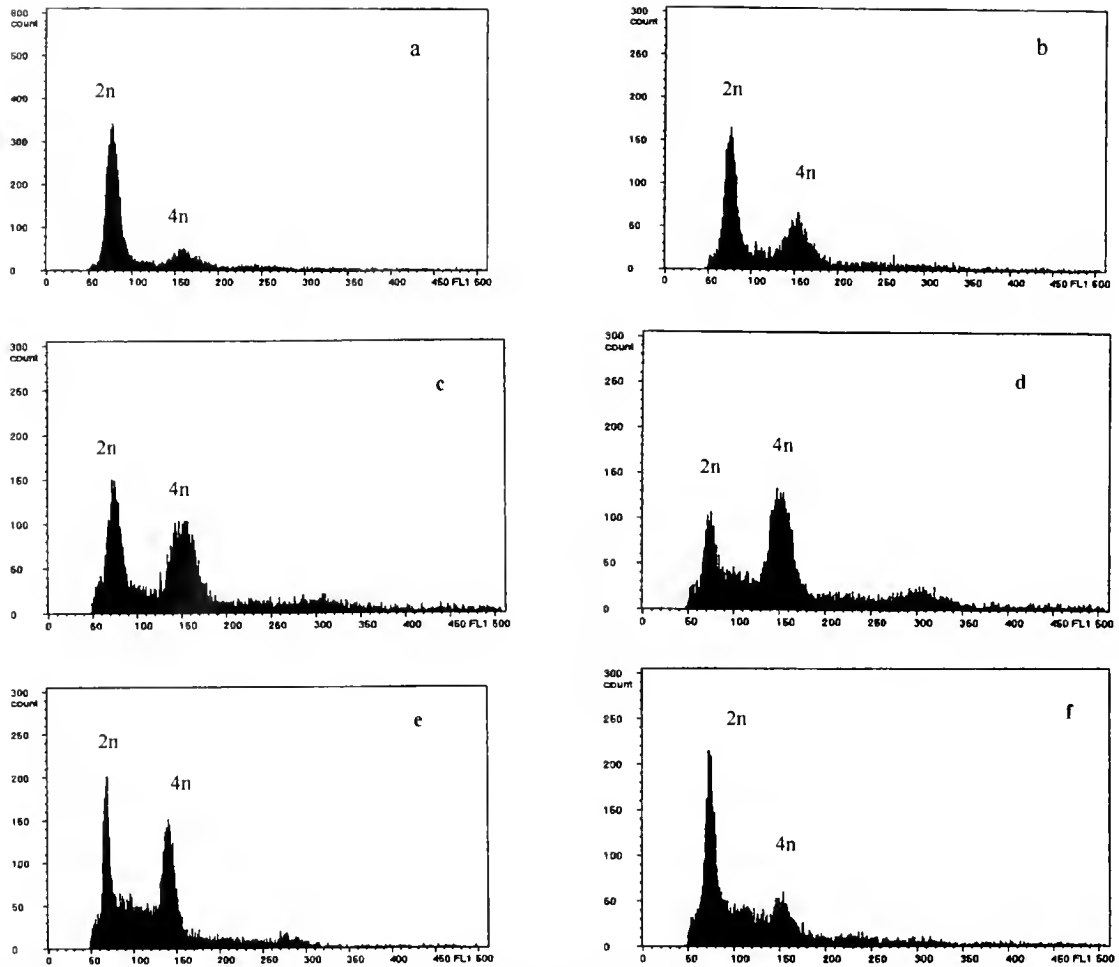
Figure 1. Flow cytometry analysis of embryos after heat shock treatment starting at different times after fertilization: (a) 90 min, (b) 96 min, (c) 102 min, (d) 106 min, (e) 108 min, and (f) 114 min.

centrated eggs were put into 1000 ml beakers containing about 600 mL hot seawater with the desired temperature. The beakers that were used to treat fertilized eggs were put into a water bath with temperature that stabilizes the treatment condition. When the treatment was almost finished, the beakers with fertilized eggs were removed from the water bath and most of the hot water in the beakers was removed. Seawater with natural temperature (15–

18°C) was added to the beakers to change the water temperature to 18–20°C. The treated eggs were then incubated at ~20°C. For each treatment, fertilized eggs without any treatment were used as the control group. Usually for each group of experiments, all fertilized eggs for the treatment were from the same gravid shrimp to exclude variation in egg quality. After ~24 h of incubation, 70–80 embryos were taken out for ploidy detection using flow cytometry,
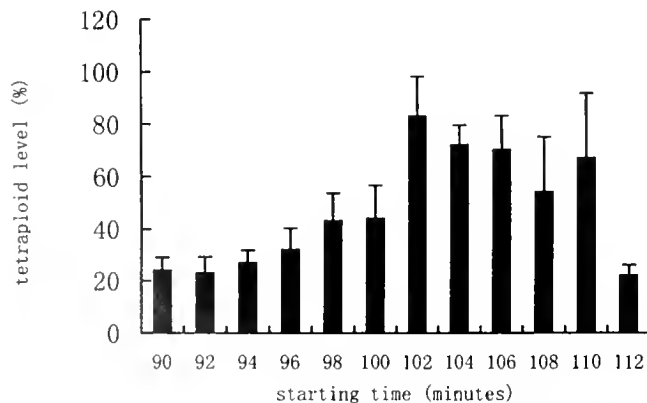
Figure 2. Tetraploid level detected at the embryo stage when a 3–4 min heat shock of 33–34°C was applied at different starting times.
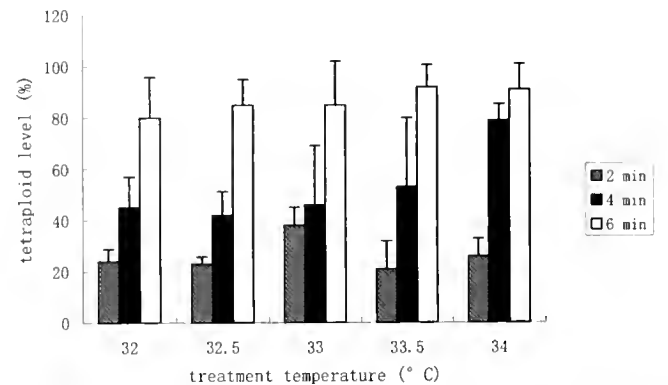

Figure 3. Comparison of tetraploid level detected at embryo stage under different heat-shock temperatures and different duration of treatment.
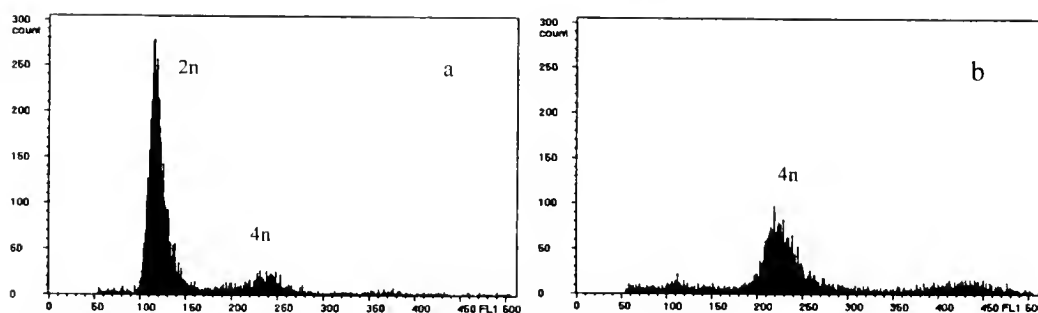
Figure 4. Flow cytometry analysis of embryos from tetraploid induction treatment under optimized conditions in Chinese shrimp *Fenneropenaeus chinensis*. (a) control and (b) tetraploid embryos.

and the remaining embryos were kept until they hatched into nauplii, about 20–30 of which were used for final ploidy analysis.

Ploidy Detection

Tetraploidy were detected using flow cytometry. For ploidy detection at embryo stage, 70–80 embryos were put together and triturated in 0.2 mL preparation buffer consisting of 2% citrate acid and 0.5% Tween 20 in distilled water. For nauplius stage, 20–30 larvae from each treatment were triturated in preparation buffer. Tissue debris was removed using nylon screen, and 0.7 mL 2 mg/L DAPI solution was added to stain the nuclei. Embryos or larvae from the control groups were treated in the same way and used as diploid controls. Percentages of triploids and tetraploids in the sample were determined by comparing areas of different peaks.

Hatching Success

For every treatment, percentages of nauplii hatched in the treated and control groups were recorded to determine the relationship between tetraploid levels and hatching levels.

Statistical Analysis

To compare the effects of different factors such as starting time, treatment intensity, and duration on tetraploid induction, F-test was used to analyze the effect of different factors on the efficiency of tetraploid induction.

RESULTS

Effect of Starting Time on Tetraploid Inducing Rate

To determine the optimal window for tetraploid induction, different starting times for treatment were tested. Flow cytometry analysis of embryos from treatment starting at different times is shown in Figure 1. Usually two peaks, diploid (2n) and tetraploid (4n or G2 phase of 2n) peaks, were present in each sample. With changes in starting time, the relative 4n area changed greatly. After numerical repeats for each treatment, the optimal starting time for tetraploid induction was determined based on data in Figure 2. The proper starting time for tetraploid induction was 98–110 min under a spawning temperature of 16°C. When starting time was at 112 min, tetraploid level dropped sharply. The window for tetraploid induction was only about 10 min. Out of this range, the treatment could not effectively inhibit mitosis 1.

Effect of Different Treatment Duration and Different Temperature on Tetraploid Rate

Using the optimized time window for the treatments, different treatment intensities (32–34°C) for different treatment duration

(2–6 min) were tested and compared (Fig. 3). Tetraploid frequency detected at embryo stage rose apparently with extension of treatment duration from 2 to 6 min at 32–33.5°C. There was no significant difference in tetraploid levels between different treatment temperatures for the same treatment duration in the range of 32–33.5°C. The data indicated that 32–33.5°C temperature could effectively inhibit the first mitosis of fertilized eggs when treatment duration was 4–6 min. Tetraploid level detected under 34°C was much higher than that at 32–33.5°C when the treatment lasted for 4 min. There was no difference in tetraploid level between 4 and 6 min treatment at 34°C. It showed that at certain range of treatment temperature, proper duration of the treatment was a key factor for tetraploid induction.

Evaluation of Effects of Different Factors on Tetraploid Level

Starting time, treatment duration and treatment temperature are major factors affecting tetraploid induction. Totally, 5 levels of starting time (79, 81, 85, 91, 96, 100 min), 3 levels of treatment duration (2, 4, 6 min), and 5 levels of treatment temperature (32, 32.5, 33, 33.5, 34°C) were tested. F-test showed that tetraploid level detected at different starting time for the treatment among different treatments and treatment duration (2, 4, 6 min) had significantly different effects among groups; and that different treatment temperature had no significant effects. At low treatment temperatures, longer treatment duration increased tetraploid induction
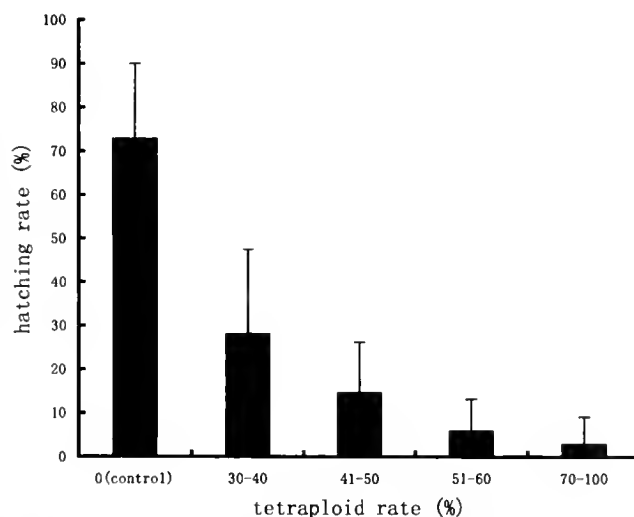

Figure 5. Relationship between tetraploid level and hatching success in Chinese shrimp *Fenneropenaeus chinensis*.

efficiency. When the spawning temperature of gravid shrimp was 16°C, the proper starting time for treatment should be 102–110 min after fertilization. If the spawning temperature was lower or higher, then the starting time for treatment should be later or earlier. After the induction condition was optimized, tetraploid level detected at embryo stage reached almost 100% (Fig. 4).

Relationship Between Tetraploid Rate at Embryo Stages and Hatching Rate

Higher treatment temperatures led to more tetraploids, they also led to reduced survival of the treated embryos. There was strong negative correlation between tetraploid induction efficiency and larval survival (Fig. 5).

Production of Tetraploid Larvae

Although tetraploid levels detected at embryo stages were high, tetraploid embryos experienced problems in hatching. The hatching success rate of tetraploids was low. In our experiments, the highest tetraploidy rate detected at nauplius stage was about 38% (Fig. 6a) while tetraploid levels detected at embryo stage was 55% (Fig. 6b). This result was obtained under spawning temperature of 15.7°C, with a 3-min heat shock at 34°C starting 110 min after fertilization. During the hatching process of tetraploid embryos, some live embryos in membrane were observed, but their morphology was abnormal. And when these abnormal embryos were selected for detection of ploidy, it was found that most of these embryos were tetraploids (data not shown).

DISCUSSION

Available data showed that the Chinese shrimp *Fennerope-naeus chinensis*, tetraploids could be produced by inhibiting the first mitosis. Reported methods for producing tetraploids in aquatic animals include inhibiting first mitosis (Thorgaard et al. 1981, Varadi et al. 1999), inhibiting the first meiosis of diploid fertilized eggs (Yang et al. 2000, Zhang et al. 2000), or inhibiting polar body I in eggs from triploids (Guo & Allen, 1994, He et al. 2000). In shellfish, there is no successful production of viable tetraploids by inhibiting mitosis I (Guo et al. 1994), although tetraploid embryos can be produced. In our experiments on shrimp, no tetraploids were produced through inhibiting meiosis. This study showed that tetraploid embryos could be produced at high rate in shrimp. How to make more embryos hatch into nauplii, however, remains a problem that must be solved. The challenge is to improve the treatment conditions so that they lead to high-level production of tetraploids without causing serious damage to treated embryos. It is also possible that tetraploid embryos have limited viability or

ability to hatch, and they can be obtained by improving hatching conditions. During tetraploid induction, the exact time of thermal or pressure shock applied to inhibit mitosis I is important. Inhibiting different processes will lead to different viability according to an analysis of tetraploid induction in fish (Pandian & Koteeswaran 1998).

This study showed that heat-shock is effective in inhibiting mitosis I in the Chinese shrimp *Fenneropenaeus chinensis*. This shrimp is a temperate species, so it is more sensitive to heat than tropical species. Heat shock has an advantage over chemical treatments, in that there is no pollution to the environment. For subtropical or tropical species, cold shocks may be more helpful. To our knowledge, *Fenneropenaeus chinensis* is the only shrimp species where tetraploid induction has been reported. Until now, there is only one report that tetraploid was produced in this species (Xiang et al. 1993). In earlier reports in this study, tetraploids were induced by cytochalasin B (CB) and ploidy was detected through chromosome counting. In this work, heat shock was used and optimal treatment was identified. The use of flow cytometry as a method for detecting tetraploidy was a key factor in our successful evaluation of heat shock treatment. Compared with chromosome counting, flow cytometry analysis allows rapid and accurate ploidy determination of many experiment groups. The application of flow cytometry techniques has greatly advanced polyploidy research in shrimp (Zhou et al. 1999).

Although heat-shocks can effectively inhibit mitosis I in shrimp, optimal conditions for the hatching of fertilized eggs need further investigation. Extending treatment duration might increase the tetraploid rate, but reduce hatching success. The proper strategy to induce triploids should be to achieve certain high levels of tetraploid and hatching rates. Hatching rates varied from brooder to brooder when the tetraploid rate was the same because of different egg quality. There is the common tendency however, that hatching success decreases when tetraploid level increases. In *Fenneropenaeus chinensis*, 40–60% tetraploid rate is preferred to obtain viable larvae. Although no viable tetraploid post-larvae were obtained, this study showed that high percentages of tetraploids could be produced by heat shock. The optimization of heat shock treatments is an important first step in successful tetraploid induction. Further work is needed to improve the survival of tetraploids so that viable tetraploid shrimp can be eventually obtained.
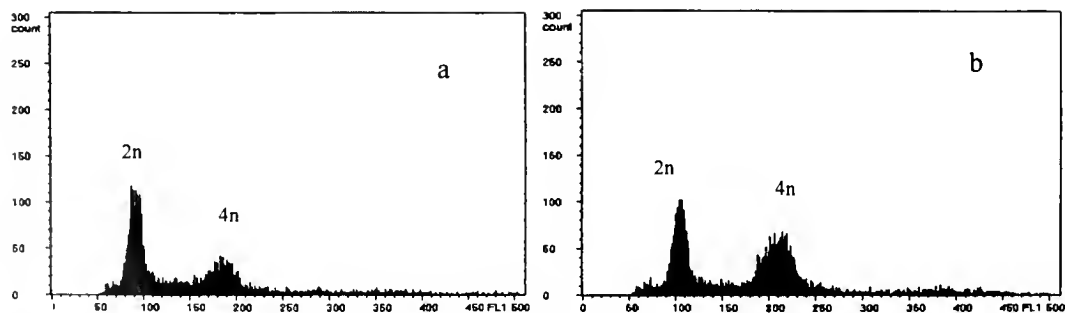
ACKNOWLEDGMENTS

Figure 6. Flow cytometry analysis at nauplius (a) and embryo (b) stages of one group treated for tetraploid induction in Chinese shrimp *Fenneropenaeus chinensis*.

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SELECTION AND USE OF DIFFERENT DIETS IN A STUDY ON CHINESE SHRIMP, *FENNEROPENAEUS CHINENSIS*

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ABSTRACT A 30-day feeding experiment was conducted to investigate the dietary selectivity in Chinese shrimp, Fenneropenaeus chinensis. Six groups of shrimp with initial body weight of 1.530 ± 0.047 g (mean $\pm$ SD, $n = 6$) were used, in which the first five groups were fed to satiation with single diets of FF, flesh of fish (Sardinella zunasi); SF, flesh of shrimp (Trachypenaeus curvirostris); CF, foot of clam (Ruditapes varigata); PW, polychaette worm (Neanthes japonica) FD, a commercial formulated diet; and the last group received MD, mixed diet. The feeding trials were conducted simultaneously and shrimp were fed to satiation. The specific growth rate (SGR), food intake (FI), food conversion efficiency (FCE), and apparent digestive ratio (ADR) were determined. The results showed that specific growth rates of dry weight, protein, and energy (SGR_d, SGR_p, and SGR_e) were highest in the MD fed group, food conversion efficiencies (FCE_d, FCE_p, and FCR_e) were highest at PW fed group. Food ingestion in terms of dry weight, protein, and energy were significantly higher in CF and MD fed groups than others. The highest ADR was observed in CF fed group. In mixed diet feeding group, percentages of the five ingested diets to the total ingested amount based on dry material, protein, and energy were: FF, 13.07%; SF, 9.60%; CF, 46.45%; PW, 30.88%; and FD, 0%: FF, 15.56%; SF, 11.44%; CF, 45.09%; PW, 27.91%; and FD, 0%: FF, 13.66%; SF, 10.48%; CF, 44.00%; PW, 31.86%; and FD, 0%; respectively. This indicates that Chinese shrimp possess the ability to discriminate different diets. The optimal foraging strategy of Chinese shrimp in this experiment was to gain energy as much as possible to meet energy needs of variable physiologic activities under the premise of maximizing growth. Additionally, the protein sparing effect of dietary E/P ratio and lipid content was also observed in this experiment.

KEY WORDS: dietary selectivity, Chinese shrimp, *Fenneropenaeus chinensis*

INTRODUCTION

Because of their economic significance to fisheries and important function in aquatic ecosystems, several studies were conducted to investigate feeding habits of shrimp and crab. The most commonly used direct method was to analyze the stomach content or foregut of the animal, from the wild or under culture condition, and evaluate its feeding habits from the composition (Chong & Sasekumar 1981, Phil & Rosenberg 1984, Cockcroft & Mclachlan 1986, Prakash & Agarwal 1989, Nunes et al. 1997, Roy & Singh 1997, Kulkarni et al. 1999, Minami 2000, Schwamborn & Griales 2000). The activity of different digestive enzymes in the animals was also used to judge their feeding habits (Biesiot & Capuzzo, 1990). In recent years, stable isotope analysis method was also applied in analyzing of the feeding habits of the animals (Newell et al. 1995, Nunes et al. 1997, Schwamborn & Griales 2000). Ivlev (1961) proposed a selection index to describe the dietary selectivity of fish. Pinn et al. (1998) used Strauss' Linear Selection Index to describe in their study the dietary selectivity of two mud-shrimp. Nevertheless, the selectivity of a diet item is affected by such factors as energy content, difficulty of foraging and handling, and so on (Sunaga 1971, Griffiths 1975, Manghagen & Wiederholm 1982, Mikheev 1984, Buskey et al. 1991, Alam et al. 1996, Mehner et al. 1998). The theory of optimal foraging is based on the evolutionary premise that individuals within a population that forage most efficiently and maximize their net rate of energy intake will possess greater fitness and contribute more genes to future generations (Calow & Townsend 1981). It has been found that the dietary selectivity of animals is partly or completely subjected to the law (Kislalioglu & Gibson 1976, Elner & Hughes 1978).

The dietary condition of shrimp is variable in wild and extensive or semi-intensive cultural waters, and the abundance and composition of diet vary greatly in different waters and time periods (Marte 1980, Luna–Marte 1982). In most cases, abundance of its preferred diet is likely to decrease to a low level because of the natural fluctuation or high feeding pressure. Hence, the shrimp cannot select the diet species in accordance with its actual preference. It is probable that shrimp might ingest the diet species that is not preferred, to release the pressure of starvation or innutrition and satisfy its growth or development. Therefore the methods mentioned previously, to analyze its feeding habit, are quite difficult to reflect or define its dietary selection or preference. Quantitative study of the preference of some diet items of animals can be really conducted in only controlled conditions when different diet items (include items differing in nutritional composition, origin, size, and so on) are provided.

In the wild environment, crustaceans, polychaette worms, and juvenile bivalves are major diet items of Chinese shrimp (*Fenneropenaeus chinensis*) (Wang, 1997). In this study Chinese shrimp, widely cultured and distributes in China, were used and five diet items were provided equally in excessive amounts to study the feeding preference of the shrimp and the strategy of its diet selectivity.

MATERIALS AND METHODS

Rearing Conditions

Chinese shrimp were kept in glass aquaria ($45 \times 30 \times 30$ cm³, water volume of 35 dm³), and each rearing unit was stocked with four shrimp. The room temperature was controlled by an air conditioner, and water temperature was $25 \pm 0.5°C$. Aeration was provided continuously and 0.50–0.67 of water was exchanged every other day. Seawater used in the experiment was filtered by composite sand filter. During the experiment, dissolved oxygen of water was maintained above 5.5 mg/L, pH was about 8.0, the water salinity was between 30–33‰, photoperiod of 14 h of light: 10 h of darkness was used.

Diets Preparation

The five diets used in the experiment were: fish flesh (FF)—the flesh of sardine (*Sardinella zunasi*) without head, scales, fins, bowels, and bones; shrimp flesh (SF)—small shrimp (*Trachypenaeus curvirostris*) without head and shell; clam foot (CF)—from (*Ruditapes varigata*); PW, polychaete worm—*Neanthes japonica*; FD formulated diet—a commercial sold shrimp diet (Sea-Horse Brand, Fujian Mawei Unite Feed Ltd. China) comprised of bean powder, fish powder, shrimp powder, compound vitamins, and compound minerals; MD, mixed diet—equal combination of the five diets. Shrimp were fed diets to satiation. Each diet item was cut into almost the same size as the formulated diet (about 4 mm in length and diameter of 2 mm) before feeding. Biochemical composition of the diets is listed in Table 1.

Source and Acclimation of Shrimp

The experiment was carried out at the Mariculture Research Laboratory, Ocean University of Qingdao, People's Republic of China. The shrimp used in the experiment were collected from the Tianheng Shrimp Farm, Qingdao. Prior to the experiment, the shrimp were transferred into aquaria and underwent a 6-day acclimatization period during which they were fed with formulated diet (FD) at satiation level twice a day (at about 6:00 and 18:00).

Experiment Design

After 24 h starvation, 96 shrimp with an initial wet weight of 1.530 ± 0.047 g (mean ± SD) were selected from acclimated animals and placed in 24 aquaria to form 6 experimental groups fed with different diets of FF, SF, CF, PW, FD, and MD. A complete randomized block design was used to arrange the 24 aquaria of 6 groups.

Sample Collection and Analysis

Three groups (eight shrimp each) were sampled from the acclimated shrimp simultaneously while experimental shrimp were selected to determine the initial body composition of the experimental shrimp. After the 30-d experiment, the shrimp of all groups were starved for 24 h and then sampled. The shrimp from the individual aquaria were pooled as a sample and there were 24 samples of final shrimp.

During the course of the experiment the daily food supply was recorded and uneaten food was collected 3 h after feeding. Feces were collected promptly. Shrimp and food were weighed to the nearest 0.001 g using an electronic balance after carefully blotted with paper towel to remove excess moisture.

After the weight was obtained all samples of shrimp, feces, and food were dried in an oven at 70°C to constant weight, homogenized with a glass mortar, and stored at −20°C. Before chemical compositions were analyzed, the samples were re-dried at 70°C to constant weight.

The N content was measured using the Micro-Kjeldahl methods and the crud protein content was calculated by multiplying Kjeldahl N content by 6.25(AOAC, 1984). Crude lipid was determined by the Soxhlet method (AOAC, 1984), ash was determined by combusting dry samples in a muffle furnace at 550°C for 12 h (AOAC, 1984), and the gross energy content of dry samples was determined by PARR1281 calorimeter (PARR Instrument Company, USA). An analysis of each sample was conducted in triplicate (three sub-samples for each sample).

Calculation of Data

Specific growth rate (SGR), food ingestion (FI), apparent digestive ratio (ADR), food conversion efficiency (FCE), and Ivlev's index of dietary selectivity (I_i) were calculated as follows:

$$SGR_W(\%/day) = 100(\ln W_t - \ln W_0)/T \text{ (Ricker 1979)}$$

$$FI_W(\% \text{ body weight/day}) = 100C/[T (W_t + W_0)/2] \text{ (Wu et al. 2000)}$$

$$ADR (\%) = 100(C-F)/C \text{ (Smith 1971)}$$

$$FCE_W(\%) = 100(W_t - W_0)/C \text{ (Matty \& Smith 1978)}$$

Where W_t and W_0 were the finial and initial wet weight of the shrimp, T was the duration of growth period in days, F was the dry weight of feces, and C was the dry weight of consumed food.

SGR, FI, ADR, and FCE in terms of dry matter (SGR_d, FI_d, ADR_d, and FCE_d), protein (SGR_P, FI_P, ADR_P, and FCE_P), and energy content (SGR_e, FI_e, ADR_e, and FCE_e) were calculated similarly.

$$I_i = (r_i - p_i)/(r_i + p_i) \text{ (Ivlev 1961)}$$

Where r_i was the portion of one diet in the total ingested diet, and

TABLE 1.

Biochemical composition and energy content of experimental diets (Mean ± SE).[a]

Composition	Diets					
	FF	SF	CF	PW	FD	MD[b]
Moisture (%)	77.23 ± 0.38	80.33 ± 1.27	79.35 ± 3.15	74.18 ± 0.80	7.70 ± 0.15	76.57 ± 0.10
Protein (%)	83.98 ± 1.12	84.13 ± 0.65	68.49 ± 0.59	63.73 ± 0.44	42.57 ± 0.50	71.14 ± 0.18
Lipid (%)	5.18 ± 0.01	5.00 ± 0.01	5.96 ± 0.01	16.32 ± 0.03	9.93 ± 0.02	8.96 ± 0.02
Ash (%)	6.41 ± 0.02	3.2 ± 0.01	5.38 ± 0.02	6.89 ± 0.02	10.75 ± 0.03	5.77 ± 0.02
Energy	22.15 ± 0.24	22.95 ± 0.04	19.89 ± 0.05	21.66 ± 0.09	19.23 ± 0.09	21.02 ± 0.03
E/P	26.38 ± 0.14	27.28 ± 0.17	29.17 ± 0.20	33.99 ± 0.17	45.20 ± 0.70	29.54 ± 0.11
L/P	0.061 ± 0.001	0.059 ± 0.001	0.087 ± 0.001	0.256 ± 0.002	0.233 ± 0.003	0.126 ± 0.001

[a] Moisture is percentage content of wet sample, moisture = 100 × (WW − DS)/WW, WW: wet weight, DW: dry weight; Protein, Lipid and Ash are percentage content of dry sample; Unit for energy content is KJ.g⁻¹ in dry sample; E/P: energy/protein ratio, unit for E/P is KJ.g⁻¹; L/P: Lipid/protein ratio, unit for E/P is g.g⁻¹.

[b] Composition of mixed diet was calculated after the experiment basing on the ingested dry weight of the first five diets, it is a weighted value according to the portions of every diet in the total ingested weight in mixed diet fed group.

p_t was the portion of one diet in the total provided diet r_t and p_t were calculated in terms of dry matter.

Statistical Analysis

Statistics were performed using SPSS10.0 statistical software with possible differences among diet treatment being tested by one-way ANOVA. Tukey's-b multiple range tests was used to test differences between treatment groups. Differences were considered significant at a probability level of 0.05.

RESULTS

Food Consumption and Feces

Table 2 lists the food consumption and feces for the six diet treatments. Shrimp fed with CF and MD consumed significantly more food than the others did. The largest amount of feces in terms of dry matter emerged in MD but it was not significantly larger than FD. However, the largest amount of feces was observed in FD in terms of protein and energy.

Growth

At the end of the experiment no significant difference existed among CF-, PW-, and MD-fed shrimp in terms of WW, DW, P, and E, and all were significantly higher (df = 5, $P < 0.05$) than the other three groups (Table 3). FF and SF were the lowest of the six groups in all terms of WW, DW, P, and E, and no significant difference existed between them (Table 3).

No significant difference was observed among CF, PW and MD in SGR_w, SGR_d, SGR_p, and SGR_e respectively, and all were significantly higher than the other three groups. Except for the high SGR_w (2.76 ± 0.06) observed in PW, the highest SGR_d (2.99 ± 0.07), SGR_p (2.91 ± 0.07), and SGR_e (3.15 ± 0.07) all appeared in MD (Fig. 1). FD was significantly higher than SF and FF in SGR_d, SGR_p, and SGR_e. Every parameter of FF and SF was lower than other groups (Fig. 1).

Food Conversion Efficiencies

Figure 2 illustrates that the FCE in terms of DW, P, and E. PW was significantly higher than other groups in FCE_d, FCE_p, and FCE_e, and it had the highest values of 22.86 ± 1.63, 22.87 ± 1.65, and 21.39 ± 1.49, respectively. FD was significantly higher than other groups except PW in FCE_p, and it was not significantly different from CF and MD though it was significantly higher than FM and SF in FCE_d and FCE_e. FF and SM had the lowest FCE

(only about 20–25% of PW). MD had significantly lower FCE than PW when four diets were ingested in different portions.

Food Ingestion of Six Diet Treatments

FI_w, FI_d, FI_p, and FI_e in CF and MD fed groups were 5.58 ± 0.24, 22.89 ± 0.84, 23.62 ± 0.87, 23.59 ± 0.86, and 5.94 ± 0.14, 23.28 ± 0.45, 24.97 ± 0.50, 24.98 ± 0.48, respectively (Fig. 3). CF and MD were significantly higher than other groups in FI for all the four terms, and no significant difference between CF and MD was observed (Fig. 3). FI_p in FD was significantly lower than the others because of its lowest protein content. FI of FF and SF fed groups were the lowest in all measures except protein (Fig. 3).

Percent Composition of Ingested Diets and Indexes of Selectivity of Five Provided Diets in MD

When five diets were provided simultaneously and in excess, Chinese shrimp ingested four diets of FF, SF, CF, and PW, and no FD was ingested (Fig. 4). Percentages of dry weight and energy consumed by shrimp of FF and SF fed groups were not significantly different from each other, but the percentage of protein consumed in SF was significantly lower than FF. Among the four ingested diets, CF had significantly higher percentage of dry weight (46.45 ± 1.63), protein (45.09 ± 1.49) and energy (44.00 ± 1.60) than the other three. Percentages of dry weight, protein, and energy of consumed PW were 30.88 ± 2.06, 27.91 ± 1.93, and 31.86 ± 2.08, respectively, and significantly higher than FF and SF.

Indexes of selectivity of five provided diets (based on dry matter) in MD treatment were FF, −0.210 ± 0.017; SF, −0.352 ± 0.016; CF, 0.397 ± 0.016; PW, 0.210 ± 0.030; and FD, −1 ± 0 respectively. It indicated that Chinese shrimp performed positive selectivity on CF and PW. Negative selectivity on FF and SF was observed, and FD was excluded under experimental conditions.

Apparent Digestive Ratio of Diets

The highest ADR in terms of dry weight, protein, and energy was in CF (92.97 ± 0.35, 98.05 ± 0.10, and 99.10 ± 0.04, respectively). ADR_d, ADR_p, and ADR_e of FD were 77.97 ± 1.92, 86.15 ± 2.06, and 91.10 ± 1.55, respectively, which are significantly lower than other groups (Fig. 5).

DISCUSSION

Feeding behaviors of shrimp and crab have been studied by methods of analyzing stomach contents or foregut, activity of digestive enzymes, and by stable isotope technique (Chong & Sase-

TABLE 2.

The dry weight (g, DW), energy (KJ, E), and protein (g, P) content of the food consumed and feces for the six diet treatments.

Parameters (mean ± SE)		Treatments					
		FF	SF	CF	PW	FD	MD
Food consumption	DW	1.383 ± 0.038[a]	1.378 ± 0.163[a]	3.897 ± 0.185[c]	2.215 ± 0.074[b]	2.020 ± 0.174[b]	4.334 ± 0.065[d]
	P	1.183 ± 0.033[b]	1.169 ± 0.138[b]	2.681 ± 0.127[c]	1.425 ± 0.048[b]	0.868 ± 0.075[a]	3.084 ± 0.048[d]
	E	30.352 ± 0.838[a]	31.610 ± 3.729[a]	77.509 ± 3.671[c]	47.990 ± 1.610[b]	38.841 ± 3.342[a]	91.014 ± 1.295[d]
Feces	DW	0.123 ± 0.020[a]	0.234 ± 0.018[b]	0.275 ± 0.023[b]	0.307 ± 0.030[b]	0.430 ± 0.031[c]	0.436 ± 0.018[c]
	P	0.066 ± 0.001[c]	0.041 ± 0.001[a]	0.052 ± 0.001[b]	0.074 ± 0.001[d]	0.114 ± 0.003[f]	0.090 ± 0.001[e]
	E	0.473 ± 0.078[a]	0.742 ± 0.057[a]	0.704 ± 0.059[a]	1.350 ± 0.133[b]	3.336 ± 0.243[c]	2.040 ± 0.084[c]

Values with different letters in the same line were significantly different (df = 5, P < 0.05) from each other.

TABLE 3.

Initial and final shrimp wet weight (g, WW), dry weight (g, DW), protein (g, P) content and energy (KJ, E) for the six diet treatments.

Parameters of Initial and Final Shrimp (mean ± SE)		Treatments					
		FF	SF	CF	PW	FD	MD
Initial shrimp	WW	1.536 ± 0.010	1.520 ± 0.040	1.525 ± 0.030	1.497 ± 0.026	1.519 ± 0.021	1.535 ± 0.008
	DW	0.360 ± 0.002	0.356 ± 0.009	0.357 ± 0.007	0.351 ± 0.006	0.356 ± 0.005	0.360 ± 0.002
	P	0.243 ± 0.002	0.240 ± 0.006	0.241 ± 0.005	0.237 ± 0.004	0.240 ± 0.003	0.243 ± 0.001
	E	6.802 ± 0.048	6.722 ± 0.170	6.755 ± 0.134	6.627 ± 0.117	6.726 ± 0.090	6.802 ± 0.036
Final shrimp	WW	2.057 ± 0.108[a]	2.204 ± 0.151[d]	3.133 ± 0.152[b]	3.418 ± 0.139[b]	2.371 ± 0.136[a]	3.335 ± 0.096[b]
	DW	0.423 ± 0.016[a]	0.448 ± 0.040[d]	0.780 ± 0.050[c]	0.855 ± 0.044[c]	0.591 ± 0.045[b]	0.882 ± 0.014[c]
	P	0.288 ± 0.011[a]	0.292 ± 0.026[a]	0.517 ± 0.033[c]	0.561 ± 0.029[c]	0.378 ± 0.029[b]	0.581 ± 0.009[c]
	E	7.753 ± 0.305[a]	7.777 ± 0.687[a]	15.193 ± 0.981[c]	16.864 ± 0.862[c]	11.229 ± 0.862[b]	17.501 ± 0.286[c]

Values with different letters in the same line were significantly different from each other (df = 5, $P < 0.05$).

kumar 1981, Phil & Rosenberg 1984, Cockcroft & Mclachlan 1986, Prakash & Agarwal 1989, Biesiot & Capuzzo 1990, Newell et al. 1995, Nunes et al. 1997, Roy & Singh 1997, Minami 2000, Schwamborn & Griales 2000). Because of the variation of food abundance and composition in natural and extensive or semi-extensive cultural water, these results cannot reflect the real food preference of the animals to some dietary organisms. Although Pinn et al. (1998) analyzed the selectivity on dietary organisms in two mud crabs by utilizing the Strauss' Linear Index, it was affected by such factors as diet density, difficulty of searching, difficulty of handling, and so on (Sunaga 1971, Griffiths 1975, Manghagen & Wiederholm 1982, Mikheev 1984, Buskey et al. 1991, Alam et al. 1996, Mehner et al. 1998). The dietary selectivity of Chinese shrimp on five diets with same availability was observed in these studies, and indicates that the Chinese shrimp possess the ability to discriminate different diets. The shrimp selected CF and PW, but ingested almost no FD (Fig. 4).

The dietary selectivity of animals are affected by many factors such as dietary energy content, abundance, and difficulty of searching, handling, digesting, and so on. In foraging, animals gain energy by ingesting prey (food) and expand energy in searching and handling prey (food). Composition of diets of some fish accorded with the prediction by the theory of optimal foraging in many experimental studies (Kislalioglu & Gibson, 1976; Tytler & Calow, 1984). Feeding behaviors of some crabs on snails and bivalves also agreed with the Optimal Foraging Theory on the whole (Elner & Hughes 1978, Boynton 1979, Hughes & Elner 1979, Kennedy et al. 1983, Lawton & Hughes 1985, Seed & Hughes 1997). Diets with almost the same encounter probability, size, and difficulty of handling were provided in this study. It was observed that the Chinese shrimp did not exhibit behaviors that maximized the net energy gain in the selection of diets under this experimental condition, and a great deal of CF, which was lower in energy content, was ingested. The correlation coefficient between ingested percent quantity and per unit energy content of diet was only 0.131, which indicated that no significant correlation existed ($n = 20$, $P = 0.589$). Poor relativity between Ivlev's index of selectivity and per unit energy content of diet was also observed ($R = 0.207$, $n = 20$, $P = 0.380$).

In this experiment, every diet provided to the shrimp was in excess but equally divided. Major factors that affect the dietary selectivity of shrimp are diet nutrition, difficulty of digesting, utilizing rate, attraction to diets, and so on. Shrimp of group MD showed distinct selectivity of different natural diets provided in the experiment. ADR of four natural diets were significantly higher than FD. The highest portion (based on dry weight) of 46.45% was observed in CF (Fig. 4), and the portion of PW (30.88%) was also comparatively higher than those of FF (13.07%) and SF (9.59%). It could be concluded from the results the shrimp preferred diets that met their high growth requirement (such as CF and PW), then they selected diets based on the digestibility (indicated by ADR), namely, they ingested more CF, which had relative higher ADR,
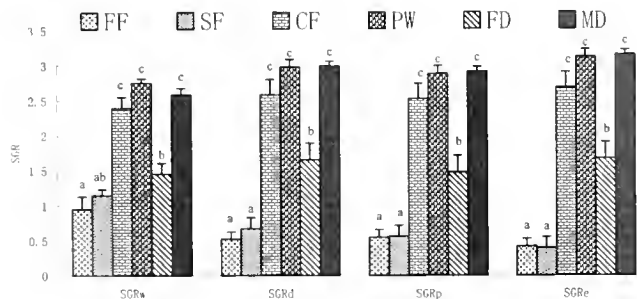

Figure 1. Special Growth Rate (SGR) of all diet treatments (Different letters above the bars denote significant differences (P < 0.05) among columns in the same cluster). Where FF = Fish Flesh, SF = Shrimp Flesh, CF = Clam Foot, PW = Polychaette Worm, FD = Formulated Diet, and MD = Mixed Diet; SGR$_w$, SGR$_d$, SGR$_p$, and SGR$_e$ are Special Growth Rates in terms of wet weight, dry weight, protein, and energy of shrimp body respectively; Vertical bar = SE (n = 4).
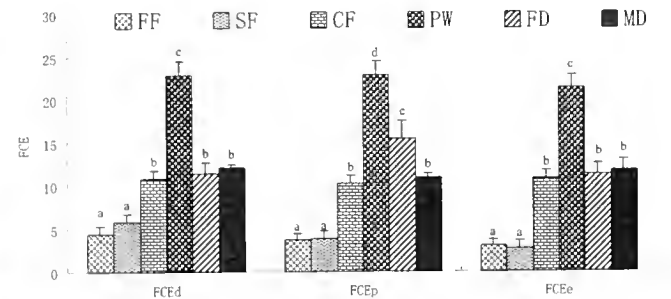

Figure 2. Food Conversion Efficiencies (FCE) in diet treatments (Different letters above the bars denote significant differences (P < 0.05) among columns in the same cluster). Where FF = Fish Flesh, SF = Shrimp Flesh, CF = Clam Foot, PW = Polychaette Worm, FD = Formulated Diet, and MD = Mixed Diet; FCE$_d$, FCEp, and FCEe are Food Conversion Efficiencies in terms of dry matter, protein, and energy respectively; Vertical bar = SE (n = 4).
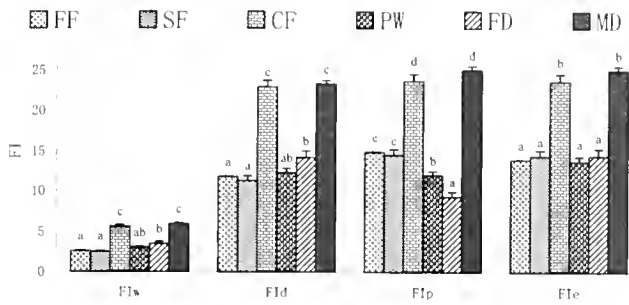
Figure 3. Food Ingestion (FI) of all groups (Different letters above the bars denote significant differences (P < 0.05) among columns in the same cluster). Where FF = Fish Flesh, SF = Shrimp Flesh, CF = Clam Foot, PW = Polychaette Worm, FD = Formulated Diet, and MD = Mixed Diet; FI_w, FI_d, FI_p, and FI_e are Food Ingestion in terms of wet weight, dry matter, protein, and energy respectively; Vertical bar = SE (n = 4).

Figure 5. Apparent Digestive Ratio (ADR) of every diet group (Different letters above the bars denote significant differences (P < 0.05) among columns in the same cluster). Where FF = Fish Flesh, SF = Shrimp Flesh, CF = Clam Foot, PW = Polychaette Worm, FD = Formulated Diet, and MD = Mixed Diet; ADR_d, ADR_p, and ADR_e are Apparent Digestive Ratios in terms of dry matter, protein, and energy respectively; Vertical bar = SE (n = 4).

than PW. The optimal foraging strategy of Chinese shrimp in this study was to gain as much energy as possible to meet the needs of variable physiologic activities, under the premise of ensuring fast growth, not to select diets to gain the highest FCE. In Group MD, the shrimp ingested a large amount of CF, which was easily digested (Fig. 5), so that they were able to ingest more diet continuously during the period to maximize the dietary energy ingestion. More studies on the effect of feeding attractants of these diets on dietary selectivity are still needed.

It was found in a few of studies that the decisive factors affecting the utilization, expressed in protein efficiency ratio (PER) and food conversion efficiency (FCE), of diets, were other ingredients when dietary protein content was above a reasonable level. These phenomena occurred in fish (Degani & Viola 1987, Viola & Lahav 1991, Erfanullah & Jafri 1995, Company et al. 1999, Morais et al. 2001, Shalaby et al. 2001, Das 1991), and also existed in shrimp and crab (Andrews et al, 1972, Colvin 1976, Sedgwick 1979, Xu & Li 1988). Xu and Li (1988) found, a protein sparing effect of lipid, in a study on the optimal protein, carbohydrate, fibrin, and lipid contents for Chinese shrimp diet that the increase of lipid content significantly promotes PER at all of the three protein contents of 36%, 40%, and 44%. Dietary protein contents of all the diets provided in this study exceeded 40% (Table 1) and could satisfy the demands for protein of Chinese shrimp. The
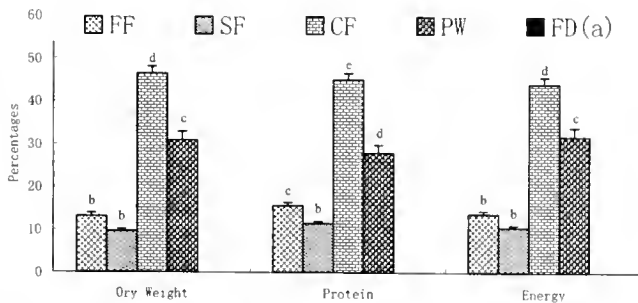
energy to protein ratio (E/P) and lipid content, however, varied greatly in different diets. The lipid contents of PW and FD were 16.32% and 9.93% respectively, and they were higher than other provided diets (Table 1). Although the lipid content in shrimp diet should not exceed 10% (Xu & Li 1988, Li 1990), it was found in this study that high lipid content had positive effect on FCE in Chinese shrimp, and the highest FCE was observed in PW, which was the highest in lipid content (16.32%). This result indicates that the shrimp could use more lipid than indicated in other reports, and diet for shrimp should be higher than 10% while protein content was high. E/P of these two diets are 33.99 $KJ.g^{-1}$ and 45.20 $KJ.g^{-1}$ respectively, which are also higher than other diets. It was probable that the protein sparing effect of these two parameters significantly improved the FCE_p of PW and FD (reaching 22.87% and 15.46%, respectively). Although shrimp of CF treatment ingested a larger amount diet in terms of DW, P, and E than PW treatment (Fig. 3), their SGR, FCE, and final body weight were not significantly higher than PW due to the lower lipid content and lower E/P ratio.

Munoz and San Feliu (1984) found in an experiment that Japanese shrimp, *Penaeus japanicus* fed on natural diets grew faster than those fed on formulated diet. In this study, the Chinese shrimp fed on FD grew significantly faster than those fed on FM and SM, and slower than those fed on CF and PW. Because the shrimp ingested no FD when it was fed simultaneously with natural diets, it was necessary that natural diets and the formulated diet should not be fed simultaneously in practice to avoid the wasting of formulated diet. Because the Chinese shrimp ingested no FD in the MD group, there was no significant difference among FD, FF, SF, and PW groups in FI_e (Fig. 3). Furthermore, FI_d of FD was significantly higher than that of FF, SF, and CF, and the FCE_p of FD was significantly higher than that of other diets except PW (Fig. 2). This result indicates that FD was less contaminating than FF, SF, and CF because of less nitrogen and organic matter loss in the water when these diets were ingested. It was found that fish culture used trash fish as feed, which results in heavier contamination than dry and artificial feeds (Wu, 1995). Considering the heavy contamination that can be caused by feeding the shrimp with natural diets in pond culture practice and the diets resource limitation (Wu 1995, Dong et al. 2000), it is reasonable to propose a high quality formulated diet to be used in culture practice.

Figure 4. Percent compostion of ingested diets in mixed diet fed group (Different letters above the bars denote significant differences (P < 0.05) among columns in the same cluster). Where FF = Fish Flesh, SF = Shrimp Flesh, CF = Clam Foot, PW = Polychaette Worm, FD = Formulated Diet, and MD = Mixed Diet. Vertical bar = SE (n = 4). Note: FD = 0(a) in all cluster.
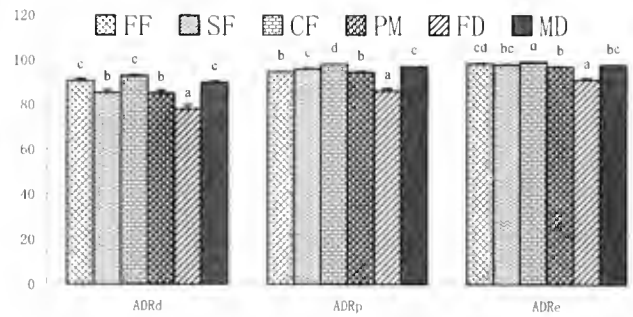
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EFFECT OF SALINITY ON SURVIVAL, GROWTH, AND OXYGEN CONSUMPTION OF THE PINK SHRIMP *FARFANTEPENAEUS PAULENSIS* (PÉREZ–FARFANTE 1967)

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ABSTRACT Survival, growth, and oxygen consumption rates of *Farfantepenaeus paulensis* postlarvae (PL) were examined at different salinities. Initially, PL 15 maintained at 30‰ salinity were gradually acclimated to 2, 5, 10, 20, and 30‰ over 5 days. Afterwards, survival, growth, and oxygen consumption rates of shrimp reared at these salinities were determined over a 42-day experimental period. Lower wet weight and cephalotorax length, and higher mortality rates were observed in shrimp reared at 2‰ salinity, especially when compared with those reared at 10‰ salinity ($P < 0.05$). In the range of 5‰ to 30‰ salinity, growth was optimized at 10‰ salinity, although this response was not significant. Salinity affected the oxygen consumption rates of *F. paulensis* postlarvae. At the beginning of the growth trial, oxygen consumption rate was markedly lower at 2‰ salinity than at 10‰ or 30‰ salinity ($P < 0.05$). This response was probably associated with a metabolic depression that preceded the shrimp death. Thereafter, oxygen consumption at 2‰ salinity showed a nonsignificant increase due to a higher variability of measurements probably associated with a better performance of surviving shrimp, which were tolerant to low salinity levels. At the intermediate salinities (5‰–20‰), oxygen consumption was higher at 10‰ salinity. At the end of the experiment, oxygen consumption reached similar and low levels irrespective of the salinity level. Oxygen consumption rate of shrimp reared at 30‰ salinity was constant and close to 5-μL mg dry weight^{-1} hr^{-1} throughout the experiment.

KEY WORDS: shrimp, *Farfantepenaeus paulensis*, growth, oxygen consumption, salinity

INTRODUCTION

Farfantepenaeus paulensis (Pérez–Farfante 1967) is a cold tolerant shrimp naturally occurring between Mar del Plata, Argentina, and Ilhéus, Brazil (D'Incao 1995). It is an important fishery resource, especially in Southern Brazil, where catches by artisanal fisheries have averaged around 3500 metric tons/yr in the last 40 years. However, unpredictable fluctuations in capture caused by climatic and oceanographic factors (Castello & Moller 1978, D'Incao 1995) usually result in a severe socio-economical problem. Some studies have examined the viability of cultivation and restocking programs with this species (Olivera et al. 1993, Wasielesky et al. 1995, Peixoto et al. 2002). The release and growth of *F. paulensis* in pen enclosures is routinely carried out at the estuary of the Patos Lagoon, Southern Brazil (Wasielesky 2000), which is characterized by abrupt and wide variations in salinity (Baptista 1984).

Salinity is one of the most important environmental factors affecting growth and survival of penaeids as it influences food consumption, conversion efficiency, and metabolic responses (Venkataramiah et al. 1972, Castille & Lawrence 1981, Dalla Via 1986, Staples & Heales 1991, Clark 1992, Brito et al. 2000). The knowledge of the species tolerance limits and optimum salinity levels is necessary to evaluate the viability of *F. paulensis* cultivation at variable environmental conditions. Furthermore, it is important to understand the effects of salinity when shrimp is reared in nursery grounds characterized by sudden salinity fluctuations and extreme environmental conditions. Salinity might have an indirect influence on the survival and growth of postlarvae when they penetrate estuarine areas, and also on the migration of juveniles back to the ocean. For example, Staples (1980) observed that reductions in salinity caused the migration of *Fenneropenaeus merguiensis* juveniles from nursery grounds to oceanic waters.

Salinity tolerance limits and the effects of acclimation to salinity on the survival of *F. paulensis* have already been evaluated

(Tsuzuki et al. 2000). However, as the optimal salinity range for growth is narrower than for survival, growth occurs when the metabolic demands for maintenance and feeding activity are satisfied. Several studies have analyzed the metabolism and activity in crustacean decapods through oxygen consumption measurements (Kutty et al. 1971, Venkataramiah et al. 1974, Venkataramiah et al. 1975, Gaudy & Sloane 1981, Du Preez et al. 1992, Villarreal & Rivera 1993). Since the rate of oxygen consumption is modified by changes in the energetic demand for biologic activities, it is expected that salinity variations would lead to changes in oxygen consumption of shrimp, as demonstrated by Kutty et al. (1971). It is also expected that changes in metabolic rates induced by salinity can affect shrimp growth and production, as pointed out by Dalla Via (1986).

In light of discussion earlier, the objective of this study is to investigate the effects of salinity on survival, growth, and oxygen consumption of *F. paulensis* postlarvae.

MATERIAL AND METHODS

General Rearing Conditions

This study was conducted at the Marine Aquaculture Station "Prof. Marcos A. Marchiori" of the Fundação Universidade Federal do Rio Grande (Southern Brazil). Postlarvae (PL) of *Farfantepenaeus paulensis* were reared at 22–25°C, 30‰ salinity, and natural photoperiod. In the initial stages of development, PL were fed with newly-hatched *Artemia* nauplii, and afterwards with *Artemia* nauplii and finely chopped meat of white clam (*Mesodesma mactroides*), fish (various fresh fish) and squid (*Illex* sp). Water of different salinities was obtained by mixing dechlorinated tap water with natural seawater. Salinity was measured with an optical refractometer (1.0‰ precision, Atago Co., Tokyo, Japan).

Survival, Growth, and Oxygen Consumption

Fifteen-day-old PL were reared in the conditions described earlier and were gradually acclimated from 30 to 2, 5, 10, and 20‰

salinity over a 5-day period, by daily reductions of 6, 5, 4, and 2‰ salinity, respectively (Tsuzuki et al. 2000). Postlarvae maintained at 30‰ salinity were used as control. After the salinity acclimation period, PL survival and growth in each salinity were examined over 6 wk by stocking 80 PL in a 100-L plastic tank. Shrimp were fed *ad libitum* twice a day with a commercial diet containing 45% crude protein (Tetra DoraMarin, Pfizer Co., USA). Every day, pH and temperature were monitored, and organic residuals were siphoned out from the bottom of the tanks when at least 10% of the water was renewed. Every two weeks, 20% of the animals in each tank were counted and individually weighed to the nearest 0.1 mg (wet weight). Cephalotorax length and dry weight (60°C for 48 h) were measured to the nearest 0.01 mm and 0.1 mg, respectively, at the beginning ($n = 48$) and at the end of the trial. At the end of the experiment (week 6), all living shrimp were weighed (wet and dry weights) and measured ($n = 26$–163). Cephalotorax length was measured using a stereoscopic microscopy (Nikon, Japan).

At the end of the salinity acclimation period, and every 2 wk during the growth trial, oxygen consumption was measured using a Barcroft–Warburg respirometer (Oser 1965). Values were expressed as μL of O_2 per mg of dry weight per hr.

Statistical Analysis

Each treatment was done in triplicate. However, no significant difference was detected between replicates and results were then pooled for further analysis. Differences between replicates and treatments were analyzed by one-way analysis of variance (ANOVA) followed by the Tukey's test. The significance level adopted was 95% ($P < 0.05$).

RESULTS

Water temperature throughout the experiment was 24.9 ± 0.1°C (mean ± SE), while mean values of pH and salinity were 7.5, 7.6, 7.6, 7.8, and 7.9 at 2, 5, 10, 20, and 30‰ salinity, respectively.

After the five-day acclimation period to different salinities, mean weights (wet and dry weights) and cephalotorax length of 20-day-old PL did not change with the acclimation salinity ($P > 0.05$). Therefore, all values were pooled and only one mean was calculated. Mean (± SE) wet and dry weight and cephalotorax length was 9.2 ± 0.2 mg, 2.0 ± 0.1 mg, and 2.1 ± 0.0 mm, respectively. Survival rates of these PL were higher than 95% and there were also no significant difference between treatments ($P < 0.05$) (data not shown). However, after two weeks of experiment, significantly lower survival rates (28.1%) were observed at 2‰ salinity (results not shown). At this salinity, only 15.8% survival was observed at the end of the growth period (Table 1).

Figure 1 shows the PL growth as wet weight at different salinities throughout the experimental period. From the second to the fourth week of experiment, a higher mean wet weight was observed in PL reared at 10‰ salinity, especially when compared with those reared at 2‰, 5‰, and 20‰ salinity ($P < 0.05$). After six weeks of experiment, wet weight of shrimp reared at 2‰ salinity was significantly lower than those reared at 10‰ salinity ($P < 0.05$). For salinities between 5‰ and 30‰, PL wet weight was higher at 10‰ salinity although this difference was not statistically significant (Fig.1, Table 1). At 2‰ salinity, PL dry weight tended to be lower at the end of the growth period, but no significant changes were detected. Cephalotorax length was significantly smaller in PL reared at 2‰ salinity than in those reared at 5‰ or 10‰ salinity ($P < 0.05$) (Table 1).

After the salinity acclimation period, oxygen consumption of PL acclimated to 2‰ salinity was lower than that observed in PL acclimated to 10‰ or 30‰ salinity. At 2‰ salinity, oxygen consumption increased after the second week and reached a maximum value at the fourth week of experiment. Afterwards, a marked drop in oxygen consumption rate occurred. At the intermediate salinities (from 5‰ to 20‰), oxygen consumption was higher at 10‰ salinity until the second week of the experiment although not statistically different ($P > 0.05$). At the end of the growth period, oxygen consumption reached similar and low levels (around 4 μL O_2 mg dry weight^{-1} hr^{-1}) irrespective of the salinity level tested. Oxygen consumption of shrimp at 30‰ salinity was constant and close to 5 μL O_2 mg dry weight^{-1} hr^{-1} throughout the experiment (Table 2, Fig. 1).

DISCUSSION

In this study, survival of *Farfantepenaeus paulensis* postlarvae (PL) was extremely low (15.8%) after a 6-wk growth period at 2‰ salinity. A similar result was verified by Cawthorne et al. (1983) when only 34% of *Penaeus monodon* juveniles survived at that salinity for two weeks. Although Tsuzuki et al. (2000) verified an increase in salinity tolerance of *F. paulensis* postlarvae with aging (from PL 15 to 30) when PL were directly transferred from 30‰ to 2‰ or 5‰ salinity, the low survival rate observed at 2‰ salinity in this study indicates that 20-day-old PL were not able to cope with low salinity levels for a long period of time (6 wk). Also, the lower shrimp growth rates observed at 2‰ salinity confirms the physiologic disturbance induced by low salinity in *F. paulensis* PL. Dalla Via (1986) suggested that reductions in shrimp growth at low salinities can be related to a higher energetic expenditure to keep the osmotic equilibrium at these saline conditions. The same author showed that exposure to 10‰ salinity for five months resulted in reduction (up to 33%) of the ash-free organic content. Therefore, in low salinity environments a significant reduction in shrimp production might be expected. However, this hypothesis can only be considered if one assumes that the food assimilation

TABLE 1.

Survival, wet and dry weights, and cephalotorax length of *Farfantepenaeus paulensis* postlarvae reared at different salinities for 6 weeks.

Salinity (‰)	Survival (%)	Wet Weight (mg)	Dry Weight (mg)	Cephalotorax Length (mm)
2	15.8 ± 4.7 (a)	102.4 ± 11.3 (a)	27.2 ± 3.7 (a)	4.8 ± 0.4 (a)
5	81.3 ± 5.2 (b)	135.2 ± 5.1 (ab)	30.8 ± 1.2 (a)	5.7 ± 0.2 (b)
10	88.3 ± 0.6 (b)	147.2 ± 4.6 (b)	33.2 ± 1.1 (a)	5.7 ± 0.1 (b)
20	82.9 ± 9.0 (b)	140.3 ± 7.6 (ab)	33.2 ± 1.9 (a)	5.1 ± 0.2 (ab)
30	70.0 ± 14.3 (b)	140.9 ± 10.0 (ab)	34.2 ± 2.6 (a)	5.1 ± 0.2 (ab)

Data are means ± SE ($n = 26$–163). Same letters indicate absence of significant difference between salinities ($P > 0.05$).
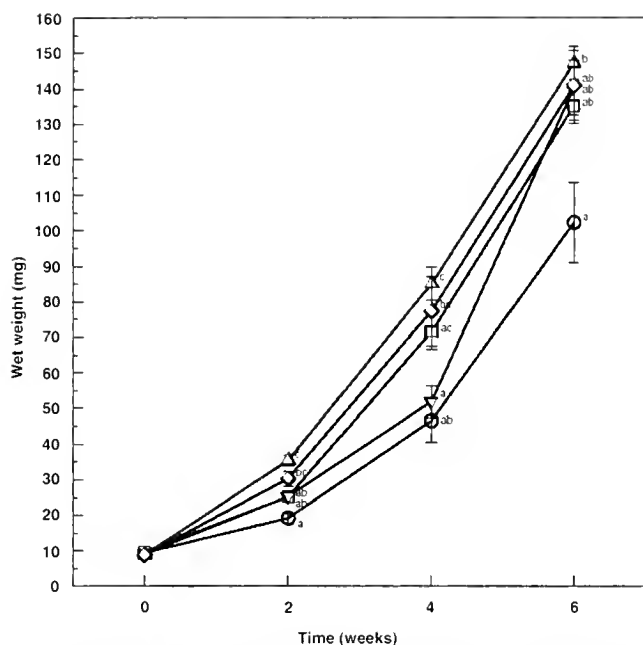
Figure 1. Wet weight of *Farfantepenaeus paulensis* postlarvae reared at different salinities. Data are means ± SE (*n* = 26–163). Different letters indicate significant differences between salinities at the same time of cultivation (*P* < 0.05). Salinities (‰): ○ = 2; □ = 5; △ = 10; ▽ = 20; ◇ = 30.

rate is not dependent on salinity. Marques & Andreatta (1998) found significant differences in dry matter consumption of *F. paulensis* reared in low salinity levels while Wasielesky et al. (2002) reported that food consumption in this species was not affected by salinity. Therefore, further investigation is needed to clarify this question.

It has been demonstrated for several penaeids that higher growth rates usually are observed at salinities ranging from 5‰ to 35‰, depending on the species and the ontogenetic phase considered. In *Litopenaeus vannamei* PL, higher growth rates occurred at 20‰ salinity when compared with those observed at 5‰ and 45‰ salinity (Huang 1983). Bray et al. (1994) reported that juveniles of the same species reared at 5‰ and 15‰ salinity achieved higher increment in wet weight than those reared at 25‰, 35‰, and 49‰ salinity. Venkataramiah et al. (1974) verified that *F. aztecus* growth was enhanced at 8.5‰ and 17‰ salinity. Henning & Lemos (1994) verified that *L. schmitti* growth was similar at 5‰ and 30‰ salinity, but higher at 10‰ salinity. In this study, a better growth rate was observed at 10‰ salinity, being significantly different from that observed at 2‰ salinity throughout the experiment. Venkataramiah et al. (1975) observed in estuaries where *F. aztecus* is naturally found, that a higher abundance occurs in salinities that are close to the optimum level estimated under laboratory conditions. The same fact is observed for *F. paulensis* distribution in the Patos Lagoon estuary, where a higher abundance is observed in areas with salinities below 10‰ salinity, although shrimp can be found in salinities between 0‰ and 31‰ (D'Incao 1991).

Before growth occurs, the metabolic demand for maintenance and feeding activity must be satisfied. The knowledge of such demands under different environmental conditions is necessary (Brett 1970). Several studies have used the oxygen consumption measurement to analyze metabolism and activity in crustacean

decapods (Kutty et al. 1971, Venkataramiah et al. 1974, Gaudy & Sloane 1981; Villarreal & Rivera 1993). Since the oxygen consumption alters with changes in the energetic demand for biologic activities, it would be expected that salinity variations could lead to changes in oxygen consumption (Kutty et al. 1971).

At the beginning of the experiment, the oxygen consumption of 20-day-old PL reared at 2‰ salinity was markedly lower compared with PL reared at the other salinities tested. This fact probably indicates that PL could be in a metabolic depression stage that would precede death. In fact, a high mortality rate (71.9%) was observed in the first two weeks of the experiment. In *Penaeus semisulcatus*, Clark (1992) also observed a decrease in the respiration rate after a salinity reduction from 40‰ to 18‰ salinity. He also noticed that shrimp were moribund and died 12 hours after being exposed to the salinity shock. Chen & Fang (1986) considered that the respiratory depression observed in *Metapenaeus ensis* after a salinity change was caused by a reduction of the water flow through the gills to resist the salinity shock, leading to a reduction of the oxygen consumption. In this study, a low oxygen consumption was observed throughout the experiment at 2‰ salinity, except after four weeks when a non-significant increase in oxygen consumption was observed. In this case, mortality rates did not significantly change after two weeks of experiment. Therefore, the increase in oxygen consumption observed after four weeks of experiment at 2‰ salinity could be attributed to a higher variability of the oxygen consumption measurements due to a better performance of surviving animals, which probably were more resistant to lower salinity levels.

Until the second week of the experiment, higher oxygen consumption rates were observed in shrimp maintained at 10‰ salinity. Concomitantly, higher growth rates expressed as wet weight and cephalotorax length were generally observed for shrimp kept at this salinity. Yagi & Ceccaldi (1984) verified in *Palaemon serratus* larvae that the oxygen consumption was maximum in salinities ranging from 25‰ to 30‰, which could be explained by a higher physiologic activity related to larvae food utilization. Moreover, between 25‰ and 30‰ salinity the energetic demand for osmoregulation seems to be lower and growth higher. It is important to emphasize that an attempt to correlate energetic expenditures for ionic and osmotic regulation with oxygen consumption rates is speculative, once the subject is still controversial. Some investigators point out that the energy expended to osmoregulation can be evaluated by oxygen consumption measurements in aquatic invertebrates (Lofts 1956, Rao 1968). In this case, oxygen consumption can be expected to increase for osmoregulators

TABLE 2.

Oxygen consumption rates (μL O_2 mg dry weight^{-1} hr^{-1}) of *Farfantepenaeus paulensis* postlarvae reared at different salinities for 6 weeks.

Salinity (‰)	Time of Cultivation (Weeks)			
	0	2	4	6
2	3.7 ± 0.6 (a)	5.3 ± 0.9 (ab)	12.3 ± 3.5 (a)	5.0 ± 0.8 (a)
5	7.9 ± 2.3 (ab)	8.2 ± 1.4 (a)	3.9 ± 0.7 (b)	3.6 ± 0.5 (a)
10	13.0 ± 2.8 (b)	12.9 ± 3.3 (ab)	5.6 ± 0.9 (ab)	4.3 ± 0.5 (a)
20	9.1 ± 2.6 (ab)	8.1 ± 1.3 (ab)	4.6 ± 0.5 (ab)	4.3 ± 0.5 (a)
30	6.3 ± 0.4 (b)	4.3 ± 0.5 (b)	4.4 ± 0.9 (ab)	4.1 ± 0.7 (a)

Data are means ± SE (*n* = 3–6). Same letters indicate absence of significant difference between salinities (*P* > 0.05).

when the osmotic difference between the hemolymph and the environment increases, resulting in an increase in the metabolic demand to keep constant the hemolymphatic concentration. Nevertheless, changes in metabolic rates related to salinity are, in most cases, too big to be attributed only to the energetic cost with ionic and osmotic regulation. In this case, it would be difficult to relate oxygen consumption rates exclusively to energetic requirements for osmoregulation (Potts & Parry 1964). Therefore, not only the anisosmotic regulation of extracellular fluids, that seems less likely the general cause of metabolic changes of the organism (Duncan 1966, Kinne 1971) should be taken into consideration, but also the isosmotic regulation of the intracellular fluids involving the mobilization of organic substances and changes in the energetic needs for ionoregulation (Wheatly 1988). Additionally, the interference

of the locomotion activity should be considered (Beamish & Mookherji 1964).

The comparatively low and stable oxygen consumption rates of shrimp reared at 30%o salinity, and the low oxygen consumption at the end of the experiment irrespective of salinity levels, indicate more economical respiration rates at salinities where animals are genetically adapted or acclimated for a longer period (Kinne 1971).

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ANATOMICAL DAMAGE TO HUMPBACK SHRIMP, *PANDALUS HYPSINOTUS* (BRANDT 1851) CAUGHT BY TRAWLING AND TRAPPING

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ABSTRACT   We compared the anatomical damage, individual size, total catch, and bycatch when humpback shrimp, *Pandalus hypsinotus* (Brandt 1851), were harvested using otter trawls, beam trawls, and traps in Simoom Sound, British Columbia. Regional body damage (RBD) and total body damage (TBD) to humpback shrimp were assessed for four major regions of the shrimp body (rostrum, carapace, abdomen, and tailfan). TBD was higher for otter and beam trawling compared with traps, with a significant difference observed between the otter trawling and half-day trapping. After standardizing trawl data by fishing effort (area swept and fishing time), TBD was significantly higher for beam trawl. RBD was significantly different across fishing methods and there were also significant differences among the various body parts. Trawl caught humpback shrimp showed the highest ratio of damaged/total individuals relative to those caught by traps. In general, the carapace and rostrum body regions were more damaged relative to the abdomen and tail fan. The survival of humpback shrimp released after trawling or trapping will depend on the extent of the body region-specific anatomical damage that has occurred and its functional importance.

KEY WORDS: damage, otter trawling, beam trawling, trapping, shrimp, fishing gear, bycatch, *andalus hypsinotus*

INTRODUCTION

Studies exploring the use of selective fishing gear are ongoing and past studies have focused on the size and shape of net meshes as well as the use of extruders in trawl nets to separate the target and bycatch species (e.g., DeAlteris & Reifsteck 1993, Suuronen et al. 1996, Richard 1999). Most studies comparing fishing gear bycatch have focused on the volume of bycatch and only a few more recent studies have focused on damage to the catch and subsequent survivability of organisms. (e.g., Mensink et al. 2000, Stevens et al. 2000, Bergmann & Moore 2001). This study, however, turns a lens to the damage to humpback shrimp, *Pandalus hypsinotus*, harvested in an inshore ecosystem in Pacific Canada with three different fishing gear types: beam trawl, otter trawl, and traps. Humpback shrimp are caught in directed trap and trawl fisheries in British Columbia and are also commonly found as incidental catch in shrimp trawl (*Pandalus* spp.) and spot prawn (*Pandalus platyceros*) trap fisheries (Boutillier & Nguyen 1999). No information has been published about fishing gear-related anatomic damage caused by these harvesting methods in Pacific Canada. In this study we focused on three objectives: 1) the relative total damage to humpback shrimp among fishing methods (total body damage [TBD]); 2) susceptibility to gear-related damage among major anatomic regions of shrimp (regional body damage [RBD]); and 3) comparison of catches of target and nontarget species among gear types.

This study was part of a larger project designed to determine whether trawling or trapping would be a preferable method of harvesting humpback shrimp, as a representative crustacean species, in an ecosystem-based management system (e.g., Jamieson & O'Boyle 2001). One of the aspects of such a management system would be to avoid "bykill" or unwanted fishing mortality of undersized shrimp or nontarget shrimp species by minimizing the practice of discarding bycatch if there were high levels of collateral damage during harvest. Previous studies have showed that shrimp trawling can result in damage to benthic habitats (e.g.,

Hansson et al. 2000). However, data to compare damage by trawling relative to other gear types are not available, and the interaction between, gear type, bycatch, and collateral damage have not been presented to date.

MATERIALS AND METHODS
Experimental Trawling and Trapping

Simoom Sound, an inlet off Fife Sound on the central coast of British Columbia was chosen as the study location (Fig. 1). Bottom salinity and temperature ranged between 31.5 to 33.5 psu and 7.5 to 8.7°C, respectively, using a Sea-Bird CTD (model SBE-911plus) deployed in October 2001. The surface sediment in Simoom Sound consists of approximately 90% silt and 21% organic content. Beam trawling, otter trawling, and trapping were used to catch humpback shrimp in Simoom Sound during November 2000 (otter trawl, trap), and February 2001 (beam trawl). Each gear type was deployed in a separate "block" of the seafloor (approximately 700 m by 400 m) characterized by relatively uniform depth and sediment type. The gear used was representative of that used in the commercial fishery and complete details on methods, vessels, and gear dimensions are given elsewhere (Ong et al. 2002). The shrimp trawl industry in British Columbia has voluntarily adopted a 100% implementation of bycatch reduction devises (BRDs) in their nets since 2000 and all trawl nets used in this study were fixed with rigid type bycatch reduction grids (Department of Fisheries and Oceans 2002).

Otter Trawling

Six otter trawls were conducted on three transects on November 14, 2001 (Fig. 1), with two trawls performed on each of the transects. The water depths ranged between 55 and 60 m. Transects lengths were between 643 and 677 m and each trawl was 10–13 min in duration, not including the time required for net haul-back. The otter trawl net measured 36.8 m long with a headrope and footrope (without a tickler chain) of 23.8 m and 30.5 m. Codend mesh size was 38 mm. Catches were sorted and counted by species and weighed to the nearest 0.1 kg. Humpback shrimp specimens used for the otter trawl damage assessment ($n = 106$)
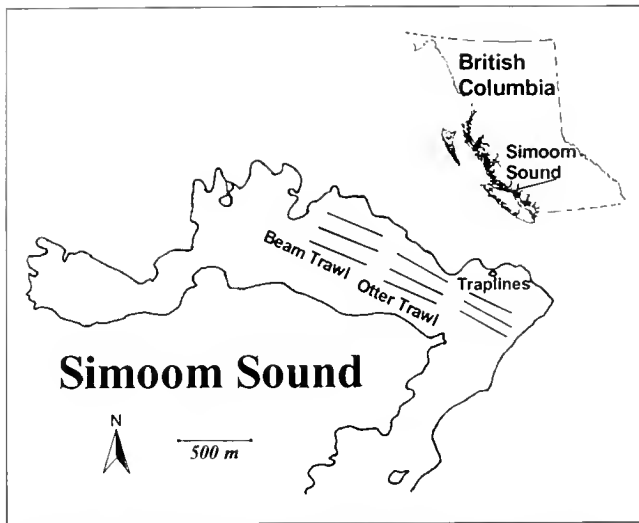
Figure 1. Map of Simoom Sound, BC, with approximate locations of beam trawl, other trawl, and traplines used in this study.

were collected from the catch after the codend contents had been placed onto a sorting table. Samples were frozen in labeled freezer bags for later analysis in the laboratory. After collection, care was taken to keep specimens flat to minimize damage because of handling.

Beam Trawling

Beam trawls were completed on three transects, west of the otter trawl lines on February 22, 2001 (Fig. 1), with two trawls conducted on each of the three trawl lines. The trawl duration, length, and depths ranged among 15–17 min, 513 and 660 m, and 46 and 55 m, respectively. The beam trawl net measured 26.6 m in total length, with a headrope and footrope length of 14.0 m and 16.5 m. Codend mesh size was 44 mm. Beam trawl catches were sorted by species, then counted and weighed to the nearest 0.1 kg. Humpback shrimp specimens used in the beam trawl damage assessment (n = 132) were collected and frozen using the same techniques described for otter trawling.

Trapping

Traps were set out twice, east of the otter trawl lines, during November 15–16, 2000, on three transects (62–75 m deep; Fig. 1). Two time periods were used. The first set of traps remained submerged for approximately 6 h (half-day traps), during the day, and the second set of traps was submerged for 17 h over night (overnight traps). Approximately 40 traps were set on each trap line with spacing of about 15 m between each trap. The three transects measured from 558 to 660 m long. Most sets included traps outside the defined block of sea floor because the groundline used was longer than the predefined transect length of 500 m. Traps were baited with salmon fish feed pellets, cut-up Pacific herring, *Clupea harengus pallasi*, and shiner perch, *Cymatogaster aggregata*, collected on site as bycatch from the trawling experiments. The traps were conical and measured 76.2 × 30.5 × 71.1 cm, with a stretch mesh size averaging 45 mm. Each trap weighed approximately 1.4 kg. On one overnight set, humpback shrimp were only collected from traps that fished the predetermined line. All the other hump-

back shrimp were from at least 10 traps within the predetermined line and from a few of the traps extending outside of it. The traps were emptied into a plastic tote and the catches from each trap were then identified to species and counted. Catches of all shrimp species were weighed to the nearest 0.1 kg. A subsample of the humpback shrimp catch was frozen using the same techniques described for otter and beam trawling. 139 humpback shrimp were collected from the half-day traps and 145 from the overnight traps.

Sample Processing

Humpback shrimp were thawed in the laboratory for 1–2 h. Weight, length, and sex were recorded for each individual. Lengths were recorded to the nearest millimeter using manual or electronic Vernier calipers. Carapace length was measured from the posterior-most part of the orbit to the posterior middorsal margin, and total length, from the tip of the rostrum to the tip of the telson. As several humpback shrimp from the beam trawl were in a transitional stage (from male to female phases), their total lengths were calculated based on the relationship between male carapace length and total length. Sexing was accomplished by noting the presence of eggs in the head or abdomen, and by examination of the endopods of the second pleopods (Butler 1980).

Damage Assessment

A table was constructed to delineate the principal body parts in the four major regions (rostrum, carapace, abdomen and tailfan) of the shrimp body, as shown in Butler (1980). The body parts chosen for analysis were those that would be required for the survival of a humpback shrimp if it were to be released. Each body part within each of the four major body regions was given a score from 0 to 1.0, with zero being a missing body part, and 1.0 representing a fully intact body part, with intermediate scores representing varying levels of damage, that is, the rostrum is composed of nine body parts and a summed score of 9.0 reflects zero percent damage whereas a score of 6.0 represents, (1 − [6.0/9.0]100), or 33.3% damage. Table 1 describes the codes and damage scores used for each of the body regions and Table 2 depicts the particular body parts and their accompanying functions. Humpback shrimp from each of the fishing methods were assessed for damage using this scheme (Table 1) resulting in data on RBD and TBD. TBD was assessed by summing the damage scores from all body regions and dividing the score by the total number of body parts assessed from each gear transect and expressing the resultant as a percentage. Because of time constraints, some of the trap-caught humpback shrimp were assessed using a low-resolution scheme wherein damage to the abdomen was not assessed (Ong et al. 2002). Only data from the high-resolution scheme are presented herein.

Statistical Analysis

Statistical analysis was performed on the humpback shrimp catch data using Systat© v.10 statistical software. A Bartlett's test for homogeneity of variances was performed on the data set prior to statistical analysis and for parametric analyses, proportional data was arcsine transformed (Zar 1984). A single factor analysis of variance (ANOVA) and Tukey HSD multiple comparison tests were used to test for significant differences in RBD and TBD. In cases where were parametric assumptions were not met, a single factor Kruskal-Wallis ANOVA by ranks was performed. Untrans-

TABLE 1.

List of humpback shrimp body regions assessed for damage.

Region	Code	Body Part	Damage Score
Rostrum	R1	Rostrum	0 = completely broken off, 0.5 = some damage, 1.0 = intact
	R2	Eye stalk and cornea (R)	0 = completely broken off, 0.5 = some damage, 1.0 = intact
	R3	Eye stalk and cornea (L)	0 = completely broken off, 0.5 = some damage, 1.0 = intact
	R4	Antennae 1 (L)	0 = completely broken off, 0.5 = some damage, 1.0 = intact
	R5	Antennae 1 (R)	0 = completely broken off, 0.5 = some damage, 1.0 = intact
	R6	Antennae 2 (L)	0 = completely broken off, 0.5 = some damage, 1.0 = intact
	R7	Antennae 2 (R)	0 = completely broken off, 0.5 = some damage, 1.0 = intact
	R8	Antennal scale (L)	0 = completely broken off, 0.5 = some damage, 1.0 = intact
	R9	Antennal scale (R)	0 = completely broken off, 0.5 = some damage, 1.0 = intact
Carapace	C1	Third maxilliped (L)	0 = completely broken off, 0.5 = some damage, 1.0 = intact
	C2	Third maxilliped (R)	0 = completely broken off, 0.5 = some damage, 1.0 = intact
	C3	Pereiopod I (L)	The following scores apply to all pereiopods:
	C4	Pereiopod I (R)	0 = broken off below (bob) coxa 0.1 = bob basis
	C5	Pereiopod II (L)	0.2 = bob ischium 0.3 = bob merus, 0.4 = bob carpus
	C6	Pereiopod II (R)	0.5 = bob propodus, 0.6 = damaged chela
	C7	Pereiopod III (L)	0.7 = broken off chela, 0.8 = broken off exopod
	C8	Pereiopod III (R)	0.9 = broken off epipod, 1.0 = intact
	C9	Pereiopod IV (L)	0.9 = broken off epipod, 1.0 = intact
	C10	Pereiopod IV (R)	0.9 = broken off epipod, 1.0 = intact
	C11	Pereiopod V (L)	0.9 = broken off epipod, 1.0 = intact
	C12	Pereiopod V (R)	0.9 = broken off epipod, 1.0 = intact
	C13	Carapace itself	0.5 = some damage, 1.0 = intact
Abdomen	A1	Somites I–VI	0.5 = some damage, 1.0 = intact
	A2	Pleurons I–V	0.5 = some damage, 1.0 = intact
	A3	Pleopods I (L and R)	0.5 = some damage, 1.0 = intact
	A4	Pleopods II (L and R)	0.5 = some damage, 1.0 = intact
	A5	Pleopods III (L and R)	0.5 = some damage, 1.0 = intact
	A6	Pleopods IV (L and R)	0.5 = some damage, 1.0 = intact
	A7	Pleopods V (L and R)	0.5 = some damage, 1.0 = intact
Tail Fan	T1	Telson	0 = completely broken off, 0.5 = some damage, 1.0 = intact
	T2	Uropods (L)	0 = both broken off, 0.5 = some damage, 1.0 = intact
	T3	Uropods (R)	0 = both broken off, 0.5 = some damage, 1.0 = intact

Scores for each body region are assessed based on the total score assigned to each body part in the region (e.g., Rostrum has nine body parts, minimum score = 0, maximum score = 9).

formed data were used because arcsine transformation did not change the ranks of the parameters. A parametric ANOVA was also used to compare catch weights of humpback and pink shrimp, *Pandalus eous* (*P. borealis*), among harvest methods. Four major hypotheses were tested: 1) gear-related damage to humpback shrimp was not equal among fishing methods, 2) proportional damage to the four major shrimp body regions differed with fishing methods, 3) total numbers and biomass of humpback shrimp in the catches

TABLE 2.

List of humpback shrimp body parts according to function.

Region	Body Part	Function
Rostrum	Rostrum	The head spine that helps deter small predators
	Antennae 1 (antennules)	Detect waterborne smells
	Antennae 2	For touch and to detect approaching predators
	Antennal scales	Provide stability while swimming
Carapace	3rd Maxilliped	Holds food while pieces are pulled off with claws; used when sparring with other shrimps
	Pereiopod I	If chelate, it is used to catch small prey
	Pereiopod II	Chelate leg with articulated carpus for grooming and retrieving scraps of food
	Pereiopods III to V	Walking legs; pereiopod V may have brushes used for grooming and cleaning eggs
Abdomen	Abdomen (somites and pleurons)	With tail fan, the strong muscles are used for fast backward swimming (in escape response)
	Pleopods	For forward swimming, and to brood eggs
Tail Fan	Telson	Bears the anus; involved in backward swimming
	Uropods	Involved in backward swimming

differed among fishing methods, and 4) individual weight of humpback shrimp was different among fishing methods tested.

RESULTS

Raw data on damage scores, lengths, and weights for all individual humpback shrimp together with catch data from each fishing gear are presented elsewhere (Ong et al. 2002). Specific analyses are summarized below.

TBD to Humpback Shrimp Among Fishing Methods

TBD tended to be higher in trawls than traps and the otter trawl caught humpback shrimp were significantly more damaged than those from half-day traps (9.9 ± 5.0% vs. 2.0 ± 1.1%; $P = 0.023$). Other comparisons were not statistically significant ($P > 0.0$; Table 3). A comparison of standardized TBD data between otter and beam trawl methods resulted in a significant difference (5.7 ± 4.1% vs. 23.6 ± 8.6%; $P < 0.001$). TBD to trap-caught humpback shrimp was standardized by soak time. There was no significant difference ($P > 0.05$) in total percent damage per hour between traps set out for 6 h in daytime compared with 17 h overnight (Table 3). There were also marked differences in the proportion of individual humpback shrimp that received any damage to the 32 body parts observed. Trawl caught humpback shrimp received the highest ratio of damaged/total individuals (otter 89.9%; beam 78.8%; overnight traps 45.0%; half-day trap 37.4%).

RBD to Humpback Shrimp Among Fishing Methods

Considering all three fishing methods, the carapace was the most damaged (Table 3) and showed the greatest variability of the four body parts, however, there were differences between gear types, as explained below carapace damage was represented by disfigurement, depression, partial tear-off and detachment from the thorax. There was very weak negative correlation ($r^2 = 0.049$) between the carapace length of humpback shrimp and percent carapace damage across all fishing methods.

There were significant differences ($P = 0.047$) in carapace damage between fishing methods with otter trawls (16.4% ± 10.0) and beam trawls (10.3% ± 0.4) resulting in higher proportions of carapace damage than humpback shrimp harvested by overnight trap (4.5% ± 1.8) or half-day traps (2.9% ± 1.9). However, there were no pair-wise significant differences ($P > 0.05$) assessed with Tukey tests (Table 3).

Damage to the rostrum differed among fishing gear ($P = 0.003$; Table 3). Damage to the rostrum of humpback shrimp harvested by otter trawl was significantly greater (12.0% ± 2.6) compared with both overnight traps (5.6% ± 2.4; $P = 0.02$) and half-day traps (2.6% ± 1.2; $P = 0.002$). The rank of the proportional damage to the rostrum of humpback shrimp was the same as reported for the carapace, with otter trawl (12.0% ± 2.6) incurring the most damage followed by beam trawls (7.2% ± 0.9), overnight traps (5.6% ± 2.4), and half-day traps (2.6% ± 1.2), respectively (Table 3).

Regardless of fishing method, the abdomen and tailfan of humpback shrimp were less damaged than the carapace and rostrum (Table 3). There was a significant difference ($P = 0.028$) in the damage to the abdomen between gear types, with the beam trawl causing at least five times more damage than any other fishing method, and significantly more than the overnight traps (Tukey test, $0.01 < P < 0.025$; Table 3). There were also significant differences in the amount of damage to the tailfan among fishing methods ($P = 0.034$). Tailfan damage from the otter trawl catch was the highest (3.0% ± 1.9), with the beam trawl (1.8% ± 0.9), half-day traps (0.2% ± 0.2), and overnight traps (0.2% ± 0.2) following, respectively (Table 3). As with carapace data, there were no pair-wise significant differences ($P > 0.05$) when assessed with Tukey tests (Table 3).

RBD to Humpback Shrimp by Each Fishing Method

Damage to specific humpback shrimp body parts differed within otter trawl activity ($P = 0.002$; Table 4). The carapace received the highest damage scores and was significantly more damaged than the abdomen (Tukey test, $P = 0.023$) (Table 4).

The damage assessments for humpback shrimp caught by beam trawl were similar to those revealed in the otter trawl catch (Table 4). There were significant differences ($P < 0.001$) in the damage to various shrimp body parts (Table 4). The carapace of the beam trawl caught humpback shrimp had significantly more damage

TABLE 3.

RBD and TBD data

Body Region	Otter Trawl	Beam Trawl	Traps Overnight	Traps Half-Day	ANOVA Gear Type	TUKEY Gear Type, P Values
RBD: Carapace	16.4 ± 10	10.3 ± 0.4	4.5 ± 1.8	2.9 ± 1.9	$H = 7.95$, $P = 0.047^*$	NS
RBD: Rostrum	12.0 ± 2.6	7.2 ± 0.9	5.6 ± 2.4	2.6 ± 1.2	$F = 11.14$, $P = 0.003^*$	O vs HT, 0.002 O vs OT, 0.02
RBD: Abdomen	0.2 ± 0.2	1.7 ± 1.7	0.0 ± 0.0	0.3 ± 0.0	$H = 9.13$, $P = 0.028^*$	B vs OT, $0.01 < P < 0.025$
RBD: Tailfan	3.00 ± 1.9	1.8 ± 0.9	0.2 ± 0.2	0.2 ± 0.2	$H = 8.76$, $P = 0.034^*$	NS
TBD	9.9 ± 5.0	7.4 ± 0.7	3.4 ± 1.4	2.0 ± 1.1	$F = 5.58$, $P = 0.023^*$	O vs HT, 0.027
TBD standardized by total catch (kg) by area swept (km²) per hour	5.7 ± 4.1	23.6 ± 8.6	ND	ND	$F = 21.2$, $P = 0.001^*$	—
TBD standardized by trap gear soak time (hr)	ND	ND	0.2 ± 0.08	0.3 ± 0.2	NS	—

Mean percent ± SD; $n = 3$ for humpback shrimp compared by gear types with ANOVA and Tukey P values; Kruskal–Wallis test applied to nonparametric comparisons, $\alpha = 0.05$.

* Statistically significant; only statistically significant comparisons given for Tukey tests.

O, otter trawl; B, beam trawl; HT, half-day traps, OT, overnight traps.

TABLE 4.

RBD (mean percent ± SD) (n = 3) for humpback shrimp caught by different gear types compared with ANOVA and Tukey P values.

Gear Type	ANOVA Body Region	TUKEY Body Region, P Values
Otter trawl	H = 14.46, P = 0.002*	C vs A, 0.023
Beam trawl	F = 40.08, P < 0.001*	C vs R, 0.031
		C vs A, <0.001
		C vs T, <0.001
		R vs T, <0.001
		R vs A, <0.001
Overnight traps	H = 9.24, P = 0.026*	R vs A, 0.05
Half-day traps	H = 8.69, P = 0.034*	NS

Kruskal–Wallis test applied to non-parametric comparisons, α = 0.05.
* Statistically significant; only statistically significant comparisons are shown for Tukey tests.
C, carapace; R, rostrum; A, abdomen; T, tail fan.

than the rostrum, abdomen, and tailfan (P = 0.031; P < 0.001; P < 0.001, respectively) and the rostrum had significantly more damage than the abdomen and tailfan (P < 0.001, P < 0.001, respectively, Table 4).

Trapping caused less damage to humpback shrimp than trawling; however, there were still significant differences among shrimp body parts (Table 4). A Kruskal-Wallis ANOVA with data from the half-day traps suggested there was a significant difference (P = 0.034) in the amount of damage assessed between body parts, but a ranked Tukey HSD test failed to reveal any significant differences (P > 0.05; Table 4). Damage to various humpback shrimp body parts from overnight traps, like the half-day traps, were significantly different (P = 0.026; Table 4). The greatest amount of damage in overnight traps was to the rostrum followed by the carapace tailfan and abdomen. The rostrum and abdomen were significantly different (P = 0.05) when tested with a ranked Tukey HSD test (Table 4).

Assessments of Humpback Shrimp Catch and Bycatch

On average humpack shrimp catches were significantly higher (P < 0.001; numbers, biomass) on the trap lines relative to the beam and otter trawl transects (Table 5). Highest catches were on the overnight traplines (582, 7.9 kg). The average weight of individual humpback shrimp was higher for trap-caught animals. (P < 0.0001). Overnight traps collected the largest humpback shrimp (13.7 ± 0.5 g) followed by half-day traps (13.0 ± 0.3 g), otter trawls (8.3 ± 0.5 g), and beam trawls (7.1 ± 1.5 g), respectively (Table 5). There were significant differences in the individual weights of humpback shrimp caught by both otter and beam trawl when compared with both half-day (P < 0.001) and overnight traps (P < 0.001; Table 5).

In addition to humpback shrimp, several other fish and invertebrate species were caught. Beam trawl and otter trawl bycatch was dominated by demersal fish, roundfish, and other shrimp species. Bycatch from the traps included decapod crustaceans, echinoderms, roundfish and smaller shrimp species (Table 6). The average bycatch of finfish was 4 ± 3 per trapline, 51 ± 18 per beam trawl, and 376 ± 218 per otter trawl. Pink shrimp were present in the catch of all harvest methods. However, the abundance of this species was significantly higher in the otter trawl catches (P < 0.0001; Table 6).

DISCUSSION

Effects on Survival

The type and extent of damage to individual shrimp will likely affect survivorship if humpback shrimp are released following their capture, as found for other Crustacea. Stevens (1990) investigated the survival of trawl-caught king crab (*Paralithodes camtschaticus*) and tanner crab (*Chionoecetes bairdi* and *C. opilio*) in the Bering Sea. After injuries to both body and legs of the crabs, survival rates in experimental tanks were about 50% and 75%, respectively, for the two crabs. Lancaster and Frid (2002) documented survival of undersize brown shrimp, *Crangon crangon*, from an UK beam trawl fishery. They reported low mortality and only the occasional loss of a telson or antennae after the catch was brought aboard and sorted by mechanical riddle. However, Bergmann and Moore (2001) suggested that post-trawling mortality of discarded decapod crustaceans have been underestimated, and showed that damaged decapods had a significantly lower longer-term survival (30%) than controls (72–83%). Mensink et al. (2000) showed that only 40% of common whelks, *Buccinum undatum*,

TABLE 5.

Mean biomass and counts (±SD) per transect for humpback shrimp catches arranged by gear types (n = 3).

Gear Type	Otter Trawl	Beam Trawl	Traps Overnight	Traps Half-Day	ANOVA Gear Type	TUKEY Gear Type, P Values
Mean catch weight (kg)	0.3 ± 0.2	3.2 ± 1.6	7.9 ± 1.5	6.6 ± 1.4	F = 20.4, P < 0.001*	B vs OT, 0.01
						O vs HT, 0.002
						O vs OT, <0.0001
Mean catch count (no.)	41 ± 22	458 ± 226	582 ± 131	508 ± 99	F = 8.9, P = 0.006*	B vs O, 0.027
						O vs HT, 0.015
						O vs OT, 0.007
Mean individual shrimp weight (g)	8.3 ± 0.5	7.1 ± 1.5	13.7 ± 0.5	13.0 ± 0.3	F = 44.0, P < 0.0001*	B vs HT, <0.001
						B vs OT, <0.001
						O vs HT, 0.001
						O vs OT, <0.0001

Biomass and counts of various harvests compared separately among gear types with ANOVA and Tukey tests, α = 0.05.
* Statistically significant; only statistically significant comparisons are shown for Tukey tests.
O, otter trawl; B, beam trawl; OT, overnight traps; HT, half-day traps.

<center>TABLE 6.</center>

Mean number of animals harvested from beam trawl, otter trawl, individual trap and trapline catches in Simoom Sound (n = 3).

Species		Beam Trawl Mean ± SD	Otter Trawl Mean ± SD	Individual Trap HD Mean ± SD	Individual Trap ON Mean ± SD	Trapline HD Mean ± SD	Trapline ON Mean ± SD
Common Name	Scientific Name						
Shrimp							
Humpback	*Pandalus hypsinotus*	441 ± 239	41 ± 23	13 ± 2	15 ± 3	508 ± 99.2	582 ± 151
Prawn	*Pandalus platyceros*	—	—	0.08 ± 0.1	0.4 ± 0.3	3 ± 4	17 ± 13
Spiny pink	*Pandalus eous (P. borealis)*	42 ± 63	6123 ± 1059	0.2 ± 0.1	0.2 ± 0.1	8.3 ± 7	10 ± 6
Two-spined crangon	*Crangon communis*	31 ± 10	1 ± 0	—	—		
Short-scaled eualid	*Eualus suckleyi*	0.2 ± 0.3	—	0.2 ± 0.2	7 ± 3	8 ± 8	267 ± 140
Flatfish							
English sole	*Pleuronectes vetulus*	0.3 ± 0.3	—	—	—	—	—
Flathead sole	*Hippoglossoides elassodon*	26 ± 12	0.7 ± 0.6	—	—	—	—
Rock sole	*Pleuronectes bilineatus*	0.5 ± 0.5	—	—	—	—	—
Arrowtooth flounder	*Atheresthes stomias*	0.2 ± 0.3	—	—	—	—	—
Selachii							
Spiny dogfish	*Squalus acanthias*	0.2 ± 0.3	—	—	—	—	—
Spotted ratfish	*Hydrolagus colliei*	9 ± 3	—	—	—	—	—
Longnose skate	*Raja rhina*	0.2 ± 0.3	—	—	—	—	—
Roundfish							
Pacific tomcod	*Microgadus proximus*	—	0.7 ± 0.6	—	—	—	—
Pacific herring	*Clupea harengus pallasi*	—	229 ± 29	—	—	—	—
Blackbelly eelpout	*Lycodopsis pacifica*	0.8 ± 0.3	—	—	—	—	—
Lingcod	*Ophiodon elongatus*	0.2 ± 0.3	—	—	—	—	—
Black cod	*Anoplopoma fimbria*	—	—	0.03 ± 0.01	0.008 ± 0.01	1.0 ± 0.0	0.3 ± 0.6
Walleye Pollock	*Theragra chalcogramma*	1.2 ± 1.6	1.2 ± 1	—	—	—	—
Sandlance	*Ammodytes hexapterus*	—	1.5 ± 2.6	—	—	—	—
Dwarf wrymouth	*Lyconectes aleutensis*	—	—	0.008 ± 0.01	0.05 ± 0.02	0.3 ± 0.6	2.0 ± 1.0
Shiner perch	*Cymatogaster aggregata*	14 ± 13	144.5 ± 96	0.05 ± 0.03	0.008 ± 0.01	2.3 ± 1.5	0.3 ± 0.6
Showy snailfish	*Liparis pulchellus*	0.2 ± 0.3	—	—	—	—	—
Staghorn sculpin	*Leptocottus armatus*	—	—	0.008 ± 0.01	0.008 ± 0.01	0.3 ± 0.6	0.3 ± 0.6
Prickleback	Stichaeidae (Family)	—	0.2 ± 0.3	—	—	—	—
Capelin	*Mallotus villosus*	0.2 ± 0.3	—	—	—	—	—
Invertebrates							
Clams	Bivalvia (class)	0.5 ± 0.5	—	—	—	—	—
Stubby squid	*Rossia pacifica*	0.2 ± 0.3	—	—	—	—	—
Squid	*Loligo opalescens*	—	0.3 ± 0.6	—	—	—	—
Flatworm	Turbellaria (class)	—	0.3 ± 0.6	—	—	—	—
Dungeness crab	*Cancer magister*	—	—	0.02 ± 0.03	—	0.7 ± 1.2	—
Decorator crab	Majidae (family)	—	—	0.008 ± 0.01	—	0.36 ± 0.6	—
Spider crab	Majidae (family)	—	—	—	0.008 ± 0.01	—	0.3 ± 0.6
Tanner crab	*Chionoecetes bairdi*	—	—	0.008 ± 0.01	0.008 ± 0.01	0.3 ± 0.6	0.3 ± 0.6
Sunflower star	*Pycnopodia helianthoides*	—	—	0.03 ± 0.1	0.05 ± 0	1.3 ± 0.6	2.0 ± 0.0

Trapline data calculated for 40 traps. ON, overnight trapline sets of approx. 17 h; HD, half-day trapline sets of approx. 6 h; — indicates no catch.

harvested by a beam trawl survived after a 6-week experimental period. Over 95% of whelks harvested in baited traps survived, suggesting that trapping was relatively benign compared with trawling. Other models assessing collateral mortalities to benthic megafauna have suggested that 5–39% of annual mortalities in fisheries on the Dutch continental shelf can be attributed to trawl discards, with half of the species observed showing values of greater than 20% annual mortality (Bergmann and van Santbrink 2000). Future studies should calibrate damage assessment with survivorship to determine the level of resolution required to create meaningful damage indices.

Possible Causes of Damage

It is likely that the relatively severe damage to humpback shrimp from otter trawling was caused by a combination of factors.

Otter trawl gear was towed at higher speeds for a relatively long distance (643–677 m), had a larger net opening, a finer cod-end mesh and caught more non-target species than trapping or beam trawling. The otter trawl yielded the smallest catch of humpback shrimp of the three gears, but exhibited the largest bycatch of finfish and pink shrimp, which likely resulted in higher internal net pressures, more scouring by other spiny crustaceans, and ultimately more damage to the humpback shrimp catch. However, when total damage to humpback shrimp caught by trawl gear was standardized by total catch weight (kg) by area (km^2) fished per time (h) TBD to humpback shrimp caught by beam trawl was higher compared with otter trawl caught animals. It is important to standardize damage data in a format that will allow for cross-comparisons to take place between investigations. We have chosen to standardize the data according to catch, fished area, and fishing

duration to incorporate effects imposed by factors such as catch size, duration of fishing activity, and boat speed.

BRDs reduction devices were shown to reduce head, carapace, and tail damage to trawl caught penaeid prawns (Salini et al. 2000). These authors also mentioned that prawn damage was higher when large animals were present in the trawls, possibly because of their trashing effect in the codend. In our study, it is possible that the trawls we used might have inflicted more damage to humpback shrimp if BRDs had not been used. Further data are needed to confirm this.

Haulup water pressure and bycatch may have also caused damage to humpback shrimps in traps. However, as the traps were hauled through the water over a short distance (62–75 m, vertically) at a relatively slow rate (about 15 m/minute) and caught fewer bycatch species these factors were lessened relative to trawling. The rostrum and carapace of humpback shrimp from the overnight traps were more damaged than half-day traps possibly because the 17 h soak allowed for more time for the humpback shrimp to collide with each other in efforts to escape. This may have been particularly important at night when shrimp are more active and may have even been attempting to swim off the bottom. Catches were also higher in the overnight sets, leading to more crowding in the traps. However, the differences in damage to the rostrum and carapace for half day and overnight traps were not evident when standardized by soak time.

Additional Damage to Humpback Shrimp from Fishing Gear

In addition to the fragmenting and crushing injuries we observed on the exoskeleton of humpback shrimp, other important but subtle anatomic damage likely occurred during or after capture by both trawl and trap. Examples would be the loss of integumental scales which function as distance receptors (Mauchline et al. 1977) and sensilla (20–500 μm in length) found on the antennae, carapace, walking legs, abdomen, telson, and uropods (Heinisch & Wiese 1987). As observed with our methods, the tailfan and abdominal region of humpback shrimp were the most resistant to damage (<2% damage), and the tailfan received more damage than the abdomen. Tailfan damage differed among gear types, with the beam trawl resulting in the most damage. The telson, which is borne on the tailfan, was reported to carry two pairs of tuft organs used as chemosensors (Mauchline et al. 1977). In our study, the highest damage scores were assigned to the carapace in all cases except overnight trapping, where the rostrum received the highest

damage. The carapace of humpback shrimp also received the highest cumulative damage scores of any body region surveyed and is likely the most critical part of the shrimp's anatomy for survival as it houses the cardiac, gastric and branchial organs. The visceral or gastric region of lobsters (*Homarus americanus*) were also identified by Ganz (1980) as particularly vulnerable to damage from otter trawling, especially during the molting phase. Because our work only assessed damage to humpback shrimp when they are not molting, the estimates presented herein are minima. Further work at different seasons would be required to investigate effects on humpback shrimp at different life stages.

Humpback Shrimp Catch and Biomass

There were considerable differences in the biomass of humpback shrimp harvested in Simoom Sound among fishing methods. Although they fished over approximately the same distance along the bottom of Simoom Sound (500–700 m) humpback shrimp catches in traps were higher than trawl catches. A similar pattern can be seen in the commercial fishery, with trap vessels usually landing more humpback shrimp compared with trawlers (average 20 metric tonnes (t) vs 10 t per year; Boutillier & Nguyen 1999). Standardization of catches by trawling and trapping vessels may be possible using fuel consumption, but data on specific vessels would be required. The mean individual weights of humpback shrimp caught by trap were significantly higher than those from beam trawl or otter trawl, suggesting that traps were selecting for larger individuals. Wright and Panek (2000) http://www.orst.edu/Dept/IIFET/2000/papers/wright2.pdf have suggested that there is an inverse relationship between the trap soak-time and the weight of prawns (*Pandalus platyceros*) harvested. However, we found no significant differences between the individual size of humpback shrimp caught in half-day versus overnight traps.

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INTRASPECIFIC AGGREGATION STRUCTURE OF A SHOAL OF A WESTERN MEDITERRANEAN (CATALAN COAST) DEEP-SEA SHRIMP, *ARISTEUS ANTENNATUS* (RISSO, 1816), DURING THE REPRODUCTIVE PERIOD

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ABSTRACT   The deep-sea rose shrimp, *Aristeus antennatus*, constitute an important fishery resource in the Western Mediterranean Sea. The spatio-temporal behavioral pattern of *A. antennatus* is well-known, with the species forming seasonal aggregations on the middle slope at depths between 400 and 900 m. These aggregations form between late winter and early summer. The object of the present study is to determine the internal structure of shoals of the western Mediterranean (Catalan coast) rose shrimp along the slope on the grounds where the species is fished (from 400 to 1000 m) at the time of peak density during the reproductive period. Interactions between fishing and research vessel have been used to sample synchronically and bathymetrically the shoals of the deep-sea shrimp to determine intra and interspecific shoal structures. The results of this study on *A. antennatus* have specifically shown that (1) The pattern shrimp shoal distribution is such that density rises rapidly in the portion located in the shallower distribution range of this species and then gradually decreases at greater depths; (2) the distribution of this resource straddles both sides of the ecological boundary located at 900 m, though with changes in the sex-ratio and individual size; (3) species coexisting with this shrimp species are concentrated at depths other than the depths of peak shrimp density; (4) commercial trawlers deploy according to the abundance pattern of the resource; and (5) the reproductive portion of the stock is heavily exploited.

KEY WORDS: Aristeus antennatus, Mediterranean Sea, population structure, aggregation, sex ratio, size frequencies, fisheries, shoals

INTRODUCTION

The deep-sea rose shrimp, *Aristeus antennatus* (Risso, 1816) (Crustacea, Decapoda, Dendrobranchiata, Aristeidae), represents an important fishery in the Western Mediterranean Sea (Sardà & Martín 1986, Demestre & Lleonart 1993, Bianchini & Ragonese 1994, Carbonell et al. 1999). This species is a characteristic component of the demersal muddy bottom community on the middle slope at depths between 400 and 1,200 m (Cartes & Sardà 1993), where Cartes & Sardà (1992) and Maynou & Cartes (2000) have defined it as a nektobenthic species of moderate-to-high swimming mobility. However, the distribution of this species is also fished frequently between 400 and 800 m in other Mediterranean areas (Bianchini & Regonese 1994, Carbonell et al. 1999, Papaconstantinou & Kapiris 2001, Cau et al. 2002). The distribution of this species is nonetheless considerably broader, reaching at least to depths of 2250 m (Sardà & Cartes 1992, 1993), indicating that the species is eurybathic with a distribution considerably broader than that of other decapod crustacean species.

The spatiotemporal behavioral pattern of *A. antennatus* is well known, with the species forming seasonal aggregations on the middle slope at depths between 400 and 900 m. These aggregations form between late winter and early summer (Tobar & Sardà 1987, Demestre & Martín 1993, Sardà et al. 1994). Towards the end of summer, the shrimp shoals tend to break up and move inside submarine canyons, with the shrimp being fished at shallower depths (400–700 m) along the margins of the canyons, locations that are less accessible to trawlers (Sardà 1993, Sardà et al. 1994, Sardà et al. 1997).

During the period in which this species forms aggregations (late winter to early summer), shoals consist of reproductive adult females. Copulation takes place at the start of the aggregation stage (Relini Orsi, 1980, Sardà & Demestre 1987) with a percentage of

males in the population of less than 20% (Sardà & Cartes 1992, Demestre & Martín 1993, Sardà et al. 1994). Tursi et al. (1996) reported that during copulation in late winter, males can be 50% of the population in Ionian Sea. Studies conducted on the catchability of shoals of this species (Sardà & Maynou 1998) have suggested that the shoals take on an elongate shape parallel to the coast. It is exactly at this time when the shrimp stock bears the brunt of fishing effort (Tudela et al. 2003), because shoal formation is at its peak on the part of the slope most readily accessible to trawlers and females attain maximum size, that is, biomass concentration is also at its peak. In addition, marketability of this species is also highest at this time.

Studies on schooling in pelagic species (Swartzman et al. 1994, Nonacs et al. 1994, Nottestad et al. 1996) particularly using echo-sounding, and in species in captivity (Pitcher 1983, Pitcher et al. 1985), have been common, but there have been very few such studies on benthic or benthopelagic species. Gordoa & Duarte (1991) considered some *Merluccius* species and reported size-dependent schooling behavior. Macpherson & Duarte (1991) also related size and depth for different fish species and discussed the possible existence of a general size-depth relationship. On the whole, studies on schooling and shoaling behavior have been quite diverse in terms of methodology used, and they have also dealt with a range of different aspects. Furthermore, although shoaling of coastal prawns and migratory displacements relating to their life cycles are well known (García & Le Reste 1987), our literature review has not disclosed any similar studies on shoaling patterns for species dwelling at depths below the margin of the continental shelf.

Accordingly, the object of the present study was to determine the depth structure of shoals of the Catalan coast rose shrimp, *Aristeus antennatus* (Risso, 1816), along the slope on the grounds where the species is fished (from 700 to 1000 m) at the time of peak density (aggregation). Bearing in mind, however, that the depth distribution for this species extends across several community boundaries (down to at least 3000 m in depth; Sardà 2001), the

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role of the noncommercially exploited portion of the population on the population as a whole has also been discussed. Shoaling structure has been considered in terms of both intraspecific aspects, such as density, size range, and sex ratio, and interspecific aspects, i.e., density relationships between the rose shrimp and other fish and crustacean species dwelling in the same faunal assemblage, on the basis of depth. Our goal has been to underscore the importance of understanding the intra and interspecific structure of aggregations of marine species as a significant factor in establishing the actual level of vulnerability to exploitation by fisheries. In addition, over and above a simple discussion of the results presented here, this article aspires to be an example of studies of this kind and thus also includes a consideration of ecological and fisheries aspects in the discussion, relating them to the specialized literature.

MATERIAL AND METHODS

A study was conducted jointly by the *R/V García del Cid* and commercial trawlers on 21 to 23 June 2000 on the "Serola" fishing grounds located off Barcelona (Northwest Mediterranean Sea), where mature females of the deep-sea rose shrimp typically aggregate at that time of year (Fig. 1).

To be able to obtain an instantaneous view of the aggregation structure of a shoal of this deep-water species, operations must be completed in the shortest possible time to avoid variations in response to sudden environmental changes affecting community structure. The weather was sunny and good over the 48 h in which sampling was performed and remained stable over the course of the survey.

The fishing vessels operating in this area are trawlers from the port of Barcelona specialized in the shrimp fishery, with engine power ratings ranging between 800 and 1100 horsepower and lengths between 17 and 21 m. Five fishing trawlers conducted fishing operations during their normal operating hours at depths

between 780 and 850 m. Haul duration was typical for the fishery, namely, two hauls daily lasting about 3.5 h each. Landings by these trawlers were recorded on June 22, 2000, by a surveyor at the wharf. Trawler headings and locations were monitored continuously using the Automatic Radar Plotting Aid (ARPA) radar system on board the research vessel, which made it possible to follow the courses of their hauls from start to finish (Fig. 1).

The *R/V García del Cid* is 38 m in length with an engine power rating of 1100 horsepower. It operated concurrently with the trawlers in the same area, but over a broader depth range, between 700 and 1 200 m (Fig. 1). A total of 11 daytime and nighttime hauls were conducted on June 21 to 23, 2000, at least two hauls in each of the 700-, 800-, 900-, 1000-, and 1200-m depth intervals. Depths, towing speed, starting time, and ending time were recorded for each haul (Table 1). The horizontal mouth opening of the gear between the wings (13.5 m) was also recorded using remotely operated Scanmar sensors.

Haul duration was 1 h to ensure that the sampling data would be discrete and suitable for use in discriminatory analysis. Biologic data collected consisted of the number and individual weight of all specimens caught. Standard carapace length (CL in mm), individual weight in g, sex, and maturity stage were recorded for all rose shrimp specimens. The data from fishing trawlers and the data from the research vessels are not directly comparable and only relative comparisons were undertaken. These data were presented in different graphics and with different units. Only biologic data and size frequencies obtained on board the research vessel were used in data treatment to avoid potential deviations. Only those females in an advanced gonad maturity stage (maturity stages IV and V according to the gonad coloration scale published by Relini & Relini (1979) as expanded by Demestre & Fortuño (1992) were classified as mature). Percentage size frequency values were compared using multivariate analysis to evaluate similarity of the surface areas of the bars and using the Kolmogorov–Smirnov test ($P < 0.05$) to compare the cumulative frequency values.

The sampling protocol used, using short hauls yielding data that were highly discrete in terms of time, provided a "snapshot" of the resource. This strategy has furnished good results when used in studies of the density and spatial distribution of benthic (González-Gurriarán et al. 1993, Maynou et al. 1996) and benthopelagic (Carter et al. 1993) crustaceans. Samples of longer duration would have entailed the risk of introducing new variables, principally in relation to changes in weather, which would definitely be a potential source of noise in the data analysis. On the basis of the results of previous work conducted in this same area published by Tobar & Sardà (1987), Demestre & Martín, (1993), Sardà et al. (1994), and Sardà et al. (1998), shoals of *A. antennatus* are continuously present at the sampling depths from late winter to early summer.

Biomass in number and individual weight standardized to km², on the basis of the area swept by each haul, have been graphically represented using the sampling data collected by the research vessel. Measurements were effected individually and overall and on the total of fish and other shrimp species. In the case of the commercial trawlers, which were not equipped with remotely controlled monitoring systems, shrimp landings ($kg \cdot h^{-1}$) were weighted on the basis of the length of the working day (7 $h \cdot d^{-1}$). Specialized personnel was on board of each commercial vessel weighting the shrimp caught. Also bills of sales in auction was collected to compare data on board. Figure 1 includes trend lines fit visually to facilitate interpretation and discussion of the data.
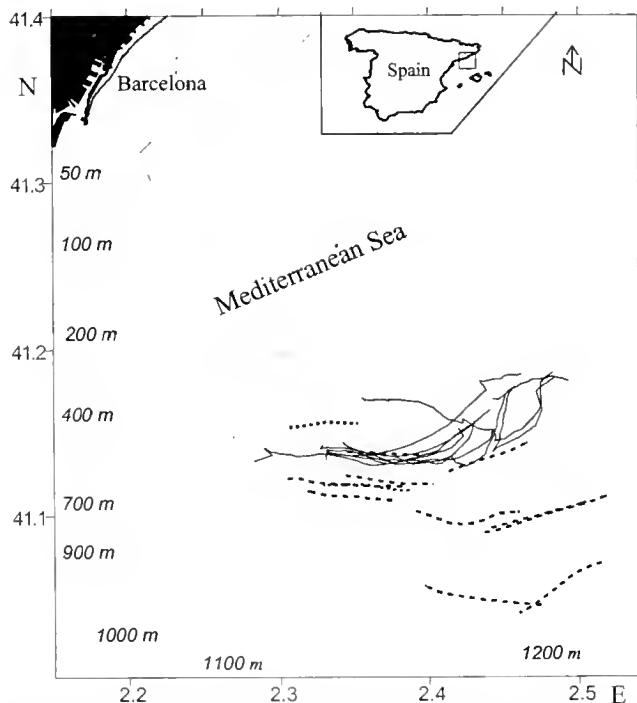

Figure 1. Study area showing the transects on which hauls were performed by the research vessel (dotted lines) and the trawlers (solid lines).

TABLE 1.

List of hauls effected.

Haul Code	Depth (m)	D/N	Local Starting Time (h)	Local Ending Time (h)	Starting Position	Ending Position	Swept Area (km²)
H1	700	D	15:03	16:05	41° 09' 01" N 02° 18' 04" E	41° 08' 42" N 02° 22' 01" E	0.06290
H2	800	D	17:52	18:47	41° 08' 08" N 02° 20' 32" E	41° 08' 27" N 02° 16' 49" E	0.06806
H3	900	N	21:43	22:56	41° 07' 44" N 02° 21' 37" E	41° 07' 43" N 02° 25' 43" E	0.08001
H4	1000	N	3:53	5:20	41° 06' 07" N 02° 23' 06" E	41° 08' 16" N 02° 29' 13" E	0.09779
H5	1200	D	8:03	9:16	41° 05' 53" N 02° 28' 56" E	41° 04' 06" N 02° 25' 42" E	0.06826
H6	750	D	12:17	13:18	41° 08' 30" N 02° 21' 21" E	41° 08' 40" N 02° 17' 50" E	0.06645
H7	1000	D	15:32	16:33	41° 07' 08" N 02° 24' 34" E	41° 08' 15" N 02° 28' 51" E	0.09005
H8	1200	N	19:33	21:09	41° 04' 23" N 02° 27' 05" E	41° 05' 04" N 02° 21' 55" E	0.08116
H9	750	N	0:18	1:42	41° 08' 38" N 02° 21' 01" E	41° 08' 51" N 02° 16' 09" E	0.07038
H10	800	N	3:44	4:40	41° 08' 18" N 02° 20' 36" E	41° 08' 14" N 02° 23' 58" E	0.07006
H11	900	D	6:52	7:54	41° 07' 34" N 02° 24' 44" E	41° 08' 40" N 02° 27' 59" E	0.06714

D/N, day–night hauls.

A matrix consisting of species (columns) and hauls (rows) was constructed for community analysis. Species that occurred only in a single haul and species occasionally represented by only a single individual in some hauls have not been included. The data were log transformed ln (x+1) and used in multivariate cluster analysis. The linear correlation value was used as the similarity index and UPGMA as the aggregation algorithm.

The abundance ratios for rose shrimp to other crustacean and fish species were calculated by dividing the number of rose shrimp individuals by the total number of individuals of all species in the other two groups, crustaceans and fishes, respectively. Diversity was calculated using Simpson's index, a good discriminator for indicating dominance by a given species or group of species (May, 1975), which is the case of the rose shrimp here, the predominant species in the present study. This diversity index has been recommended for use in comparisons of marine communities (Lambshead et al.1983).

RESULTS

Abundance and Distribution

Shrimp abundance on the basis of the samples collected by the research and commercial vessels have been depicted in biomass (Fig. 2a and b) and number of individuals (Fig. 3). These figures show that the lowest catches, with densities of about 20 ind. km⁻², were made at around 700 m in depth, whereas the highest catch densities, of around 1700 ind.·km⁻², were made at 800 m. Individual density levels then tapered off progressively with increasing depth. These results clearly define a specific structure across the shoal with depth, with rose shrimp density augmenting sharply in the shallowest portion of the shoal and then gradually falling off towards the deepest portion.

The yields obtained by the trawler that effected tows at a depth of around 800 m (Fig. 2b) were 2-fold those of the three trawlers operating at greater depths and 5-fold those of the trawler operating at a shallower depth. Trawler deployment thus mirrored the distribution of the shrimp resource being fished: one vessel operating at 700 m, where shrimp density was lowest; one vessel operating at 800 m, where shrimp density was highest; and three vessels operating at more than 800 m, where biomass began to taper off. This spatial deployment of the fishing trawlers was dictated by the amount of space that had to be left between them to ensure proper maneuverability. The first vessel to reach the fishing grounds begins to work at the depth the skipper deems best to achieve the highest yields. Vessels arriving later will then take up a position next to the vessels already present, though always on the deeper side, where rose shrimp density will tend to be lower still profitable.

Day-Night Shoal Structure

Figures 2a and 3 depict the hauls conducted in the daytime (hollow circles) and nighttime (solid circles). The distribution pat-
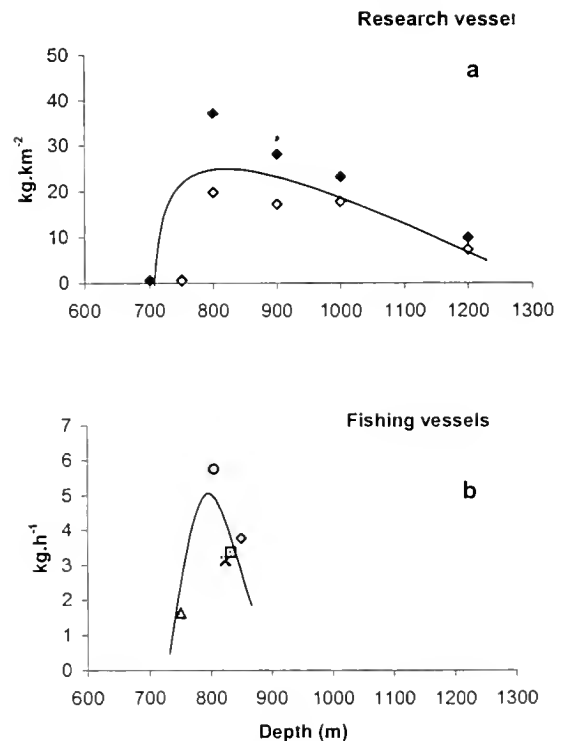

Figure 2. Catches in weight by depth taken by the research vessel (a) and the trawlers (b). Hollow symbols, day samples. Solid symbols, night samples. Grey points, different fishing vessels.
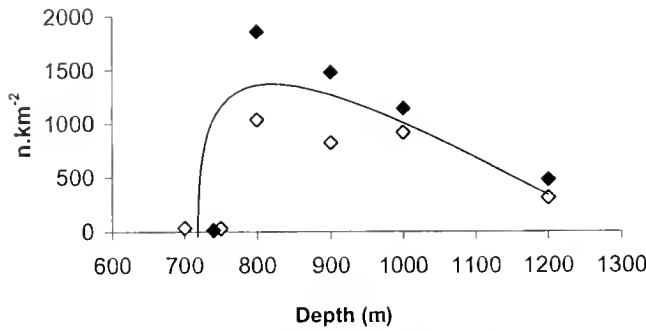
Figure 3. Densities by depth made by the research vessel. Hollow symbols, day samples. Solid symbols, night samples.

tern can be observed to differ according to depth. In the depth range between 750 and 900 m catches were higher at night than in the daytime, which suggests that part of the population migrate to the upper portion of the slope at night. The low individual densities at 700 m were insufficient to allow any reliable inferences concerning daytime–nighttime movements. Differences between daytime and nighttime catches appeared to decrease with depth; however, it should be noted that commercial day-night catch data were

unavailable for comparison with the experimental catch data, because commercial trawlers are not allowed to operate at night. This observations coincides with the migrations of decapods suggested by Cartes et al. (1993).

Size Frequencies

Figure 4 shows the size frequencies for females at the different sampling depths. The bell-shaped size frequency curves tended to flatten out and have wider tails with depth both in the daytime and at night. At depths around 800 m the population tended to consist of females with a modal mean size of 40 mm CL, ranging from a minimum of 20 mm CL to a maximum of 51 mm CL. A similar structure was observed at 1000 m. However, from 1000 m, there was a change in the size frequency distribution, with the proportions of both the smallest sizes and the largest sizes increasing. This trend was quite distinct at 1200 m, despite the low number of individuals, however due to the low occurrence of *A. antennatus* in this depth, the number of individuals caught was considered sufficient for a good size spectrum on this depth. Because of the small number of individuals caught at 700 m, it was not possible to construct a sufficiently representative size structure for that depth.

The similarity analysis for the size frequencies (Table 2) indicated significant differences ($P < 0.05$) between the size frequen-
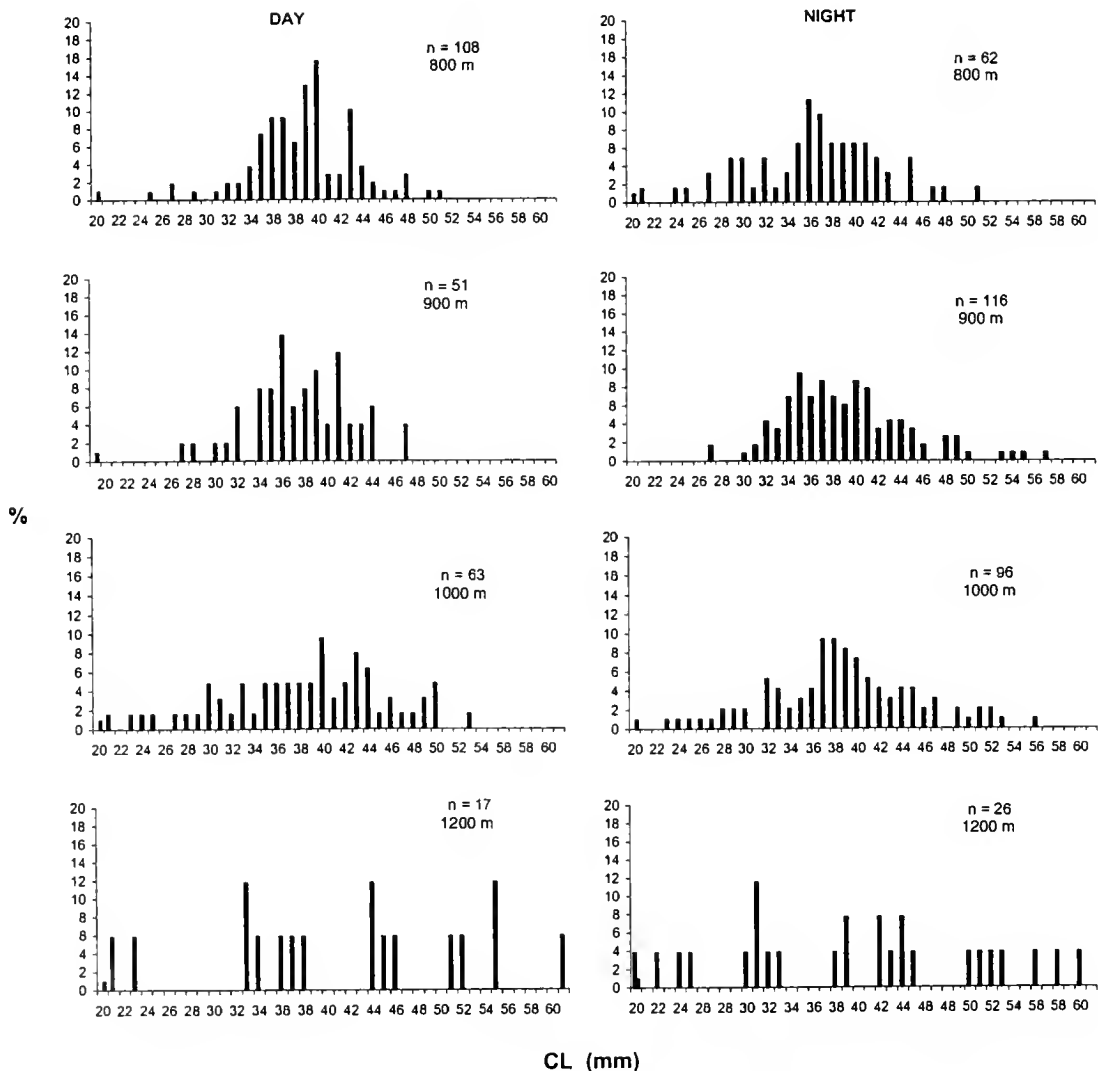
Figure 4. Size frequencies for females by depth and by daytime-nighttime. CL, carapace length.

TABLE 2.

Similarity between frequency values.

H3	900 m	N	0.724						
H4	1000 m	N	0.717	0.728					
H5	1200 m	D	0.306*	0.373*	0.378*				
H7	1000 m	D	0.653	0.677	0.697	0.349			
H8	1200 m	N	0.380*	0.365	0.460	0.469	0.426		
H10	800 m	N	0.694	0.736	0.649	0.305*	0.657	0.310*	
H11	900 m	D	0.683	0.723	0.670	0.333*	0.638	0.386*	0.711
			H2 800 m N	H3 900 m N	H4 1000 m N	H5 1200 m D	H7 1000 m D	H8 1200 m N	H10 800 m N

H, haul code; D, day; N, night.

* Significant differences (*P* < 0.05) *Kolmogorov–Smirnov* test.

cies for the 800–900 m and 1000–1200 m depth intervals but not between daytime and nighttime. There was a tendency towards greater spread of the sizes at deeper depths, with higher proportions of both juvenile and larger individuals. It is interesting to note that the most relevant changes in the size structure of the *A. antennatus* population were linked to the community boundary (below 900 m) for the species, as will be discussed in the following section. Males exhibited a trend similar to that of the females, with greater proportions of the extreme sizes (small and large individuals) at depths greater than 900–1000 m (Fig. 5). However, because of the low occurrence of males in the population at the time of year when the study was carried out, no reliable analysis of the level of significance was possible.

Sex Ratio

The sex ratio (Fig. 6) was characterized by the low presence of males at depths of 800 m (<20%) and 900 m (<5%.). The proportion of males increased progressively from 1000 m, gradually rising to nearly 40%. Virtually 100% of the adult females were mature, and all bore a spermatophore on the telycum, which confirms that the shoal was at the spawning stage, as demonstrated in various earlier papers (Sardà & Demestre 1987, Demestre & Fortuño 1992, Sardà et al. 1994).

Assemblages Differences

The increase in biomass with depth (Figs. 7 and 8) was caused principally by the presence of very large species, e.g., *Alepocephalus rostratus*, *Mora moro*, and *Lepidion lepidion*, which are typical of the community below 900 m, and their occurrence also raised abundance levels (Table 3). According to the results of the cluster analysis, the main discriminating factor was the presence of deep-water fish and crustacean species in the depth interval considered in this study, such as *Bathypterois mediterraneus*, *Mora moro*, *Nezumia aequalis*, *Acanthephyra eximia*, *Geryon longipes*, *Munida tenuimana*, *Paromola cuvieri*, and *Sergestes arcticus*, as opposed to species that are characteristic of shallower depths, such as *Hymenocephalus italicus*, *Phycis blennoides*, *Trachyrhynchus scabrus*, *Schyliorhinus canicula*, *Aristeus antennatus*, *Pasiphaea multidentata*, *Pasiphaea sivado*, and *Polycheles typhlops* (Fig. 9). Trawls nos. 1, 2, 9, 6, and 10 made up a group comprising the 700- and 800-m depth intervals. Trawls nos. 3, 5, 4, 7, 11, and 8 made up a group comprising the depth intervals between 900 and 1200
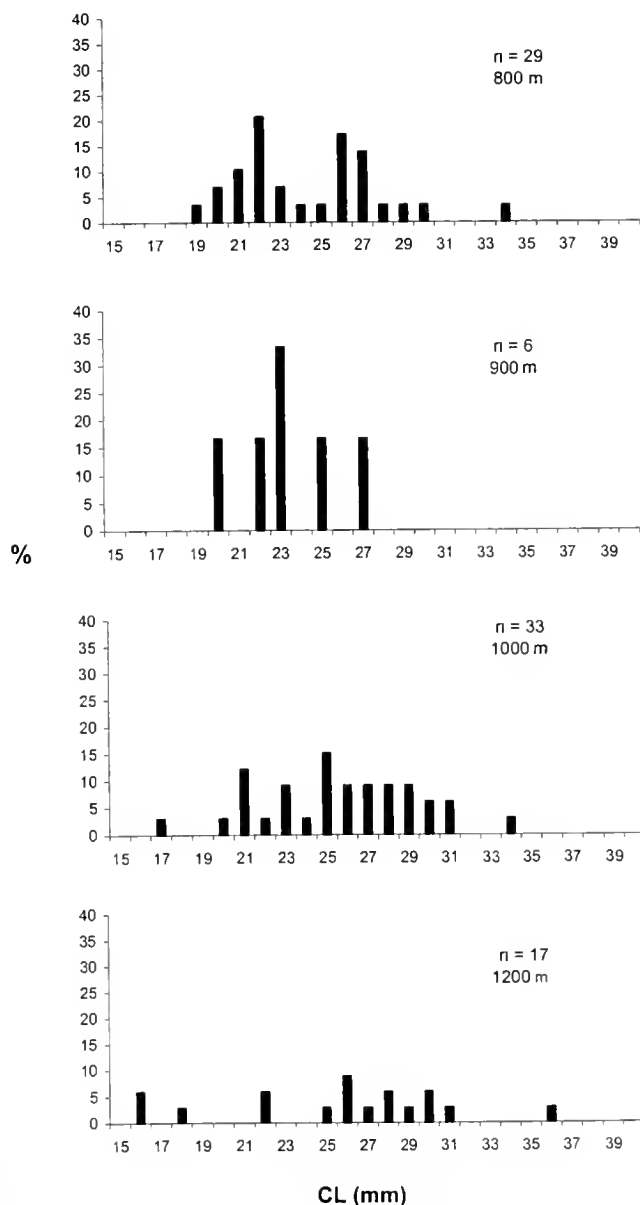

Figure 5. Size frequencies for males by depth. CL, carapace length.
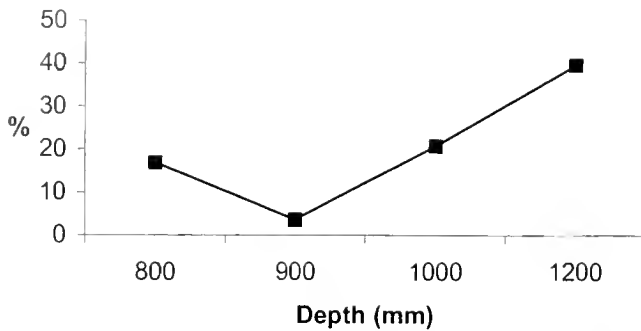
Figure 6. Sex ratio by depth expressed as the percentage of males.

Figure 8. Biomass by depth.

m. It is important to bear in mind that, unlike most other species, distribution of the deep-water rose shrimp is virtually continuous from 700–800 m to more than 1200 m (Fig. 2). Accordingly, this species is represented and attains high abundance levels in both of the assemblages revealed by the cluster analysis.

Density-Dependent Exclusion

The density of other species was lowest between 800 and 1000 m, where shrimp shoal density was highest. As *A. antennatus* density decreased with depth, the density of other species increased progressively (Fig. 10), which suggests a high degree of density-dependent exclusion between the shoal of shrimps, the dominant species, and the distribution of other species at the same depth. This was also reflected by diversity, which displayed little variation between trawls, with Simpson index values between 0.09 and 0.19 (Fig. 11). High index values are indicative of lower diversity due to dominance by one or just a few species. Index values were highest (reflecting single species abundance and low diversity) and displayed less dispersion for the depth intervals between 800 and 1000 m, as a consequence of the greater occurrence of shrimps in those intervals. Diversity index values were most variable for the extreme depth intervals sampled (700 and 1200 m), bearing out the preceding results.

DISCUSSION

A number of workers have described the composition of the community supporting the deep-sea rose shrimp, *Aristeus antennatus*, fishery in the Western Mediterranean Sea separately for crustaceans and for fish (Abelló et al. 1988, Stefanescu et al. 1992, Cartes & Sardà 1993, Stefanescu et al. 1994). This community, dwelling over muddy bottoms on the middle slope, is composed
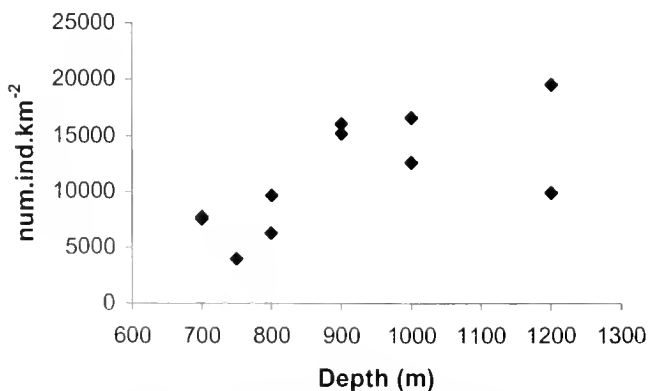
Figure 7. Individual abundance by depth.
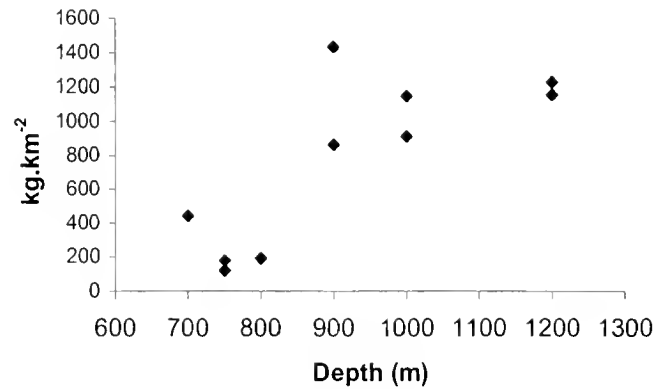
principally of the target species, *Aristeus antennatus*, along with other species of no commercial interest, e.g., *Geryon longipes*, *Polycheles typhlops*, *Lepidion lepidion*, *Alepochephalus rostratus*, and *Trachyrhynchus scabrus*. In any case, *A. antennatus* is an interesting species as compared with the other species dwelling in the community because of certain specific biologic characteristics, namely, (1) its broad depth distribution, making it a highly eurybathic species, and (2) even though fishing pressure has been extremely high over the past 40 y, the population seems to be in a healthy state of exploitation. Cartes & Sardà (1993) defined three main zonations for the deep-sea decapod fauna in the Western Mediterranean: the upper middle slope above 670 m; the lower middle slope between 850 and 1200 m; and, below this last-mentioned depth, a transition zone to the lower slope community (down to 2000 m). However, sampling between 650 and 900 m in that study was inadequate, and the depth limit between the upper middle slope and lower middle slope assemblages could not be accurately determined. Based on the samples collected in the present study, the boundary between the upper middle slope and the lower middle slope would appear to be more exactly situated at around 900 m related exclusively for *A. antennatus*. The above-mentioned boundaries represent genuine barriers to distribution for different decapod crustacean and fish species, but not for *A. antennatus*, which enjoys a continuous distribution from 550 and at least 3000 m (Sardà et al. 1993, Sardà 2001). However, we must consider here that the definition of boundary is a controversial question, often depending on the sampling adequacy and data used in the analysis. Haedrix & Merret (1990) and Koslow (1993) and Stefanescu et al. (1993) and Moranta et al. 1998, provided respectively different results investigating in the same areas, however only fishes are considered in these studies. In this paper we present clusters including crustacean and fishes, reaching similar results as Morales-Nin et al. (2003) with a first boundary around 800 m depth. However, as has been observed in the present study, changes in the internal population structure of this species are apparent, linked to a community boundary existing at around 900 m. Similarly, the findings presented here have demonstrated that the main stock, in terms of fishable biomass, is distributed chiefly between 700 and 1,000 m during the period of gonadal maturation from late winter to early summer. This portion of the *A. antennatus* population consists primarily of females, with low proportions of males (<10%) and medium-sized individuals. The highest fishing effort is expended during the reproductive period of females. All these aspects would appear to suggest that this species can be expected to quickly become overexploited, but this does not seem

TABLE 3.

Species abundances (number individuals km^{-2}).

Species	Haul Code Depth (m)	1 700	9 700	6 750	2 800	10 800	11 900	3 900	4 1000	7 1000	5 1200	8 1200
Pisces												
Alepocephalus rostratus		0	0	60	0	0	3098	462	2516	2254	689	4731
Antonogadus megalokynodon		97	71	0	15	14	0	0	0	0	0	0
Bathypterois mediterraneus		0	0	0	0	0	0	0	20	11	0	123
Benthocometes robustus		0	0	0	0	300	0	0	0	11	0	12
Coelorhynchus coelorhynchus		0	782	301	44	414	0	50	0	0	234	554
Chauliodus sloani		16	14	0	0	29	0	0	0	0	0	0
Epigonus telescopus		48	14	0	15	0	0	0	0	0	0	0
Hymenocephalus italicus		403	85	45	29	0	0	0	0	0	0	0
Lampanyctus crocodilus		48	71	15	73	29	119	37	10	111	73	0
Lepidion lepidion		113	384	150	191	271	1162	1850	1391	1199	1143	1799
Myctophidae		16	57	15	0	43	0	0	0	11	0	0
Mora moro		0	171	120	59	157	149	75	61	477	308	308
Nettastoma melanurum		0	0	0	0	0	0	25	61	0	29	25
Nezumia aequalis		16	28	0	88	29	447	612	654	644	293	357
Notacanthus bonapartei		64	355	75	59	71	60	137	1023	167	15	74
Phycis blennoides		225	57	120	59	29	149	137	20	44	15	0
Symphurus nigrescens		48	14	30	0	14	0	0	0	0	0	0
Trachyrhynchus scabrus		1208	298	316	720	571	1549	2125	1166	655	469	789
Selaceans												
Etmopterus spinax		0	0	0	0	0	30	0	0	11	59	74
Galeus melanostomus		113	242	241	309	200	387	525	123	144	322	308
Scyliorhinus canicula		48	28	0	15	0	0	0	0	0	0	0
Crustaceans												
Acantephyra eximia		0	14	15	0	0	119	62	757	155	483	838
Aristeus antennatus		32	568	30	1028	1856	819	1350	1135	888	308	456
Geryon longipes		113	43	45	88	143	194	187	317	122	132	271
Monodaeus couchi		0	0	0	0	14	0	0	0	0	0	271
Munida tenuimana		16	227	0	59	200	89	37	61	33	293	961
Paromola cuvieri		16	43	0	15	57	209	50	51	67	88	99
Pasiphaea multidentata		129	28	150	59	29	30	87	20	0	29	49
Pasiphaea sivado		0	0	15	0	0	0	175	0	0	15	0
Plesionika acanthonotus		0	57	0	0	14	119	0	20	67	0	25
Plesionika martia		741	28	211	59	29	30	50	0	0	0	0
Polycheles thyphlops		290	227	75	176	428	179	137	102	144	44	25
Pontophilus norvegicus		0	0	0	0	57	89	87	41	144	59	25
Sergestes arcticus		0	0	0	0	14	30	0	0	56	147	0
Sergia robusta		16	0	0	0	0	89	0	0	11	59	0

to be the actual condition of the stock (Demestre & Lleonart 1993). Accordingly, perhaps the biology and internal population structure of this species may somehow include the necessary features to avert potential overexploitation.

The community boundary at around 900 m described here is mainly the result of the upper limit to the depth distribution range for such species as *Alepocephalus rostratus, Lepidion lepidion, Nezumia aequalis, Acanthephira eximia,* and *Geryon longipes,* species with high abundance and biomass levels. Also, the ARPA log system results indicate that this same depth is the maximum fishing depth at which commercial trawlers operate following the *A. antennatus* shoals. Therefore, this community boundary could be a direct effect of the high fishing pressure down to the said depth of 900 m. On the other hand, at the present time no technical constraints preventing fishing operations at deeper depths exist, yet fishermen seem to be aware that there is a community boundary at that level and thus do not operate at deeper depths, in the knowledge that yields of *A. antennatus* there will be insufficient.

The deep-sea rose shrimp presents a well-defined distribution pattern across this boundary at 900 m. The number of individuals making up the shoal rose sharply from 750 to 800 m, that is, over a depth interval of around 500 m, spatially equivalent to about one mile, given the bottom configuration at the study location. From 900 m shrimp abundance fell off gradually over a distance of about 5 or 6 miles down to a depth of around 1200 m, though shrimp distribution continues over a distance of several dozen miles out to the bathyal zone (Sardà et al. 1993, Maynou & Cartes 2000). Shoals were tongue-shaped situated parallel to the depth profile, with peak abundance in the shallower portion (Sardà & Maynou 1998). Small daily or weekly variations in shoal location caused the fishing trawlers to relocate operations over the depth of peak shrimp abundance (Sardà & Maynou 1998). To date trawlers have not undertaken shrimp fishing operations at deeper depths for technical reasons: insufficiently large winch size, distance to the fishing grounds offshore, unfamiliarity with the bottoms, etc.; even so, in recent years trawlers have been observed to expand their fishing depth gradually down to 1000 m. Nevertheless, shrimp specimens caught experimentally at depths below 1000 m have been shown to
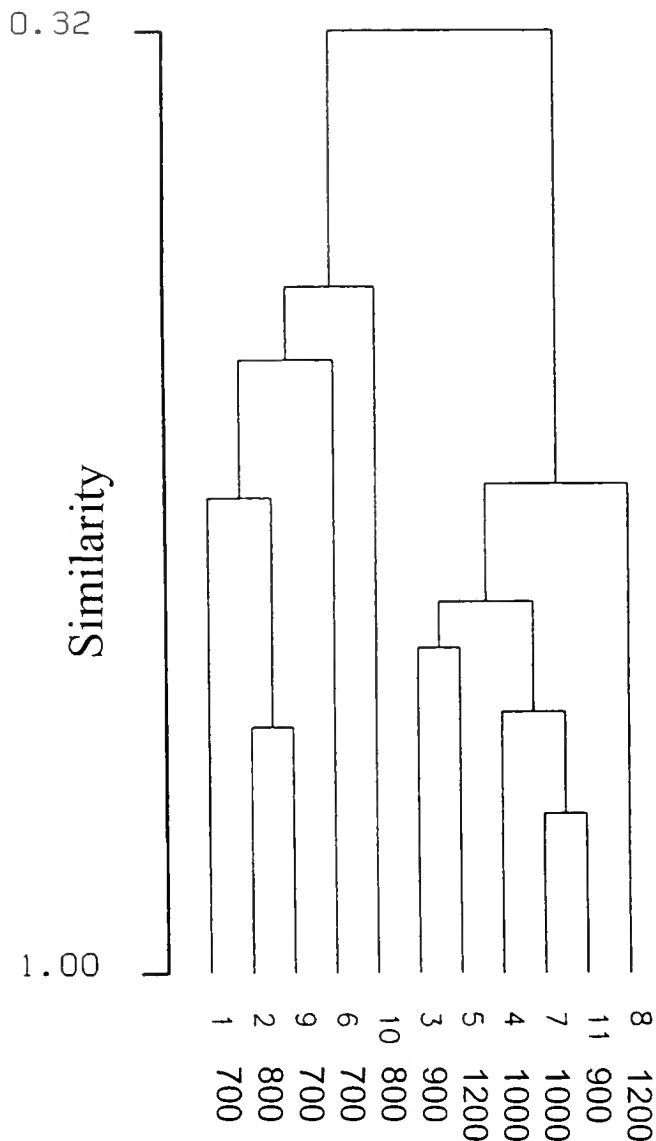
Figure 9. Cluster illustrating the similarity between hauls carried out at different depths.

he, on average, smaller in size (Sardà et al. 1994). The size frequencies set out herein bear out that observation. Furthermore, the proportion of males increases (Sardà et al. 1993), with males being smaller than females as a consequence of this species' sexual dimorphism, as illustrated in Figure 5. These features lower the commercial attractiveness of the deeper shoals and cause the fishing trawlers to stay within the more commercially profitable depth range. Trawlers have never been recorded on the deeper portion of these fishing grounds (Sardà et al. 1998), suggesting that the shoal structure described here remains unchanged at this time of year. The literature contains no discussion of the role of this unexploited or pristine portion of the stock (below 1000 m) in relation to the exploited portion of the stock at shallower depths. Furthermore, stock assessment studies (Demestre & Lleonart 1993, Martínez-Baños 1997, García-Rodríguez & Esteban 1999) have suggested that despite the high level of fishing pressure to which they are subjected as a target species, shrimp stocks are not overexploited. The stability of the population in the face of fishing pressure would seem to support the assumption that the stocks are replenished by
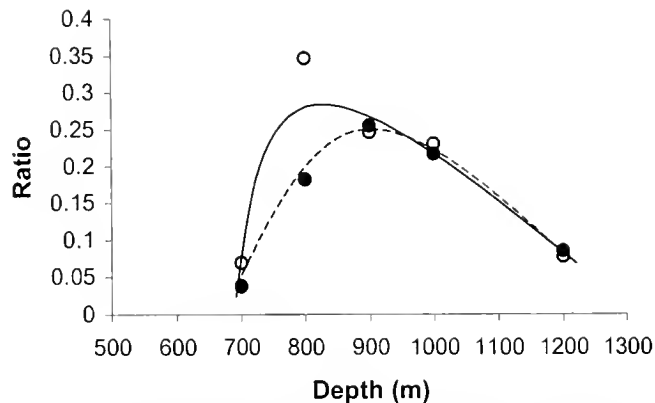
Figure 10. Ratios for the number of shrimp individuals in relationship to other species. Hollow circles, fishes (solid line); solid circles, crustaceans (dotted line).

an influx of individuals from the pristine populations located in deeper waters.

Based on the size frequency data, modal size at first maturity for males would appear to be in the neighborhood of 21 mm CL (Sardà & Demestre 1987, Sardà & Cartes 1997). Mean size of males appeared to be located mainly in the 800 m depth interval, where they coexist with sexually mature females. The implication is that mating takes place principally between adult females and two-year-old males around the time of first maturity (Demestre & Fortuño 1992), with smaller and larger males being less aggregated and mainly present at deeper depths, down to 1200 m (Sardà & Cartes 1997). To date there is no further evidence to suggest that these depths are recruitment zones or are subject to lower predation and thus are more conducive to juvenile development, as postulated for deep-water species by Gage & Tyler (1990).

There is little information on daytime and nighttime variations in catches, the only data available being provisional data for the Gulf of Taranto (Maiorano et al. 1999 and unpublished data), the area off Sardinia (Sabatini et al. 1999 and unpublished data), and the area off Algeria in North Africa (A. Campillo and A. Nouar,
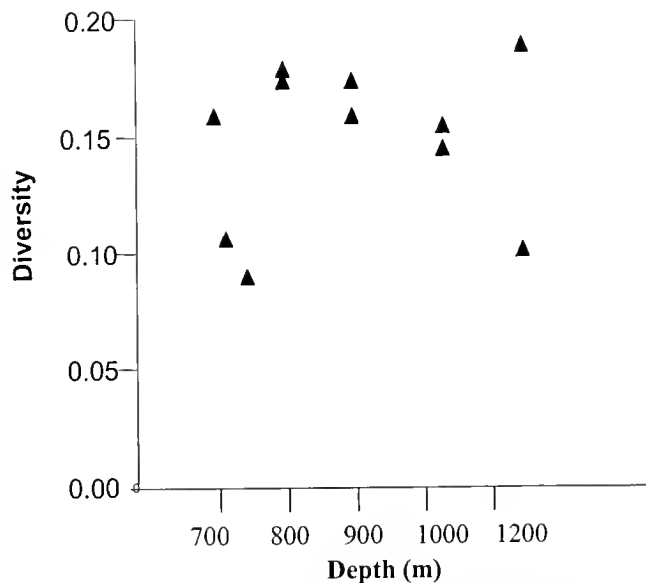
Figure 11. Plot of Simpson's diversity index by depth.

personal communications). The literature reviewed contains no further studies on this topic. According to the researchers just mentioned above, the shrimp population carries out nocturnal migrations up the depth profile and can thus be caught at shallower depths at night. A similar pattern was observed over the depth interval considered in the experiment reported here, with nighttime catches being somewhat higher than daytime catches and the difference peaking in shallower waters at around 800 m. The difference decreased with depth, suggesting the possible involvement of an effect related to light levels (directly or indirectly), with the population located at deeper depths thus being less affected by decreases in luminosity or by the effect of light on migratory mesopelagic organisms dwelling in the higher layers in the water column. Studies of stomach contents would be needed to be able to establish relationships between the vertical migrations of mesopelagic species and food availability at the different depths and in the water column. The present results corroborate the hypotheses described in Cartes et al. (1993). These authors observed that certain nektobenthonic species seem to undergo migrations along the bottom to shallower areas of the slope at night. Moreover vertical migrations into the water column above would seem to be an unlike explanation, in view of the small share of planktonic prey items in the nocturnal diet of *A. antennatus* (Cartes 1991).

The main question requiring elucidation is why shrimp shoals present the characteristic structure described, in terms of both shoal morphology and size and sex composition. The presence of a lower proportion of larger males and of females of different sizes with increasing depth might be attributable to the adaptation of the metabolisms of large individuals to deeper habitats, which are more oligotrophic and less favorable to high biomass levels composed mainly of reproductive females. An alternative hypothesis could be that adult females in advanced stages of gonadal maturity have certain nutritional requirements that are best filled at depths between 800 and 1000 m, where there may be some sort of environmental features at certain times of year that trigger the population structure observed. Puig et al. (2001) have proved this hypothesis for shrimps of the genus *Plesionika*. They observed characteristic distributions of berried and juvenile females at certain depths, associated with the presence of nepheloid layers in the same area in spring and fall. However, no such relationship has yet been demonstrated for *A. antennatus*.

Cartes & Sardà (1989) and Maynou & Cartes (1997, 1998) consider this species to occupy one of the lower positions in the benthopelagic food chain but to be atypical among deep-sea decapod crustaceans in that it exhibits a relatively high proportion of full stomachs as compared with other deep-sea decapod crustaceans. The high metabolic and growth rate demonstrated for this species by Company & Sardà (1998, 2000) is likewise indicative of this. Furthermore, more mobile species tend to have higher metabolic rates, that is, they have higher energy requirements, which translates into a higher daily ration (Koslow 1996). Given the reduction in food sources in deep-sea habitats, causing dietary overlap and competition for food (Gage & Tyler 1990), it seems reasonable to suppose that *A. antennatus* will have specific nutritional requirements during spawning and will therefore tend to adopt a distribution at optimum depths to fulfill those requirements. This could be one of the main reasons for the high level of dominance found for this species in the depth interval studied. In the Catalán Sea total consumption by bathyal decapod crustacean assemblages is higher on the upper middle slope (400–900 m) than

on the lower middle slope (900–1200 m). The generally lower food consumption by decapod crustaceans with depth is consistent with the commonly accepted notion that food availability also declines with depth, which holds both for the suprabenthos (one of the main sources of food for benthic decapod crustaceans) and for mesopelagic decapods and euphausiid crustaceans and other crustacean taxa (Carpine 1970, Cartes 1998, Cartes & Maynou 1998, Mura et al. 1998). The reduction in food resources takes place around the zonation boundary located at 900 m, with deep-water rose shrimp shoals being located above that depth.

Temperature did not appear to be a determining factor in these processes, in that temperature in the Mediterranean is constant at around $13 \pm 0.5°C$ below 200 m (Hopkins 1985), hence the population structure and behavior of *A. antennatus* can be considered temperature-independent. In the deep-water habitat that concerns us here, food availability in the deep-sea food web would seem to be the principal limiting factor (Gage & Tyler 1990).

In conclusion, the results of this study on the Catalan Sea, have specifically shown that the shoals of *A. antennatus* during the reproductive period has the following structure:

(1) The pattern shrimp shoal distribution is such that density rises rapidly in the portion located in the shallower portion and then gradually decreases with greater depth;

(2) The distribution of this resource straddles both sides of the ecological boundary located at 900 m, although with changes in the sex-ratio and individual size;

(3) Species coexisting with this shrimp species are concentrated at depths other than the depths of peak shrimp density;

(4) Commercial trawlers deploy according to the abundance pattern of the resource;

(5) The reproductive portion of the stock is heavily exploited;

(6) There is substantial evidence that ecological aspects need to be taken into account when evaluating the dynamics of exploited populations with a view to sustainable management.

On the whole, the following salient aspects would appear to merit consideration: Fishing on the shrimp stock takes place mainly during the season of aggregation and maturation of reproductive females, which heightens the population's vulnerability to fishing activity. This factor needs to be taken into account for purposes of assessment and management. Nevertheless, studies published by Demestre & Lleonart (1993), Martínez-Baños (1997), and Tursi et al. (1996) have reported the status of exploitation of this species to be near the maximum sustainable yield (MSY) in different parts of the Mediterranean Sea. This is where the unexploited portion of the stock inhabiting the lower-middle slope (from 900 m to at least 2200 m) comes into play. This portion of the stock may act as a reserve, contributing additional biomass to the exploited portion of the stock and thereby preventing overexploitation. However, it should be noted that this hypothesis has not yet been demonstrated and that studies focusing directly on this aspect are needed.

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SEAFOOD DEALERS' SHRIMP-PURCHASING BEHAVIOR AND PREFERENCES WITH IMPLICATIONS FOR UNITED STATES SHRIMP FARMERS

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ABSTRACT   The US shrimp-farming industry has been expanding in the southern United States in response to the strong market demand for shrimp. However, US farmers have difficulty competing on price with imports in frozen shrimp commodity markets. This study identified the shrimp-purchasing behavior and preferences of seafood wholesalers and retailers in nine southeastern US states to provide shrimp farmers the market information needed to develop successful marketing strategies. Results of a mail survey of the seafood dealers, including a conjoint analysis experiment, are presented and discussed. There appears to be a potential market for fresh, farm-raised shrimp in a variety of sizes, but there is considerable dealer resistance to the whole or live head-on shrimp form. Shrimp farmers interested in successfully marketing to seafood dealers may be required to process their product to offer shrimp tails, rather than whole shrimp.

KEY WORDS: shrimp, buyer preferences, marketing, conjoint analysis

INTRODUCTION

According to the "Top Ten Seafoods" summary prepared by the National Fisheries Institute, shrimp was the leading seafood consumed in the United States in 2001, surpassing tuna for the first time in a decade (National Fisheries Institute 2001). Per capita consumption of shrimp was 3.4 pounds/person in 2001, or 23% of total US seafood consumption. Demand for seafood in the United States far exceeds the amount produced by US commercial fishermen and aquaculture producers. In 2001, 882.6 million pounds of shrimp, about 85% of the total supply, were imported into the United States, primarily from Southeast Asia. These imports were valued at $3.6 billion and accounted for 37% of the value of total edible fishery product imports (National Marine Fisheries Service 2001). Domestic farmed shrimp production accounts for less than 5% of the total US supply (Harvey 2002).

Interest in the shrimp farming industry has been growing rapidly in Florida and other southern states in response to the excess domestic market demand for shrimp. The most viable candidate shrimp species for large-scale culture in Florida appears to be the Pacific white shrimp, *Litopenaeus vannamei*, because of its market popularity, fast growth, adaptability to diverse salinities, and ability to be cultured at high densities.

In the past, expansion of marine shrimp culture in Florida has been constrained by high coastal land prices, competing uses of coastal land, and concerns over potential environmental damage to sensitive coastal ecosystems. However, aquaculture researchers in Florida have successfully acclimated the marine shrimp *L. vannamei* to hard freshwater at the age of 3 weeks (12–15 days postlarvae). The freshwater found in much of central and south Florida and other southern states contains the mineral balance to support this species. Farmers with hard freshwater wells are now able to demonstrate the technical feasibility of raising shrimp from postlarvae to commercial market size in inland locations. Attempts to demonstrate economic and market feasibility are ongoing.

US shrimp farmers, including those in Florida, wish to harvest and market their shrimp as quickly as possible. However, US farm-raised shrimp cannot compete effectively on price with imports in fresh-frozen shrimp commodity markets for the most popular forms and sizes. Furthermore, although some domestic farms will undoubtedly develop processing capability, the equipment, packaging, and marketing required to assure the success of value-added products and to satisfy food safety requirements (HACCP) are beyond the capability or interest of many small farmers. Thus, shrimp farmers in Florida and other southern states are particularly interested in the potential for marketing their product to seafood dealers (retailers and wholesalers) as live shrimp or fresh, head-on shrimp.

This research is part of a more comprehensive study designed to identify and characterize the most attractive direct markets for fresh, farm-raised shrimp. The specific objectives of this research were 1) to identify the shrimp-purchasing behavior, preferences, and attitudes of seafood dealers (wholesale and retail) in the southeastern United States and 2) to characterize marketing challenges and opportunities associated with the seafood dealer market.

LITERATURE REVIEW

Relatively little published information specific to domestically cultured shrimp is available relating to preferences and purchase behavior of seafood dealers. Most recent research has been focused on wild-caught and farmed finfish. This paucity further emphasizes the need for reliable market research information for farm-raised shrimp.

Dore (2000) provided an overview of the shrimp distribution system in the United States. The retail food business in the United States is dominated by supermarkets. Specialty retail seafood markets are typically located on the coasts or in large cities, and many of these combine retail sales with a wholesale or restaurant business. Similarly located, specialty seafood wholesalers primarily supply restaurants. Although some retail food stores do buy through wholesale grocers, most supermarkets are supplied through their own purchasing departments, with smaller chains more likely to buy direct.

The wholesale, retail, and food service sectors of the seafood industry create significant economic activity within many non-coastal areas of the country; this is becoming even more pronounced given the rapid development of inland aquaculture (Adams 1998). Market analyses for several aquaculturally produced finfish have demonstrated a strong retailer and wholesaler preference for highly processed product (fish fillets), consistent with a

noted consumer preference for convenience and ease of preparation (Golz & Nelson 1999, Wirth et al. 1990).

Shrimp are sold in a variety of fresh or frozen product forms, including whole (head-on) or tails, shell-on or peeled, and round or split and deveined. Growth in US consumption of shrimp is primarily in the form of raw headless, raw peeled, or cooked peeled shrimp (National Marine Fisheries Service 1996). Head-on shrimp is increasingly important in Europe, but the US market for this product is still very small and consists primarily of oriental restaurants (Dore 2000). Shrimp prices vary according to a wide variety of factors, including size, supply, quality, origin, species, and color (Yokoyama et al. 1989). Many species of shrimp are consumed in the United States, but white shrimp are generally preferred (Keithly et al. 1993). Sales and shipments are reported by size categories of shell-on shrimp tails, defined by count per pound. Customary commercial size classifications in the United States are U/15 (under 15 shrimp/lb), 16/20, 21/25, 26/30, 31/35, and so on.

General information concerning retailer and wholesaler shrimp purchase behavior was extracted from three small studies conducted in Hawaii and Florida. These were the only readily available reports containing information specifically relating to shrimp dealers. Shang (1990) interviewed 63 fish distributors in Hawaii and found that the shrimp dealers sold shrimp in six forms: frozen head-off, frozen peeled and deveined, breaded, canned, dried, and fresh. Frozen head-off was the most important category, accounting for about 70% of the total volume sold; fresh shrimp accounted for only 1% of the total volume. Dealers preferred large shrimp for frozen tails and frozen peeled and deveined shrimp. Firms that indicated foreign imports as their major supply source most often cited "best price" as their reason, firms that relied on US supply sources did so for "best quality" or "steady supply."

Schumann (2000) surveyed Florida shrimp broker/distributors regarding their willingness to purchase live shrimp. Only about 10% of respondents reported that they currently purchase live shrimp but about 35% indicated that they would probably purchase live shrimp in the future. Shrimp buyers indicated a willingness to pay $3.50–$4.80/lb for farmed shrimp in 1999 and an interest in marketing full shrimp farm production capacity.

The Florida Department of Agriculture and Consumer Services (2001) interviewed US seafood wholesalers at the Boston Seafood Show concerning their shrimp purchase behavior. About one half (49%) of the dealers indicated they purchase only frozen shrimp and 62% purchase only head-off shrimp forms. Almost one half (46%) of the dealers currently purchase farm-raised shrimp, and 74% indicated a willingness to purchase white shrimp farmed domestically in fresh water and to purchase shrimp directly from a shrimp farmer. At the time of the interview, these dealers also had the opportunity to evaluate Pacific White shrimp farmed in freshwater; 83% of the dealers rated the shrimp as good or excellent overall.

Although published information specific to domestic buyer preference for shrimp is very limited, a strong demand appears to exist for high-quality, reasonably priced shrimp. The shrimp farmer is ideally situated to provide a consistent supply of fresh shrimp and can adapt production to meet buyer demands for size, quality, quantity, and timeliness. However, head-off forms seem to dominate shrimp sales and consumption, so shrimp farmers may encounter some resistance to direct marketing of whole shrimp.

METHODS AND MATERIALS

This study was designed to characterize preferences within the domestic seafood dealer market and identify opportunities and challenges associated with marketing to seafood dealers. A questionnaire was developed and administered by mail to 3038 seafood dealers, identified by Standard Industrial Classification code, in nine southeastern U.S. states (AL, AR, FL, GA, LA, MS, NC, SC, and TN). The mailing list included the entire population of seafood wholesalers, retailers, and processors in the nine states, as identified by American Business Information, a commercial provider of business directories. The questionnaire solicited information concerning the location and size of the seafood business, business sales structure, shrimp-buying practices, and preferences for various shrimp product forms and attributes. A conjoint analysis was conducted to quantify the utility value and relative importance of key shrimp product attributes that are within the control of shrimp farmers: size, refrigeration state, form, and price. A thank-you/reminder postcard was mailed to each dealer approximately 4 days after the survey mailing.

Conjoint Analysis

Conjoint analysis has become a popular marketing research tool for designing new products. A conjoint analysis allows for a buyer's overall preferences for a product to be disaggregated among the complement of that product's features. This requires knowledge of the overall evaluations of a set of alternative products that are prespecified in terms of levels of different features (Green & Srinivasan 1978). Using conjoint analysis, a researcher can analyze a heterogeneous product market and obtain results that can be highly disaggregated to homogeneous groups of buyers. Alternatively, aggregating results for buyers who have similar preference or utility functions can be useful in modifying current products or services and in designing new ones for selected market segments (Green & Wind 1975).

The features and feature levels that define the conjoint design must be carefully selected. The features correspond to product characteristics that have been demonstrated or are hypothesized to influence purchase behavior. The feature levels are sample values for each of the selected factors, and the levels should span the realistic range of each feature. Table 1 summarizes the four features (size, refrigeration state, product form, and price) and feature levels selected for the conjoint analysis experiment in this study. The levels for price were selected based on published retail prices

TABLE 1.

Shrimp features and feature levels for conjoint analysis.

Feature	Feature Levels
Size (tail count/lb)	Extra large (16–25/lb)
	Large (26–35/lb)
	Medium (36–50/lb)
State	Fresh (never frozen)
	Frozen
Form	Whole (head-on)
	Shell-on tails
	Peeled & deveined tails (P & d)
Price	$3.00/lb
	$5.50/lb
	$8.00/lb

for fresh, shell-on shrimp tails over the range of sizes included in the conjoint analysis. These prices were then adjusted to compensate both for retail mark-up and for the conversion from shell-on tail weight to round weight, to arrive at realistic wholesale prices for whole (head-on) fresh shrimp.

The conjoint experiment uses a full-profile approach in which respondents rate a set of hypothetical products defined by a specified level for each feature. In a full-factorial design, in which every possible combination of feature levels is rated, the number of products to be rated quickly becomes very large and the task becomes unrealistic for survey participants. A fractional factorial design is generally used instead, in which an orthogonal subset of feature level combinations is selected. The orthogonality permits estimation of all single-factor, or main, effects, although information concerning feature interactions is lost (Green 1974). Every level of each feature occurs with every level of every other feature, thus unrealistic combinations of feature levels may occur in the design.

The orthogonal design was developed using CONJOINT DESIGNER, a software package developed by Bretton-Clark. Only nine hypothetical products were required to represent the orthogonal design described in Table 1, as opposed to 54 for a full-factorial design. In addition, the experiment included one "holdout" product defined to closely resemble realistically marketable farm-raised shrimp. Holdout products are used to validate results as well as to gather data on particular products of interest (Herman 1988). The coefficients of the conjoint model are estimated using only the products that determine the orthogonal design, without use of any holdout products. The actual ratings of the holdout products can then be compared with those predicted by the conjoint model as an indication of the predictive validity of the model. The 10 shrimp products presented to the survey participants are described in Table 2.

Several important product characteristics, such as farm-raised vs. wild-caught, raw vs. cooked, and domestic vs. imported, were deliberately omitted from the conjoint experiment to limit the number of tasks required of the survey respondents. Seafood dealers were asked to rate each of the products shown in Table 2 on a scale of 0–10, where 0 was the least desirable combination of product attribute levels, and 10 was the most desirable combination of product attribute levels.

Model Specification

A conjoint preference model is used to estimate the influence of various product features on preferences indicated by the respon-

dents. The specification of the conjoint preference model as described by Wirth et al. (1990) involves two steps. First, the functional form for each product feature must be specified. Next, the functional forms for each feature are combined into a conjoint preference model for estimation.

There are three ways to model a buyer's utility function for each product feature: a part-worth or dummy variable function model, a linear vector model, and a quadratic or ideal-point model. Green and Srinivasan (1978) provide a detailed theoretical discussion of the three functional forms. The most general and most commonly used utility model is the part-worth model, which is especially appropriate for qualitative variables. The part-worth model requires separate estimates of the contribution or part-worth of each level of a feature. Quantitative features with two or three feature levels, such as price, can be modeled using the part-worth model, the vector model, or the ideal-point model. For this study, the part-worth function model was used to model all four shrimp product features: size, state, form, and price. The part-worth model provides the greatest flexibility in the shape of the utility function for each of the product features. However, this model also requires estimation of the greatest number of parameters (perhaps reducing the reliability of the estimates).

In conjoint analysis, a buyer's utility for a particular product, as represented by the preference rating, is modeled as the sum of the buyer's utilities for each product feature. The part-worth function model posits that for a set of t features, where y_{jp} denotes the level of the pth feature for the jth product, the preference S_j is given by the following:

$$S_j = \sum_{p=1}^{t} f_p(y_{jp}) \qquad (1)$$

where f_p is the function denoting the part-worth of different levels of y_{jp}. In practice, $f_p(y_{jp})$ is estimated only for the selected set of feature levels, with values for intermediate levels obtained by linear interpolation (Green & Srinivasan 1978). The general model for this study can be expressed as follows:

$$\text{Rating} = f(\text{Size, State, Form, Price}) \qquad (2)$$

where

rating = preference rating given to the hypothetical shrimp products by survey respondents,

size = shrimp size (extra large, large, or medium),

state = refrigeration state (fresh or frozen),

form = shrimp product form (whole, shell-on tails, or peeled and deveined tails), and

price = product price ($3.00/lb, $5.50/lb, $8.00/lb).

This study used mean deviation coding for the dummy variable specification and the coefficients were estimated using ordinary linear regression. This dummy variable coding technique is mathematically equivalent to traditional dummy variable coding, but the coefficient for the base level is easily calculated as the negative sum of the coefficients for the other k-1 levels. The intercept is the overall mean preference rating, and dummy variable coefficients measure deviation from the mean rating (Harrison et al. 1998).

RESULTS AND DISCUSSION

Mail Survey

A four-page questionnaire was mailed to 3,038 seafood dealers in the nine states comprising the southeastern United States. A

TABLE 2.

Hypothetical products rated by seafood dealers for conjoint analysis.

Product No.	Size	State	Form	Price
1	Medium	Frozen	P&d	$8.00/lb
2	Medium	Fresh	Tails	$3.00/lb
3	Large	Fresh	Whole	$8.00/lb
4	Extra large	Frozen	Tails	$8.00/lb
5	Medium	Frozen	Whole	$5.50/lb
6	Extra large	Frozen	Whole	$3.00/lb
7	Large	Frozen	Tails	$5.50/lb
8	Large	Frozen	P&d	$3.00/lb
9	Extra large	Fresh	P&d	$5.50/lb
10 ("holdout")	Large	Fresh	Whole	$5.50/lb

total of 253 (8.3%) surveys were returned as undeliverable. Two hundred and fifty (250) of the remaining 2,785 surveys were completed and returned, giving an effective response rate of 9.0%. The survey included questions concerning the location and size of the seafood business, business sales structure, shrimp buying practices, and the conjoint experiment described in the Methods and Materials section.

Almost half (46%) of the responding dealers were located in Florida, followed by Louisiana (16%), Georgia (11%), and North Carolina (10%). The businesses were fairly evenly distributed between rural, suburban, and urban locations (22–35%), with fewer in resort areas. Most (87%) of the seafood dealers can be classified as small businesses, with 25 or fewer employees.

Dealers were asked to describe their business in terms of the percentage of their total sales in each of four specified categories: wholesale to wholesale, wholesale to retail, retail, and other. For this report, dealers were classified as "wholesalers" if they indicated that more than 50% of their total sales were wholesale to wholesale and/or wholesale to retail. Similarly, dealers were classified as "retailers" if they indicated that more than 50% of their total sales were retail. All other dealers were classified in a "combination/other" category. Respondents were fairly evenly split between the "wholesaler" and "retailer" designations, but approximately 70% of responding dealers reported some retail sales, suggesting that many seafood dealers are diverse, selling in multiple markets.

Dealers were then asked several questions about their current shrimp-buying practices. Of those responding, 85% indicated that they currently purchase shrimp and reported their total annual shrimp purchases. About two thirds of dealers who buy shrimp purchase 50,000 pounds or less annually. Almost 10% buy more than one million pounds annually.

These dealers were also asked to list the percentage of their total shrimp purchases in each of several specified sizes and product forms. Figure 1 shows the percent of responding shrimp buyers who indicated they currently purchase *any* shrimp in the specified sizes and forms. The results indicate that shrimp dealers carry the full range of shrimp sizes from 16/20 count to shrimp smaller than 41/50 count. Figure 2 shows the shrimp product forms currently being purchased by responding shrimp dealers. The majority of

shrimp dealers (85%) carry shrimp tails, but 50% of shrimp dealers purchase some whole, head-on shrimp. More than 25% of shrimp dealers also purchase peeled & deveined (p&d) tails and/or peeled & undeveined (pud) tails.

The dealers were asked several questions specific to US farm-raised shrimp. Of the dealers responding, 73% were familiar with aquaculture and 54% indicated they currently buy farm-raised shrimp, although the country of origin was not identified. Seventy-five percent would offer domestic farm-raised shrimp if it were readily available and 72% would be willing to purchase shrimp directly from a farmer. Only 38% of dealers were familiar with Pacific White shrimp raised in fresh water, but 55% would be willing to purchase these shrimp.

Figure 3 shows the percent of dealers in each sales category that indicated willingness to buy shrimp directly from a US shrimp farmer. About 18% of dealers classified as "wholesalers" for this study specifically stated that they were *not* willing to buy directly from shrimp farmers, whereas only 7% of "retailers" were unwilling to buy direct. Because of survey length constraints, dealers were not specifically asked about their willingness to buy whole shrimp directly from farmers.

Willingness to buy directly from farmers does not appear to be directly correlated with any of the other basic dealer characteristics recorded in this survey, including business location (state, or rural vs. urban), company size, and shrimp volume. However, willingness to buy directly from a shrimp farmer is likely contingent on many factors not measured in this study due to survey length constraints, such as the dealer's proximity to the farm, product quality and quantity available, and the level of services (e.g., packing, grading, delivery) provided by the farmer.

These results do suggest that US shrimp farmers should find a ready dealer market for their product, especially with seafood retailers. Farmers who are willing and able to perform the processing, storage, transportation, and other marketing functions normally performed by seafood wholesalers may receive prices higher than normal farm-gate prices and capture a share of the farm-retail price spread.

Finally, dealers were asked to rate various shrimp product features from 0–10, with 10 indicating the feature is "most important" in their shrimp purchase decisions. Table 3 shows the mean rating
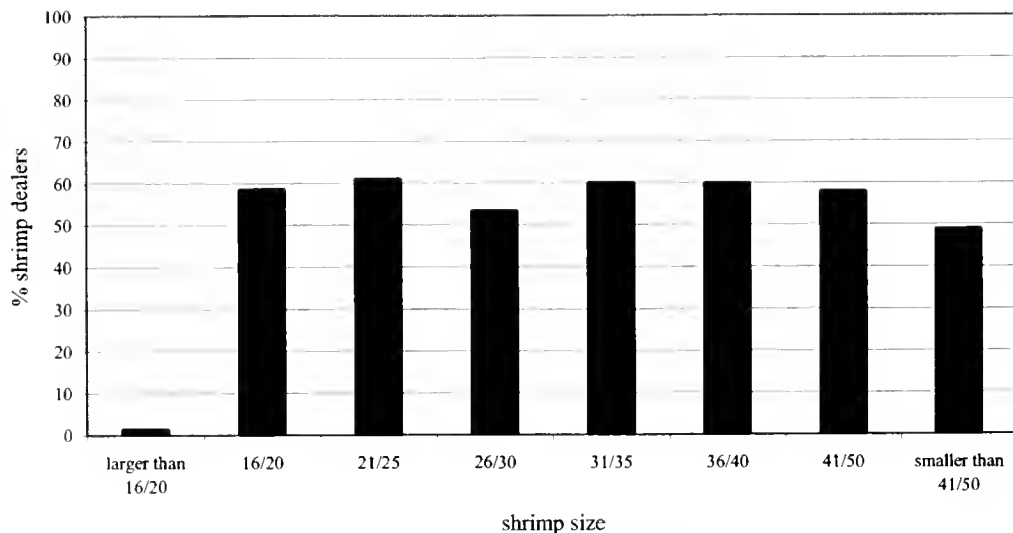

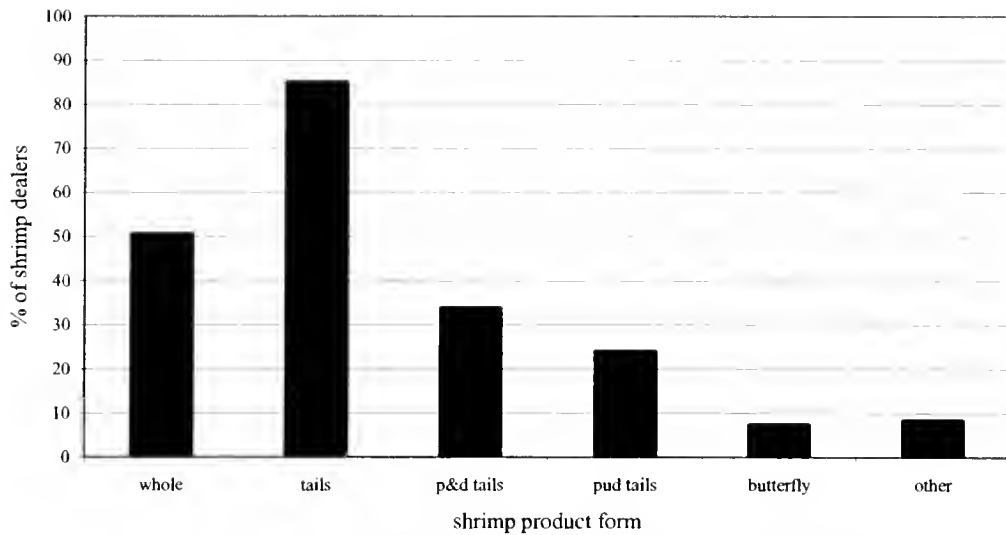Figure 1. Percent of shrimp dealers currently buying any shrimp in specified sizes.

Figure 2. Percent of shrimp dealers currently buying any shrimp in specified forms.

and ranking of each product feature for all dealers combined and for those identified as wholesalers or retailers. Ratings were consistent among wholesalers and retailers. Quality, freshness, and smell were the three most important shrimp product features to the responding dealers, each with mean rating greater than 8.5. Unfortunately, from the perspective of US shrimp farmers, production source (imported vs. wild-caught vs. farm-raised) and country of origin appear to be relatively unimportant to dealers. Dealers also do not consider the whole (head-on) shrimp form, or fresh (never frozen) state to be very important.

Conjoint Analysis

The seafood dealers were asked to rate the 10 hypothetical shrimp products shown in Table 2 on a scale of 0–10, with 0 indicating least preferred and 10 indicating most preferred. These products were designed to permit quantification of seafood dealer preferences for four shrimp product features that are within the control of shrimp farmers: size, refrigeration state (product), form,

and price. The first nine products comprised the orthogonal fractional factorial design for the analysis. The tenth "holdout" product was selected to represent the most feasible whole shrimp product for shrimp farmers to market directly, without processing.

The conjoint model parameters were estimated using ordinary least squares regression; results are shown in Table 4. Coefficients were estimated for the entire sample of dealers, and for subgroups of dealers who attributed more than 50% of their total sales to wholesale (wholesale-to-wholesale and wholesale-to-retail combined) or to retail. The coefficients for all dealers combined were statistically significant at $P = 0.05$, except for the coefficients for state = fresh, and for price = $5.50/lb (significance varies for dealers in each sales category). The regression constant was estimated at 3.83 for all dealers, and is interpreted as the mean preference rating, with feature level coefficients measuring deviation from that rating in response to a particular product attribute. The adjusted R-Square value computed for this model, interpreted as the proportion of the variability in the dependent variable (rating) that can be explained by the variability in the independent vari-
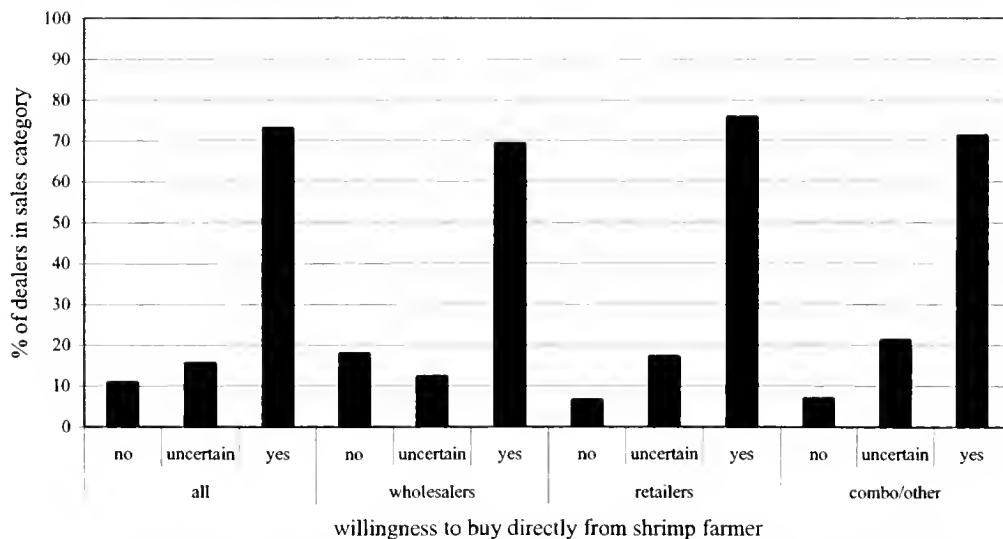
Figure 3. Dealer willingness to buy directly from shrimp farmers, within each sales category.

TABLE 3.

Mean rating and ranking of shrimp features in dealer
purchase decisions.

Product Feature	Mean Rating (Ranking)			
	All	Wholesale	Retail	Combo
Quality	9.51 (1)	9.57 (1)	9.61 (1)	9.38 (1)
Freshness	8.82 (2)	8.88 (2)	9.03 (3)	8.44 (5)
Smell	8.75 (3)	8.66 (3)	9.12 (2)	8.31 (6)
Price	7.73 (4)	8.04 (5)	7.78 (4)	7.06 (10)
Color	7.61 (5)	8.04 (4)	7.44 (5)	7.44 (8)
Size	7.51 (6)	7.80 (6)	7.27 (7)	8.13 (7)
Consistent size	7.37 (7)	7.70 (7)	7.10 (8)	8.94 (3)
Taste	7.17 (8)	7.19 (8)	7.38 (6)	8.56 (4)
Consistent taste	6.93 (9)	6.97 (9)	6.94 (9)	9.31 (2)
Tails	6.49 (10)	6.42 (11)	6.79 (10)	7.13 (9)
Raw	5.88 (11)	5.86 (12)	6.08 (11)	5.33 (12)
Frozen	5.82 (12)	6.63 (10)	5.79 (12)	4.27 (15)
Fresh	4.64 (13)	4.22 (15)	5.00 (13)	4.33 (14)
Whole	4.23 (14)	4.29 (14)	4.32 (14)	4.19 (16)
Country of origin	4.19 (15)	4.32 (13)	4.14 (15)	5.44 (11)
P&d	3.44 (16)	3.53 (18)	3.23 (16)	4.56 (13)
Wild caught	3.40 (17)	3.73 (17)	3.16 (17)	4.06 (17)
Nutritional value	3.14 (18)	4.11 (16)	2.74 (19)	2.38 (19)
Farm raised	3.00 (19)	3.36 (19)	3.01 (18)	2.31 (20)
Imported	2.73 (20)	3.26 (20)	2.65 (20)	2.93 (18)
Cooked	1.50 (21)	2.04 (21)	1.25 (21)	0.93 (21)

TABLE 5.

Utility of shrimp product feature levels to seafood dealers.

Feature	Level	Utility			
		All	Wholesale	Retail	Combo
Size	Extra large	0.49	0.66	0.26	0.90
	Large	0.28	0.34	0.34	0.07
	Medium	−0.76*	−1.00*	−0.59*	−0.97*
State	Fresh	0.06	−0.14	0.14	0.07
	Frozen	−0.06*	0.14*	−0.14*	−0.07*
Form	Whole	−0.92	−0.87	−1.03	−0.87
	Tails	1.51	1.36	1.66	1.40
	P&d	−0.59*	−0.49*	−0.63*	−0.54*
Price	$3.00/lb	0.66	0.99	0.54	1.09
	$5.50/lb	0.23	0.19	0.20	0.65
	$8.00/lb	−0.90*	−1.19*	−0.74*	−1.74*

* Calculated.

tional factorial design were used to determine the utility and relative importance of each attribute.

In the conjoint model, each level of each feature is considered an independent variable contributing to the overall rating of the product described by those feature levels. Of course, many combinations of these variables are logically impossible (for example, it is not possible for a shrimp product to be simultaneously large and extra large), hence the variables are not independent in reality. This confounds interpretation of the significance of the coefficients for computation of feature relative importance. It is unlikely that any of the specified features or levels genuinely have no importance at all in buyer decisions. It is customary to calculate the relative importance of the features, shown in Table 6, based on the calculated coefficients of all feature levels without regard to their individual levels of significance (Wirth et al. 1990, Harrison et al. 1998), and that practice is observed here. The calculated relative importance of each attribute is generally affected very little by this convention.

Product form was the most important shrimp feature for all dealers, contributing almost 50% to the preference rating. Tails were strongly preferred, and contributed more to the product utility value than any other feature or feature level. Price contributed almost 30% to the rating and was slightly more important than

ables (size, state, form, and price) is 0.096. The low value is due in part to the highly cross-sectional nature of the data. Aggregating responses across individuals introduces additional variation due to differences in each respondent's subjective rating for the same product (Harrison et al. 1998). The F-statistics indicate that all models were statistically significant at the $\alpha = 0.05$ level.

The regression coefficients provide a direct measure of utility for the levels specified for each feature. The effects coding technique used in this study constrains the utility of the levels of each feature to sum to 0, so the utility of the base level for each attribute is easily calculated (Table 5). The relative importance of each attribute is then the range of utility over all levels of that attribute, expressed as a percentage of the sum of the utility ranges for all attributes. Only ratings of the nine products included in the frac-

TABLE 4.

Shrimp dealer conjoint model coefficients, estimated by linear regression.

	All			Wholesale			Retail			Combo		
	Coeff.	SE	P	Coeff.	SE	P	Coeff.	SE	P	Coeff.	SE	P
Constant	3.83	0.10	0.00	3.75	0.17	0.00	3.91	0.14	0.00	4.41	0.39	0.00
Size extra large	0.49	0.14	0.00	0.66	0.23	0.00	0.26	0.19	0.17	0.90	0.52	0.09
Size large	0.28	0.14	0.04	0.34	0.23	0.14	0.34	0.19	0.07	0.07	0.52	0.89
State fresh	0.06	0.10	0.55	−0.14	0.17	0.42	0.14	0.14	0.31	0.07	0.39	0.86
Form whole	−0.92	0.14	0.00	−0.87	0.23	0.00	−1.03	0.19	0.00	−0.87	0.52	0.10
Form tails	1.51	0.14	0.00	1.36	0.23	0.00	1.66	0.19	0.00	1.40	0.52	0.01
Price $3.00/lb	0.66	0.14	0.00	0.99	0.23	0.00	0.54	0.19	0.00	1.09	0.52	0.04
Price $5.50/lb	0.23	0.14	0.09	0.19	0.23	0.40	0.20	0.19	0.28	0.65	0.52	0.21
F (model)	29.51		0.00	12.46		0.00	15.34		0.00	3.31		0.00
Adj. R-Square	0.10			0.11			0.09			0.10		

<div style="text-align:center">

TABLE 6.

Relative importance of shrimp product features to seafood dealers.

</div>

Attribute	Relative Importance (%)*			
	All	Wholesale	Retail	Combo
Size	23.3	26.1	17.9	26.4
State	2.2	4.4	5.5	1.9
Form	45.3	35.2	52.0	31.9
Price	29.1	34.3	24.7	39.8

* Relative importance does not sum to 100% because of rounding.

size. As expected, the highest preference was for the lowest price and the largest size. Refrigeration state had no significant effect on the product rating, suggesting that dealers are completely indifferent to the shrimp refrigeration state in their shrimp-purchasing decisions. Results were fairly consistent between all dealers combined and the wholesaler and retailer groups, except that form was more important and size was less important to retailers.

The model can be validated by comparing the actual mean dealer ratings with the ratings predicted by the model for the "holdout" product #10 (large, fresh, whole shrimp for $5.50/lb). The buyer utility for the product is the base utility level plus the sum of the utility values for each selected product feature. The predicted utility for the "holdout" product #10 was calculated as 3.48. The actual dealer mean rating for product #10 was 3.08 with a standard deviation of 4.08. Thus the model's predicted rating is quite accurate.

CONCLUSIONS

The demand for seafood in the United States far exceeds the amount produced by US commercial fishermen and aquaculture producers. The US shrimp farming industry has been expanding rapidly in the southern United States in response to the excess market demand for shrimp. Shrimp farmers wish to harvest and market their products as quickly as possible, at the lowest possible costs, so the shrimp product form leaving the farm is likely to be live shrimp or fresh, head-on shrimp. One marketing alternative, especially during the early stages of industry development, is for shrimp farmers to market their products directly to seafood dealers. This research was designed to identify and characterize the shrimp-purchasing behavior of seafood dealers (wholesale and retail) in the southeastern United States and to identify challenges and opportunities associated with the seafood dealer market.

The results of the seafood dealer survey and conjoint analysis of dealer product ratings suggest that the shrimp dealer market is not an especially good candidate for direct sales of whole, farm-raised shrimp. The large majority of dealers are willing to buy farm-raised shrimp direct from the farmer but dealers revealed a strong preference for shrimp tails, rather than whole shrimp. The small percentage of dealers willing to purchase whole shrimp would only be able to support a small volume of shrimp products in a niche market.

This research also reveals other potential barriers to the dealer market. Price is extremely important to dealers, contributing 30% to the shrimp purchase decision. Shrimp dealers attach little importance to farm-raised vs. wild-caught or to country of origin, so dealers may be unwilling to pay higher prices for domestic farm-raised shrimp, compared with shrimp from other sources. Dealers are also completely indifferent to the shrimp refrigeration state (fresh vs. frozen) in their shrimp-purchasing decision, suggesting that domestic shrimp farmers cannot obtain any competitive advantage or product differentiation by selling fresh, never frozen, shrimp, which is the farmers' preferred refrigeration state for marketing purposes.

Overall, the results of this study indicate a potential dealer market for fresh, farm-raised shrimp in a variety of sizes, but there is considerable resistance to the whole or live, head-on shrimp form. The mail survey and conjoint experiment results suggest that shrimp farmers interested in successfully marketing to seafood dealers may be required to process their product to offer shrimp tails, rather than whole shrimp. Each shrimp farmer will have to compare his own costs versus returns for both whole shrimp and shrimp tails before choosing the product form and outlet that yields the highest profit margin.

ACKNOWLEDGMENTS

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OLFACTORY DETERRENTS TO BLACK DRUM PREDATION ON OYSTER LEASES

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ABSTRACT Black drum (*Pogonias cromis*) predation is a serious threat to oyster production on Louisiana leases, and leaseholders hypothesize that black drum carcasses suspended above leases deter predation. We conducted experiments under laboratory and field conditions to test whether the scent of dead con-specifics deterred black drum predation. Preliminary experiments indicated that fish >70 cm total length were effective predators, and oysters <70 g wet total weight were preferred, and we used these sizes in subsequent experiments. Salinity did not affect feeding rates. Experiments in 30,000 L raceways indicated that scent did not significantly lower feeding rates. Parametric analyses of factorial experiments on oyster leases at two sites in Barataria Bay, Louisiana, during the fall and spring (periods of the year when fish feeding is most intense), indicated that scent reduced feeding rates by 10% to 20%, but only at one site in one season. Nonparametric analyses corroborated seasonal differences indicated by parametric analyses, but not the scent effect. We therefore conclude that scent from dead con-specifics is not an effective control strategy under most conditions. Dredge hauls during experiments suggested mortalities to all predators ranging from 63.1% to 92.5% within the first 4 weeks after seeding. The relative mortalities to black drum, southern oyster drills (*Stramonita haemastoma*) or possibly *Perkinsus marinus* infections varied among sites, as did temporal patterns of mortality within and among seasons.

KEY WORDS: black drum, oysters, deterrents, olfactory

INTRODUCTION

Oyster reefs (*Crassostrea virginica*) are important components of Gulf of Mexico coastal ecosystems, providing shelter for several economically important invertebrates and larval fish, improving water quality, and stabilizing shorelines (Bahr & Lanier 1981, Zimmerman et al. 1989). Oyster production in the northern Gulf of Mexico is greatest in an "optimal" salinity band ranging from 5–15 psu in coastal marshes. Survivorship is physiologically constrained at lower salinities, while predation losses are too high to sustain populations at higher salinities (Melancon et al. 1998), except at intertidal sites where oysters have a refuge from predation (Roegner & Mann 1995, O'Beirn et al. 1996, Brown & Stickle 2002).

Oysters spawn in the northern Gulf of Mexico as water temperatures rise above 25°C in the spring, and then resume spawning as temperatures drop below that level in late summer and fall (Supan 1983, Banks & Brown 2002). Warmer temperatures in summer months are also associated with increased prevalence of the parasitic protozoan *Perkinsus marinus* ("Dermo"), which can result in mortality as high as 50% in oyster populations, especially at higher salinities (La Peyre et al. 2003).

The Gulf of Mexico oyster industry was developed in the mid-eighteen hundreds and was further spurred by the development of the oyster dredge in the early nineteen hundreds. Oysters are harvested from public areas (mostly reserved as "seed grounds" where leaseholders can collect small oysters to plant on their leases) and private leases. Seed oysters are planted in the fall, and typically harvested in their second spring when they reach market size. Louisiana currently has approximately 8700 leases covering 419,000 acres under cultivation. Louisiana's oyster production averages almost one third of the national production, and the Gulf of Mexico region produces 60% of the national production, worth almost 50 million dollars annually.

Coastal zones producing maximal oyster yields are however changing, as coastal erosion results in saltwater intrusion, shrinking the optimal salinity band (Melancon et al. 1998). As salinities increase, predation by black drum *(Pogonias cromis)* and southern oyster drills (*Stramonita haemastoma*) increases, as does prevalence of Dermo. The importance of black drum as predators was clearly indicated by a survey of Louisiana oyster leaseholders (Louisiana Department of Wildlife and Fisheries [LDWF] 1999) indicating significant loss in 55% of the leases. Seed oysters may be stressed during transport from seed areas to oyster leases, and they produce scents that attract black drum. Almost 80% of leaseholders who had recently seeded oysters reported significant losses to black drum.

Black drum inhabit near-shore and estuarine waters in the Gulf of Mexico (Simmons & Breuer 1962), mature at the end of their second year, and spawn in coastal passes from February through March. The larvae are transported into estuaries where the juveniles mature. The largest black drum in the Gulf of Mexico are over 40 years old, and reach 105 cm in length and 29 kg in weight (Sutter et al. 1986, Beckman et al. 1990). Black drum consume over 30 oysters per night (Sutter et al. 1986), with small "seed" oysters planted in leases preferred over natural reefs (Cave 1978, Cave & Cake 1980, Dugas 1986). Oysters are consumed by fish greater than 40 cm in total length, and oyster sizes consumed increase with length. Juvenile black drum feed on a variety of invertebrates (Pearson 1929, Gunter 1945, Darnell 1958). Production losses may be as high as 1500 sacks per lease in some parishes (LDWF 1999). Most oysters are lost in March, when groups of fish return to coastal leases to feed after spawning, or in October, immediately after small seed oysters are bedded.

Our long-term goal is to develop deterrents to black drum predation on oyster leases. We first performed preliminary experiments to understand basic aspects of the predator-prey interaction, such as the vulnerability of different size classes of oysters, the role that drum body size plays in determining feeding rates and prey-size selection, and how the predator-prey interaction is affected by salinity. Based on prior experimental work (Cave 1978), or analyses of diet (Dugas 1986, Luquet 1992), our hypothesis was

that larger fish would be more effective predators, and small oysters most at risk. We also expected reduced feeding rates with lower salinity, because leases in coastal areas experience higher mortality (LDWF 1999). These experiments also determined which sizes of predators and prey and salinities were used in the laboratory olfactory cue experiments discussed in the next paragraph.

Second, we compared feeding rates in the laboratory, with or without a black drum carcass; our hypothesis being that alarm substances deter predation, as leaseholders report that carcasses suspended above leases reduce losses (P. Vujnovich Jr., Pers. Comm.). Alarm substances have been shown in other cases to cause avoidance behavior in fish (reviewed in Smith et al. 1994, Mathis et al. 1995, Chivers & Smith 1998). If scent cues reduce feeding rates, scent, or components of scent, could be added above the lease as a deterrent.

Third, to determine if olfactory cues were practical deterrents under field conditions, we conducted experiments on commercial leases in Barataria Bay, Louisiana. With the help of a leaseholder, we planted leases with seed oysters with or without drum carcasses. Comparison of predation rates between control and experimental plots determined whether scent was an effective deterrent. We placed oysters in trays and recorded their survival at 2 distances from scent sources in plots to determine how effective scents were at a distance, and the role of current in displacing scents. We also made hauls with a dredge to independently estimate losses to predation. This design was replicated on two leases, and in both the fall and spring.

MATERIAL AND METHODS

Preliminary Experiment

Predation rates and size preference of black drum were measured in experiments conducted from December 1999 to March 2000 at the LDWF Lyle St. Amant Marine Laboratory on Grand Terre Island, Louisiana. Black drum captured by hook and line or trot lines were held for 5 days at ambient salinities with oyster prey for acclimation, and were then starved for 2 days before experiments to standardize hunger levels. Experiments were conducted with a single fish for 5 days in 2000-L tanks with biologic filters. Barataria Bay water was mixed with fresh water to achieve average salinities of 13 (±0.4, standard error of the mean) and 36 (±0.3) psu; water temperature averaged 15°C (±1.0). Oysters were provided in three sizes (10 <50 g total wet mass, 10 between 51–150 g, and 7 >151 g) and were replenished daily. We used two size classes of black drum (30–70 cm [average = 51.4 ± 0.7] and >70 cm [90.6 ± 0.9] total length). Because prey sizes were presented in unison to the fish, they were not independent treatments. We therefore used a multivariate analysis of variance (Peterson & Reynaud 1989). Effects of predator and prey size, and salinity were evaluated in the factorial arrangement of treatments (2 predator × 2 prey sizes × 2 salinities).

Laboratory Experiment

These experiments were conducted during October to April of 1999 to 2000 (to avoid low dissolved oxygen concentrations) at the Grand Terre Laboratory in large outdoor raceways (concrete block tanks measuring 10 × 3 × 1 m deep) holding 30,000 L of water. Tanks initially received filtered seawater (ambient salinity averaging 27.9 ± 0.7 psu, and temperature averaging 22.4 ± 0.7°C) from Barataria Bay, and water was re-circulated through oyster chip filters during experiments. Two black drums, greater than 70 cm total length, were held in each tank. Fish were measured, tagged, and acclimated in the tank for 1 week before starting experiments. Temperature, dissolved oxygen, and salinity were measured daily. Seventy-five small and 25 medium oysters were added initially. Broken shells or missing animals were counted daily and oysters replenished.

For each 5 day long experiment, two tanks were randomly selected as experimental tanks, and two as controls. Experimental tanks had a single black drum carcass suspended in a burlap bag at one end of the tank. After each experiment, fish were removed; the tank was drained and scrubbed, and refilled with filtered water from Barataria Bay. Fish used in experimental treatments were used next in controls (or the reverse) and were either then released or sacrificed and used for the scent deterrents. Numbers of oysters consumed were compared between the two treatments with a one-way analysis of variance.

Field Experiment

Whereas laboratory experiments aid in understanding predator behavior under controlled conditions, field experiments determine if olfactory cues deter predation under natural conditions and are feasible for industry use. We therefore made arrangements with a leaseholder to conduct experiments on two leases in Barataria Bay with a history of predation problems. The leases were in Lake Grand Ecaille in southeast Barataria Bay (29°35′06″N, 89°33′47″W) with historically high predation levels (Mr. Peter Vujnovich, Jr., pers. comm.), and in Creole Bay in west Barataria Bay (29°35′73″N, 89°34′10″W), a site with intermediate historical levels of black drum predation (Fig. 1). The field experiments were replicated twice, once in October 2000 and again in March 2001.

At each site, four 60-m diameter, circular plots were seeded by the leaseholder with oysters at densities typical for leases. Each plot was randomly assigned as a control or treatment, and plots were located 100-m apart to minimize influence from other treatments. Immediately after seeding, two black drum carcasses were enclosed in burlap bags and suspended from a PVC pole in the center of the experimental plots, and plastic "oyster grow out" trays (60 × 50 × 10 cm) each with a minimum of 100 oysters were placed in all four plots to assess predation rates. Three trays were near the center, and one tray was set at the end of three equidistant rays (Fig. 2). Trays were inspected for predation, oysters replenished, and the deterrent sources renewed at 1-week intervals, for 4 weeks. The number of oysters gaping (a sign either of oyster drill predation, Brown & Richardson 1987, or possible mortality to Dermo), or missing (presumably either consumed or at least handled and removed from trays by black drum) were recorded at each date. Separate trays (2 per plot) enclosed with 3 cm Vexar and retrieved at the end of the experiment, indicated that natural oyster mortality, or mortality caused by handling, averaged only 2%, and so we have assumed that gaping oysters were mostly the result of predation by oyster drills. Oyster drills were quite common on trays when they were retrieved, averaging from 2.7/tray at the coastal site to 8.5/tray at the estuarine site.

Each plot was also sampled with a dredge (0.3 × 0.6 m opening) to assess oyster densities. Three 30-m long dredge hauls (parallel to each axis on which trays were set) were taken in each plot at the start, after 2 weeks, and at the end of the experiment (after 4 weeks), and all oysters were pooled for each plot and date.

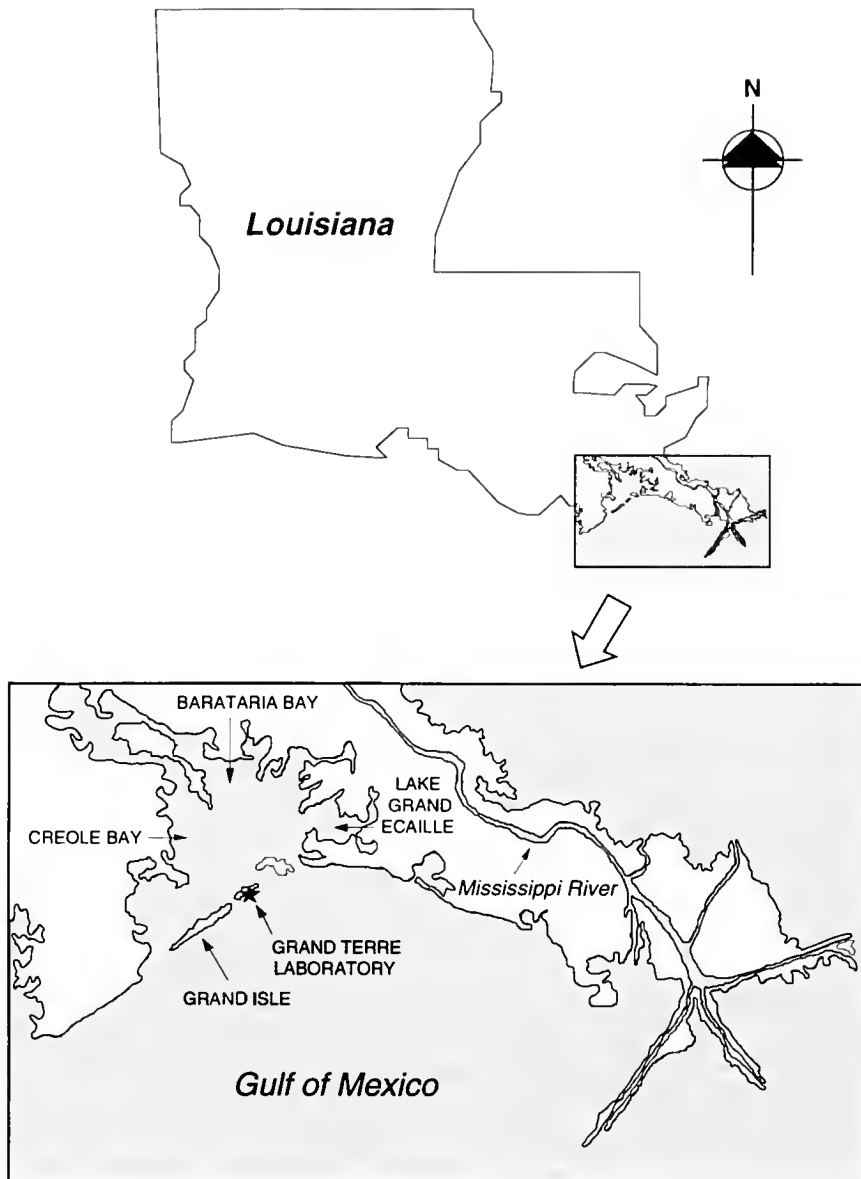Data were analyzed in repeated measures factorial analyses of

Figure 1. Map of Barataria Bay, with both sites where experiments were conducted on commercial oyster leases indicated.

variance. Data from trays for each date, or the three dredge hauls, were the repeated variables in the two way design (presence or absence of scent versus two seasons) conducted separately for each site. Preliminary statistical analyses at all sites indicated no significant effect of distance (e.g., center versus edge of plot, $P > 0.08$ in all cases) so all trays in a plot were considered replicates. Dependent variables were percent of oysters surviving ($n = 12$ trays for both plots in each treatment at each site at each date), percent mortality because of black drum, percent of oysters gaping, and numbers of oysters retrieved from dredge hauls ($n = 2$ plots per treatment per site per date). In several cases, data were not normally distributed, even after log transformation. We therefore performed a nonparametric factorial test, a 2-way (season × scent treatment) Sheirer-Ray-Hare extension of the Kruskal Wallis test (p. 446, Sokal & Rohlf, 1995). This test is essentially a two-way analysis of variance performed on the ranked data that provides H statistics that test the treatment and interaction effects. Each of the 4 weeks was considered replicates in this analysis.

RESULTS

Preliminary Experiment

Black drum size had a highly significant effect on feeding rate (Table 1, Wilk's $\lambda = 0.81$, $F = 7.9$, $P = 0.008$), with larger fish consuming on average 3.8 oysters and smaller fish only 0.9 oysters per week. Oyster size was also important (Wilks's $\lambda = 0.64$, $F = 9.4$, $P = 0.0006$), with a Tukey's *a posteriori* test indicating that the 5.2 small oysters consumed on average was significantly greater than the 1.7 medium oysters consumed. No large oysters were consumed by the fish. In contrast, salinity neither had an effect on feeding rates (Wilk's $\lambda = 0.99$, $F = 0.43$, $P = 0.52$), nor were any of the interactions between the main treatment effects significant. Based on these experiments, we only used fish larger than 70 cm total length in later experiments, and small or medium sized oysters as prey. Because salinity had no consistent effect, we used ambient salinity water for the laboratory scent experiments.
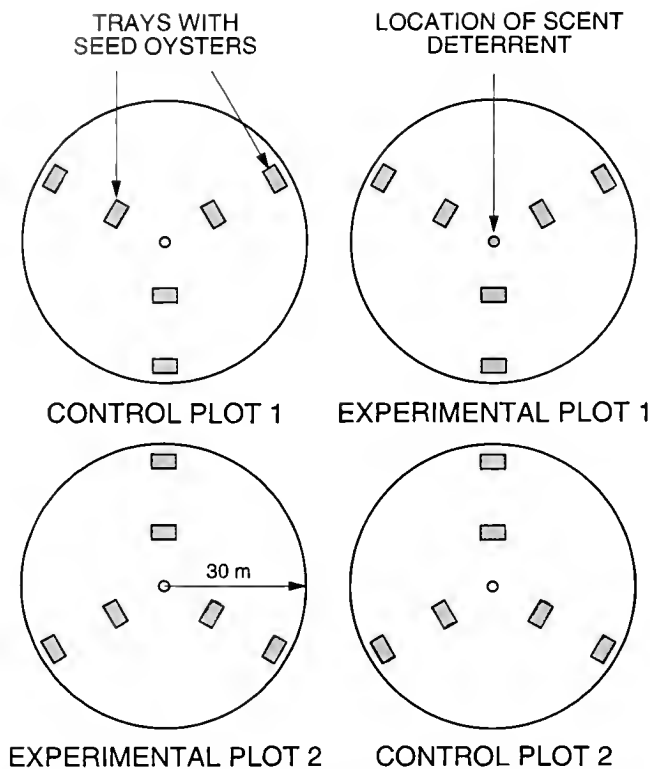
Figure 2. Layout of the four circular plots at each of the two sites. Two plots were controls, and two plots had centrally-located black drum carcasses renewed weekly during the 4-week long experiments. Locations of trays containing seed oysters are also indicated. Distances among plots are not to scale.

Laboratory Experiment

Under laboratory conditions, the presence of the scent of dead con-specifics depressed feeding rates (Fig. 3) but not significantly ($F_{1,12} = 0.9$, $P = 0.37$). Feeding rates were quite variable among replicates, and overwhelmed differences between the two treatments.

Field Experiment

At Creole Bay (Table 2), the repeated measures analysis of variance indicated a strong difference in oyster survival among weeks, and a significant interaction between time and season. The general pattern was for survival rates to increase with time (Fig. 4), although the shape of the curves differed between the fall and spring experiment. Evidently black drum were quickly attracted to the seeded leases, but moved away later (especially in the fall) as seed oysters were depleted on the lease. The significant season

Figure 3. Number of oysters consumed ($\bar{x} \pm SE$) in the control and scent treatments in the laboratory experiment. There were 6 replicates in each treatment.

main effect still however indicates that mortality rates were much higher overall in the spring than in the fall. Comparison of Tukey's *a posteriori* tests indicated that survival rates differed among seasons for each of the 4 weekly samples. In contrast, the scent treatment was not significant, nor were any of the interactions of scent and other treatments significant. The nonparametric test also indicated a strong difference between seasons ($H = 8.5$, $P < 0.01$), but an insignificant treatment ($H = 0.3$, $P > 0.05$) and interaction ($H = 0.1$, $P > 0.05$) effect.

At Lake Grand Ecaille, there were also differences among weeks in oyster survival (Table 2), with a significant interaction between time and season. In the fall, survival rates were high initially, but dropped considerably, probably caused by movement of black drum onto the lease (Fig. 5). The significant season main effect again suggested different mortality rates among seasons, and survival was essentially zero in most of the weeks during the spring, probably caused by high predation rates by black drum present at the site from the initiation of the experiment (Fig. 5). Comparison of Tukey's *a posteriori* tests indicated significant differences in survival between seasons, for each of the weeks. There was also a significant treatment effect at this site however, although the increased survival in scent treatment plots averaged only 10–20%, and occurred only in the fall, when mortalities were

TABLE 1.

Average feeding rates ($\bar{x} \pm SE$, N in parentheses) for 2 size classes of black drum feeding at 2 salinities on 3 size classes of oysters.

Fish	Salinity	Small	Medium	Large
<70 cm	13‰ (5)	1.8 ± 0.6	0	0
	36‰ (4)	3.5 ± 1.2	0	0
>70 cm	13‰ (6)	10.0 ± 4.5	4.0 ± 2.6	0
	36‰ (3)	5.7 ± 4.7	2.7 ± 2.7	0

TABLE 2.

F values from two-way repeated measures analyses of variance of oyster survival at two sites in Barataria Bay, Louisiana, in two seasons. The repeated measures are four samples through time at each site and season.

Source of Variation	Creole Bay	Lake Grand Ecaille
Time	80.8**	92.4**
Time × Season	22.9**	94.9**
Time × Scent	0.4	11.7**
3 way interaction	0.1	3.2*
Season	117.9**	1009.0**
Scent	1.0	13.7**
Scent × Season	0.03	5.4*

* Significant at $P < 0.05$

** Significant at $P < 0.01$
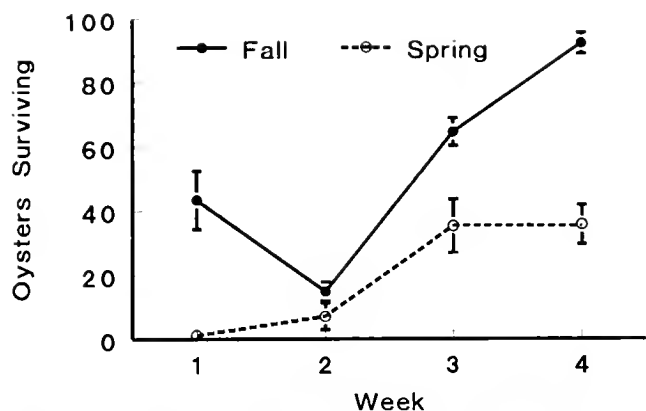
Figure 4. Percent survival of oysters ($\bar{x}$ ± SE, pooled over both scent treatments, $n = 24$) in samples collected during 4 weeks at Creole Bay in two seasons.

TABLE 3.

F values from two-way repeated measures analyses of variance of oysters collected in dredge hauls at two sites in Barataria Bay, Louisiana, in two seasons. The repeated measures (= time) are three dredge hauls through time at each site and season.

Source of Variation	Creole Bay	Lake Grand Ecaille
Time	13.1**	32.7**
Time × Season	2.7	8.0
Time × Scent	1.2	1.8
3 way interaction	1.3	6.6
Season	7.4*	7.0*
Scent	0.3	0.1
Scent × Season	2.9	1.6

* Significant at $P < 0.05$.
** Significant at $P < 0.01$.

much lower than in the spring (Fig. 5). However, *a posteriori* tests indicated treatment plots differed from controls only for the last week of the experiment in the fall. The nonparametric test again indicated a significant season effect (H = 15.1, $P < 0.01$), but not a significant treatment (H = 0.4, $P > 0.05$) nor interaction effect (H = 0.1, $P > 0.05$).

Oysters collected in dredge hauls also declined dramatically through time (Table 3, Fig. 6) corroborating the high mortality rates suggested by the data from the trays deployed in plots. Oyster densities also differed between the two seasons, but there were neither scent treatment effects nor interactions. Oyster survival (estimated by dividing final mean densities by initial densities) varied from 7.5% in the fall at Creole Bay to 8.9% in the spring. At Lake Grand Ecaille, 36.9% of the oysters survived in the fall, but only 9.7% in the spring. Thus these data also suggest, as did the data from trays, that mortality rates were higher in the spring at the coastal site. Nonparametric tests were not necessary here as data were normally distributed (SAS, Inc. 1988, procedure Univariate).

Mortality caused by black drum (again as estimated by the fraction of shells missing from trays) varied with time and season (Table 4, Fig. 7). At Creole Bay in the fall, mortality due to drum peaked during week 2, and was always at least 50%. In the spring, mortality caused by fish steadily declined. At Lake Grand Ecaille, mortality due to black drum steadily increased through the experi-

ment in the fall, and was constant and near 100% in the spring. Conclusions from nonparametric tests were again similar: no significant treatment effects occurred at Creole Bay, but a significant seasonal effect (H = 43, $P < 0.01$) occurred at Lake Grand Ecaille.

The percentage of oysters gaping in trays also varied with time, and was dependent on season at one of the sites as well (Table 4). At Creole Bay, percentage of oysters gaping was fairly consistent through time in the fall, but increased with time in the spring. At Lake Grand Ecaille, percent of shells gaping was high initially in the fall, but declined through time. Gaping shells were essentially absent in all but the final week in the spring, explaining the strong seasonal effect at this site. The nonparametric tests re-enforced these conclusions; none of the treatment contrasts were significant ($P > 0.05$) at Creole Bay, but there was a significant seasonal effect (H = 15.1, $P < 0.01$) at Lake Grand Ecaille.

DISCUSSION

Predator Prey Interaction

Our data indicate that large black drum are much more significant predators of oysters than smaller fish, and that smaller oysters are much more at risk. These findings corroborate earlier laboratory feeding data (Cave 1978), studies of the diet of field caught fish (Pearson 1929, Gunter 1945, Darnell 1958, Dugas 1986), and results of surveys of oyster leaseholders (LDWF 1999). Oyster

Figure 5. Percent survival of oysters ($\bar{x}$ ± SE, $n = 12$) in samples collected in 2 scent treatments during 4 weeks at Lake Grand Ecaille in two seasons.
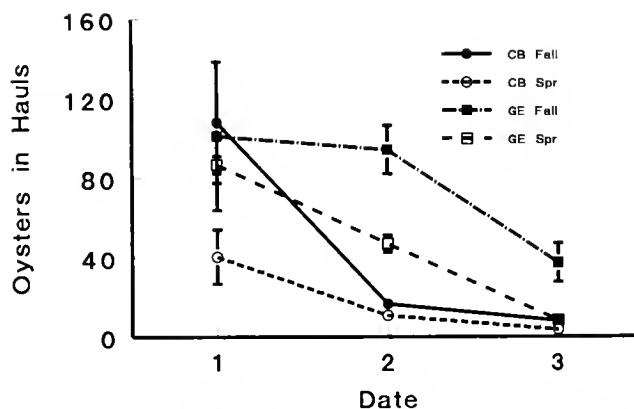
Figure 6. Number of oysters collected in hauls ($\bar{x}$ ± SE, $n = 4$ plots, pooled over both scent treatments) collected at three dates during field experiments at both sites in two seasons.

TABLE 4.

F values from two-way repeated measures analyses of variance of oyster percent gaping and mortality caused by black drum at 2 sites in Barataria Bay, Louisiana, in two seasons. The repeated measures (= time) are four samples through time at each site and season.

Site	Source	% Gaping	% by Black Drum
Creole Bay	Time	36.7**	50.7**
	Time × Season	7.9**	6.3**
	Time × Scent	0.1	0.1
	3 way interaction	0.5	0.8
	Season	3.2	0.9
	Scent	0.1	0
	Scent × Season	1.1	1.5
Lake Grand Ecaille	Time	21.5**	50.5**
	Time × Season	17.6**	51.8**
	Time × Scent	4.1*	3.2*
	3 way interaction	1.4	1.1
	Season	48.7**	128.3**
	Scent	0.4	0.1
	Scent × Season	0.7	0.4

* Significant at $P < 0.05$.

** Significant at $P < 0.01$.

leaseholders reported much higher predation rates on leases seeded with oysters the previous fall in comparison to inactive leases, or active leases that were not seeded the previous fall. Two mechanisms could explain this higher loss. First, our experiments suggest these small, individual oysters are easily consumed by fish. In comparison, naturally occurring oyster reefs along the Louisiana coast occur inter-tidally, where predation pressure by fish, stone crabs and oyster drills is reduced because of greater aerial exposure, and where oysters grow in aggregations that are hard for fish to feed on (Brown 1997, Brown & Stickle 2002). Second, oysters that are transported on boat decks from state-maintained seed areas on the eastern side of the Mississippi River to commercial leases that are often 40–80 Km away undoubtedly experience stress, and injured oysters produce scents that attract black drum (LDWF 1999).

Surprisingly, we found no evidence that reduced salinities altered feeding rates. Perhaps salinities below the 13 ppt used in

Figure 7. Percent mortality ($\bar{x} \pm SE$, $n = 12$) caused by black drum at two sites in two seasons in the field experiments on leases.

these experiments reduce predation. This salinity occurs at the so-called "conch line" in Lousiana estuaries, north of which oyster drills are not considered serious predators (Bahr & Lanier 1981, Butler 1985). We also found evidence from the field experiments that losses to oyster drills were higher in the fall particularly at Creole Bay, whereas fish predation rates were higher in the spring at Lake Grand Ecaille. Black drum are apparently replacing nutrient reserves lost during spawning in the previous winter, and by voraciously feeding in groups, far outweigh any advantages of oyster growth during the winter months.

Our field experiments certainly corroborate that predation can be a considerable mortality source for oysters planted in leases. Losses to drum occurred at high rates in both seasons, but were particularly high at Lake Grand Ecaille in the spring, where essentially all oysters were handled and consumed in most of the weeks. There was also a tendency for mortality to be high initially at Creole Bay, and for the reverse pattern to occur at Lake Grand Ecaille in the fall. These different temporal patterns are probably explained by black drum either being resident on the site at the start of the experiment (for example at Creole Bay in the spring), or being attracted to the site after the leases were seeded (for example at Lake Grand Ecaille in the fall).

Other mortality sources for oysters include predation by southern oyster drills, and mortality to Dermo infections. We hypothesize that Dermo infections in these experiments caused minimal mortalities for four reasons: (1) southern oyster drills were recovered in high numbers on trays, especially at the estuarine site in the fall; (2) oysters in covered cages survived well (average of 98%) in all experiments; (3) temperatures were not extreme, as experiments were conducted in the spring and fall, not summer months when prevalences are higher in Louisiana oyster reefs (Cook et al. 1998, La Peyre et al. 2003), and (4) Dermo prevalences were low (weighted incidence of 0.2–0.9 on Mackin scale) in seed grounds where oysters were collected to seed our experimental plots (P. Banks, LDWF, Pers. Comm.). Assuming gaping shells were the result of southern oyster drill predation, mortality to these invertebrate predators appeared to be greatest in the fall, not spring months, and occurred more frequently at the more estuarine site.

Importance of Scent

Laboratory experiments did indicate some reduction in feeding in the presence of scent of a dead conspecific, but the effect was overwhelmed by differences in the feeding rates of individual fish in each of the independent experimental replicates. Observations indicated that fish were not fully acclimated to the laboratory settings even after a week. Although raceways were large, fish repeatedly rubbed against walls, and the concrete surface produced scrapes on the fish skin. Cave (1978) kept fish for long periods in aquaria similar to ones in our preliminary experiments, undoubtedly resulting in better acclimation, and perhaps producing feeding rates that are more comparable to field conditions.

Our field experiments did not indicate that scent of dead black drum was a practical feeding deterrent. Increases in oyster survival occurred in scent plots at Lake Grand Ecaille, but only during the fall, when mortality rates were overall lower, and survival was increased only by 20% at the most. Black drum feed in groups, and a scent stimulus may have to be maximal to deter these voracious predators, especially when they feed on leases in the spring to
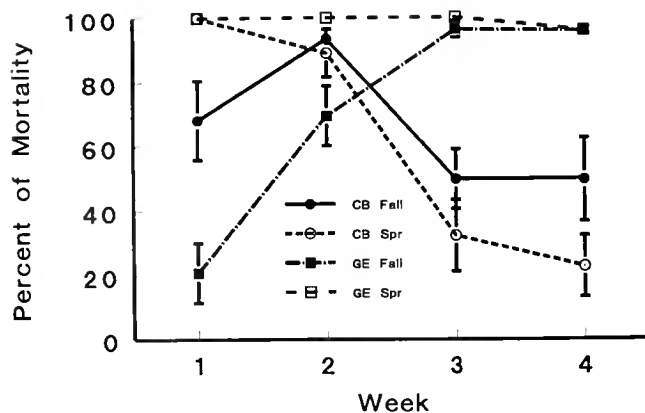
renew energy reserves. We deployed two carcasses at the center of a 700-m^2 circular plot, and it could be argued that deploying larger numbers of carcasses would be more effective. However, this is a small area (selected as the smallest area where an oyster boat could circle and wash off seed oysters onto the sediment). Furthermore, if greater numbers of black drum are fished off leases and deployed, the question arises as to whether the improved survival of oysters is caused by the scent stimulus or simply reduced fish abundance and thus reduced predation pressure.

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ABSTRACTS OF TECHNICAL PAPERS

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&

PACIFIC COAST SHELLFISH GROWERS ASSOCIATION

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CONTENTS

ASSESSING POPULATIONS OF PACIFIC RAZOR CLAMS (*SILIQUA PATULA*) ALONG THE PACIFIC COAST OF WASHINGTON STATE.

Dan L. Ayres, Washington Department of Fish and Wildlife, 48 Devonshire Road, Montesano, WA 98563; and **Ervin J. Schumacker,** Quinault Department of Natural Resources, PO Box 189, Taholah, WA 98587.

Perfect habitat for the Pacific razor clam (*Siliqua patula*) is found along the Pacific Ocean beaches in Washington State. To determine total abundance of razor clams, the newly designed Pumped Area Method recently became the method of choice. This method requires water to be pumped from the surf or a nearby lagoon. This water, as it is directed through a handheld PVC wand, is used to liquefy the sand within an aluminum ring (½ square meter in area). The razor clams found float to the surface and are removed, measured and returned. This process is repeated along a randomly selected transect with 6 rings completed every 50 feet. Each transect requires one turn of the tide (5 hours). For each mile of razor clam habitat determined to be on management beach, one transect is completed. The data collected is used to calculate the average number of razor clams per square meter. Using an estimate of the number of square meters of razor clam habitat, the total number of razor clams can be determined. The State of Washington and the Quinault Indian Nation use this jointly determined abundance estimate to co-manage the harvest of razor clams at the Copalis, Mocrocks and Kalaloch management beaches.

RELATIONSHIPS BETWEEN SUMMER MORTALITY AND IMMUNE RESPONSES IN THE PACIFIC OYSTER, *CRASSOSTREA GIGAS.*

Colleen A. Burge, Yuichi Eugene Saito and **Carolyn S. Friedman,** School of Aquatic and Fishery Sciences, University of Washington, Seattle, WA 98195.

The Pacific oyster, *Crassostrea gigas,* has experienced summer mortality events in the Pacific Northwest and Japan since the mid 1950's and in California starting in 1993. Summer mortality events have been linked to multiple stressors associated with planting times and height including extreme dissolved oxygen and temperature fluctuations. Hemocytes are integral in many important physiological processes such as nutrient digestion and transport, excretion, wound repair and pathogen defense. Cellular defense is the primary immune mechanism of marine invertebrates. Hemocytes found in hemolymph and interstitial spaces function in host defense via inflammation, wound repair, encapsulation, and phagocytosis. Hemocyte performance may contribute to observed differences in mortality between selected family lines of *C. gigas* from the Molloscan Broodstock Program (MBP) of Oregon State University. To better understand the differences in oyster performance, we examined the immune response of oysters from two different MBP families grown at a site with low mortality (Totten Inlet). These families were selected based on mortality rates: high mortality (MBP family 10-116) vs. low mortality (MBP family 10-115). The ability of hemocytes to phagocytose or engulf foreign particles, move towards a chemical stimulus (chemotaxis), and kill *Vibrio parahaemolyticus* was examined. Differences and similarities in immune responses between the two groups of oysters will be described.

POPULATION STRUCTURE AND RECOVERY DYNAMICS OF BLACK ABALONES (*HALIOTIS CRACHERODII*) AT SAN NICOLAS ISLAND, CALIFORNIA.

Melinda D. Chambers, C. S. Friedman, L. Hauser, School of Aquatic and Fisheries Sciences, University of Washington, Seattle, WA 98105; and **Glenn R. Vanblaricom,** Washington Cooperative Fish and Wildlife Research Unit, School of Aquatic and Fisheries Sciences, University of Washington, Seattle, WA 98105.

Populations of black abalone have experienced declines of 85-99% since the emergence of the disease Withering Syndrome (WS) in 1985. Black abalone populations in the California Channel Islands formerly harbored unprecedented densities. Since 1981 we have collected data that documents the change in abundance on San Nicolas Island. Recent data indicate the first recruitment event since the onset of WS with observations of individuals sized <50 mm. A drift card study conducted in August 2002 on San Nicolas Island, indicated that dispersal was largely localized. Subsequent genetic studies will be conducted throughout 2002 and 2003 to confirm speculation that genetic differentiation corresponds with geographic distance. Tissue samples will be collected from each of the California Channel Islands that support black abalone populations of high density and genetic analysis will be conducted using allozymes and mtDNA. Additionally, further drift card studies will be conducted to improve our understanding of circulation patterns in the Channel Islands and temperature data will be monitored using TidbiT stowaway devices, as elevated sea surface temperatures are tightly associated with WS symptoms in black abalone.

2002 MONITORING OF HARMFUL ALGAE IN SOUTH PUGET SOUND AND WILLAPA BAY—SPECIES OF CONCERN AND FUTURE CONSIDERATIONS.

Aimee E. Christy, The Evergreen State College, Olympia, WA 98505.

Harmful Algal Blooms (HABs) are natural phenomena and have occurred worldwide for hundreds of years. The impacts of these blooms include both economic and health concerns for shellfish growers, consumers and the local economies dependent on shellfish resources. Pacific Shellfish Institute (PSI) is a member of the Olympic Region Harmful Algal Bloom (ORHAB) Partnership working to understand HABs and reduce HAB impacts on humans and the environment. In addition to monitoring toxic algae in Willapa Bay, PSI independently monitors plankton communities at several locations in south Puget Sound. Monitoring efforts have detected numerous species of plankton that form HABs and are of special concern to the shellfish industry. Evidence exists that the incidences of problems associated with toxic algae are rising. Pos-

sible explanations for the increased frequency and intensity of blooms include natural dispersal of plankton via currents, climatic changes, nutrient enrichment and the transport of new species in ballast water. Understanding the factors that contribute to HABs, studying life cycles of local species, diligent monitoring and response efforts, innovative methods for quick and economic toxin detection and the ability to predict HAB occurrences are necessary steps in the protection of human health and shellfish resources.

LITERATURE REVIEW—IMPACTS OF URBANIZATION ON WATER QUALITY IN SHELLFISH GROWING AREAS IN PUGET SOUND, WASHINGTON. Aimee E. Christy, The Evergreen State College, Olympia, WA 98505; and Stuart D. Glasoe, Puget Sound Water Quality Action Team, Olympia, WA 98504.

In response to population growth, urbanization and worsening bacterial contamination trends throughout the region, the Puget Sound Water Quality Action Team is undertaking a study to better understand the impacts of urbanization on water quality in shellfish growing areas. A literature review was conducted to assemble available information to determine the current understanding of the relationship between urbanization and bacterial contamination in the nearshore environment. Results of the literature search indicate distinct differences in bacteria sources and transport pathways between rural and urban watersheds. The concentration and rapid transport of urban pollutants into receiving waters caused by the conversion of native vegetation to impervious surfaces and drainage networks is well documented. A number of indicators (impervious surface coverage, developed land, population, housing density) are being examined and some appear more significant than others in correlating development and bacterial contamination. Findings encourage protecting natural filtration areas, preserving buffers and native vegetation, disrupting connectivity between impervious surfaces and receiving waters, educating the public, and using innovative planning and low-impact development techniques to mimic and preserve natural hydrologic functions. Understanding the relationship between urbanization and water quality will provide the tools necessary to develop in ways that support future growth, natural resources, public health and clean water.

THE HISTORY AND DEVELOPMENT OF THE PUGET SOUND COMMERCIAL GEODUCK INDUSTRY. Marion Dumont, Univ. of Washington, Tacoma, Washington.

The commercial geoduck industry had its official beginnings in Washington State in 1970, the onset of a decade defined by conflict, transition and change. The men and women, politicians, harvesters, leaseholders and government agents were fiercely competitive and adventurous, given to quarreling and trouble making. They proved a driving force for an innovative and booming industry.

RELATIVE IMPORTANCE OF SURVIVAL AND GROWTH RATE IN DETERMINING YIELDS OF PACIFIC OYSTERS. *CRASSOSTREA GIGAS.* Ford Evans, Sean Matson, John Brake, and Chris Langdon, Coastal Oregon Marine Experiment Station and Dept. Fisheries and Wildlife, Oregon State University, Newport, OR 97365.

Data were collected on three cohorts (C-6, C-7, C-9) of unselected full-sib Pacific oyster (*Crassostrea gigas*) families. The roles which individual growth rate and survival play in determining average family yield were investigated. C-6 families were planted subtidally in Yaquina Bay, OR. C-7 families were planted intertidally in Tomales Bay, CA, and subtidally in Yaquina Bay, OR. C-9 families were planted intertidally in Totten Inlet, WA. Once market size, oysters were harvested and yield, average individual growth rate and survival recorded for each family. Phenotypic correlation coefficients (r_p) between performance characters were estimated within each cohort. Correlation coefficients of performance characters between sites were estimated for C-7. Survival was significantly correlated with yield within all cohorts ($r_p = 0.66$ to 0.98, $p<0.05$). Average individual growth rate was significantly correlated with yield in all cases ($r_p = 0.65$ to 0.93, $p<0.05$) except for C-7 in Yaquina Bay, OR ($r_p = 0.52$, $p = 0.20$). Correlations between average individual growth rate and survival tended to be higher in cohorts planted intertidally ($r_p = 0.70$ to 0.71) than in cohorts planted subtidally ($r_p = -0.130$ to 0.32). C-7 yield was significantly correlated between Tomales Bay, CA, and Yaquina Bay, OR ($r_p = 0.88$, $p<0.01$). Yield stability appeared to be driven primarily by the significant correlation of survival between sites ($r_p = 0.89$, $p<0.01$). Individual growth was not correlated between sites ($r_p = 0.32$, $p = 0.51$). These results indicate the relative importance of survival and growth rate in contributing to yields of Pacific oysters varies between sites and cohorts. The implication of these results on breeding schemes targeting oyster yield will be discussed.

LIFE HISTORY OF AN EXOTIC SABELLID POLYCHAETE, *TEREBRASABELLA HETEROUNCINATA:* FERTILIZATION STRATEGY AND INFLUENCE OF TEMPERATURE ON REPRODUCTION. Carl A. Finley, Thea T. Robbins, California Department of Fish and Game, Bodega Marine Laboratory, P.O. Box 247, Bodega Bay, CA 94923; and Carolyn S. Friedman, School of Aquatic and Fishery Sciences, University of Washington, Box 355020, Seattle, WA 98195.

Abalone culture facilities have been devastated by an exotic sabellid, *Terebrasabella heterouncinata,* following its introduction from South Africa in the late 1980s. Infestations are associated with shell deformities, increased mortality and financial losses. In addition, the potential introduction and establishment of this exotic pest into the natural environment was unknown. The development of an effective management strategy is dependent upon understanding the life history of this sabellid, including its fertilization

strategy and generation time. In the present study, red abalone, *Haliotis rufescens*, with single sabellid infestations were isolated in containers at 18°C. This first, parental generation was held in isolation until individuals produced F_1 larvae, which were subsequently isolated until individuals produced a second, F_2, generation. In a separate study, uninfested abalones were exposed to infested abalone at three temperatures typically encountered in California. Transmitted larvae were observed as they developed to specific life stages: initiation of feeding, sexual maturation and production of motile, infestive, larvae. This research demonstrated that isolated individuals are functional hermaphrodites and do pose the threat of producing fully functional offspring and that the generation time of *T. heterouncinata* is significantly temperature dependent. The aquaculture industry, UC Santa Barbara researchers and Department of Fish and Game (DFG) initiated an aggressive eradication program in 1996, and DFG policy was established in 1997 to prevent further spread of the sabellid. Culling of infested stocks and strict hygiene protocols including freshwater treatment of tanks proved effective in curbing new infestations. Results of recent eradication efforts will be described.

SUMMER MORTALITY OF THE PACIFIC OYSTER, *CRASSOSTREA GIGAS*, ALONG THE WEST COAST OF THE U.S.: PERFORMANCE OF FAMILY LINES AND ENVIRONMENTAL PARAMETERS.

C. S. Friedman, C. A. Burge, University of Washington, Seattle, WA 98195; D.P. Cheney, R. A. Elston, A. D. Suhrbier, Pacific Shellfish Institute, Olympia, WA 98501; G. N. Cherr, F. J. Griffin, A. Hamdoun, Bodega Marine Lab, UC Davis, Bodega Bay, CA 94923; and C. J. Langdon, Hatfield Marine Science Center, OSU, Newport, OR 97365.

Mortality of the Pacific oyster, *Crassostrea gigas*, has occurred in the U.S. west coast and Japan since the mid 1950's. Multiple stressors have been implicated as contributing to these mortality events. In an attempt to alleviate the >50% annual oyster mortality observed in California and variable losses in Washington state, we examined the interaction between survivorship, growth and stress response of family lines from the Molluscan Broodstock Program (MBP) of Oregon State University, and planting time and height, and selected environmental parameters. To examine differential performance between family lines and planting period three oyster families were each outplanted during Fall 1999, 2000, 2001 and Spring 2000, 2001, 2002 at 2–3 sites in California, 3 sites in Washington (Spring only), and 1 site in Oregon (Spring 2002 only). Fall plants survived significantly more than did oysters planted in the spring (p<0.05) in California. In addition, two families (one commercial strain and MBP family 10–115) outperformed MBP family 10–116 (p<0.001) at all locations. During the study period inter-annual variation in phytoplankton was more pronounced than spatial variation. While suspected harmful algal species were present throughout the study period, phytoplankton

did not appear to be directly involved in oyster mortalities. However, in California a 2000 mortality coincided with a *Gymnodinium sanguineum* bloom, while 2001 and 2002 mortalities were not associated with any phytoplankton bloom. Extreme temperature and dissolved oxygen fluctuations were repeatedly associated with oyster mortalities at the Washington and California study sites.

WITHERING SYNDROME OF ABALONE IN CALIFORNIA.

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Catastrophic declines in many abalone species in California, both wild and cultured, have been attributed to the bacterial disease, Withering Syndrome (WS). The etiological agent was recently described as a novel rickettsial bacterium, "*Candidatus Xenohaliotis californiensis*" which infects gastroepithelial cells of abalone and results in morphologic changes in the digestive gland (degeneration and metaplasia) and foot muscle (atrophy). Digestive gland metaplasia appears pathognomonic for WS. Differences in susceptibility and tissue changes were noted between species with black abalone, *Haliotis cracherodii*, being more susceptible to WS than red abalone, *H. rufescens*. Climatic variation associated with ENSO events has been demonstrated to result in development of WS in red abalone, and exacerbate disease development in black abalone. Survivors appear relatively resistant to WS and are being considered as captive broodstock in species restoration programs. Molecular tools and therapeutants have been developed and will play a key role in the abalone culture industry and captive broodstock programs, particularly for the endangered white abalone, *H. sorenseni*, which is being cultured in a WS endemic region.

ASSESSMENT AND MANAGEMENT OF INTERTIDAL CLAM RESOURCES IN BRITISH COLUMBIA.

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Intertidal clams continue to be an important resource in British Columbia, and are utilized by commercial, recreational and Aboriginal harvesters as well as the aquaculture industry. The most

important species is the Manila clam, or Japanese littleneck, although native littleneck, razor and butter clams are also landed commercially. The basis for assessment of clam resources is a statistically rigorous survey, with clearly defined protocols for design, field procedures, data management and analyses. These protocols are used by government programs, co-management programs with First Nations or Industry, and by contract biologists. How assessment data are used varies between fisheries and depends upon capacity for gathering assessment data and allocation policies. The regular commercial fishery is managed using historic expectations of production and landings that are monitored for indications of depleted legal size stocks. The depuration fishery and First Nations pilot program allocate individual beaches to harvest groups. These groups must undertake stock assessments before quotas can be determined. Beaches were initially managed experimentally using harvest rates of 25 or 50% of estimated legal biomass. Review of stock responses to harvest has led to development of a sliding scale of harvest rates determined by abundance thresholds. In the Area 7 fishery, assessment surveys are done annually on index beaches, which are used to determine trends in biomass indices for subareas that are fished. A simple feedback gain model is used to set harvest thresholds for each subarea.

FEEDING RATES OF THE MUD SHRIMP *UPOGEBIA PUGETTENSIS* AND IMPLICATIONS FOR ESTUARINE PHYTOPLANKTON ABUNDANCE. Blaine Griffen, Chris Langdon, Coastal Oregon Marine Experiment Station and Dept. Fisheries and Wildlife, Oregon State University, Newport, OR 97365; and Ted Dewitt, US-EPA – Pacific Coastal Ecology Branch, Newport, OR 97365.

The burrowing shrimp *Upogebia pugettensis* is an abundant inhabitant of Pacific Northwest bays and estuaries where it lives commensally with the clam *Cryptomya californica.* Suspension-feeding activities of the shrimp and its commensal clam, as well as particle settlement within the burrow, represent three potential causes of phytoplankton reduction within shrimp habitats. These three components together comprise what we call the "shrimp-burrow complex". Laboratory measurements of particle filtration rates indicated that shrimp were responsible for filtering the majority of phytoplankton removed by the shrimp-burrow complex; however, particle settlement in burrows and adhesion to burrow walls could also be responsible for removal of significant proportions of phytoplankton. Particle filtration efficiencies of shrimp + burrows and clams were similar to those of Pacific oysters, *Crassostrea gigas,* for particles 2 to 10 microns in diameter, indicating a potential for food competition among these species under food-limiting conditions. A population filtration model, based on field measurements of shrimp filtration rates together with data on phytoplankton concentrations and shrimp populations in the Yaquina estuary, Oregon, predicted that shrimp-burrow complexes in this estuary were capable of filtering the entire body of overlying water between one and two times daily.

PATTERNS IN INTERTIDAL HABITAT USE BY SUBADULT DUNGENESS CRAB (*CANCER MAGISTER*). K. Holsman, P. Sean McDonald, D. Armstrong, and J. Ruesink, Department of Zoology, University of Washington, Box 351800, Seattle, WA 98195.

Complex intertidal habitats characteristic of northeastern Pacific coastal estuaries provide critical nursery environments for young-of-the-year Dungeness crabs, *Cancer magister,* yet their role in supporting subsequent year classes remains unclear. As with other brachyuran species that undertake diel intertidal migrations, subadult *C. magister* (40 -130mm; 1+ and >1+ year classes), which reach densities as high as 4,300 crabs ha^{-1} in subtidal channels during low tides, may migrate during flood tides from subtidal refuges into intertidal habitats to forage. Results of a bioenergetic model for crabs in Willapa Bay, Washington, indicate that intertidal foraging may contribute significantly to the energy budget of subadult *C. magister* and may facilitate the high abundance of crabs observed in large coastal estuaries. We conducted bay wide trapping surveys in intertidal oystershell, eelgrass, and bare mud habitats in order to elucidate patterns of habitat use by subadult crabs, and underwater video was used to observe tidal migrations in these habitats. Significant differences in crab abundance and the magnitude of migrations were observed across habitats, with lowest densities of *C. magister* occurring in older shell beds concurrent with high densities of red rock crabs (*C. productus*). Observations of tidal migrations using underwater video suggest that the physical structure of plants may hinder crab movement in eelgrass beds since the number and size (carapace width, CW) of crabs migrating was smallest in this habitat. Although the density of prey species may be less in open mud or sand habitats, the lack of structural hindrance and interspecific competition may render open mud the most valuable intertidal habitat to subadult crabs. The importance of intertidal habitats to subadult crabs has direct implications in coastal estuaries of the Northeastern Pacific where anthropogenic and biotic modification of intertidal areas threaten the productivity of intertidal habitats and may adversely impact estuarine populations of *C. magister.*

SEASONAL UTILIZATION OF INTERTIDAL HABITATS BY FISH IN A WASHINGTON STATE COASTAL ESTUARY. Geoff Hosack, David Armstrong, 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; Brice Semmens and Jennifer Ruesink, Dept. of Zoology, University of Washington, Seattle, WA 98195.

Estuaries are regarded as important nursery areas for juvenile marine and anadromous fish. Estuaries also support fisheries and aquaculture and the effects of these activities on fish habitat are

becoming increasingly scrutinized under the *Endangered Species Act* and *Magnuson-Stevens Act* . We are conducting a study to evaluate the importance of the intertidal environment for juvenile fish within Willapa Bay, Washington with respect to aquaculture. Our objectives are to compare commercially cultivated and uncultivated habitats in order to: (1) elucidate potential habitat preferences among juvenile fishes, (2) establish possible mechanisms for habitat preferences, and (3) evaluate the function of intertidal habitats for fish foraging, predator avoidance, and mobility behaviors. One-meter high hoop nets were deployed over three habitats (oyster culture, eelgrass and unvegetated open mud/sand) to determine habitat preference at three locations in 2001. They were also deployed monthly to determine seasonal presence/absence of fish species and at three different tidal elevations in 2002. Preliminary results show that intertidal use by the majority of species exhibits a pronounced increase during late spring and early summer. Few significant differences in habitat use were found, but a prototype two-boat surface trawl was designed and tested in 2002 to further investigate potential differences in utilization of these low intertidal habitats and adjacent subtidal channel by juvenile Chinook salmon (*Oncorhyncus tshawytscha*). Finally, chinook salmon and shiner perch (*Cymatogaster aggregata*) have been marked with acoustic tags and held in a large enclosure to observe fine-scale movement over a suite of intertidal habitats.

BIOMASS SURVEYS AND ACTIVE MANAGEMENT OF INTERTIDAL RAZOR CLAMS (*SILIQUA PATULA*) AT BEACHES NEAR MASSETT, HAIDA GWAII, CANADA.

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Intertidal razor clam populations and biomass were estimated for commercial clam beaches near Massett, Haida Gwaii for the period 1994 to 2000 using a three stage sampling design. Clams were collected by fluidizing the substrate in a 0.5 m^2 sampling cylinder. Population was estimated for three size fractions (Shell Length (SL) >4 mm, >20 mm and >90 mm, the latter being the commercial size limit) at three beach sections on 18.8 km of beach accessible to the commercial fishery. Calculations varied considerably in some years depending on assumptions about transect length and beach area. There was a record catch of 237 t in the fishery in 2000 that led to concerns by managers about possible overfishing. However surveys indicated that the biomass of clams >90 mm at the start of 2000 was 1876 t (SE 157 t). Biomass was shown to have been at a historic high in 2000 with large numbers of two year old clams in the population, most of which were expected to recruit to the fishery in 2001. The fishery had been passively managed using size limits for many years. However an

examination of razor clam gonads showed that only 50% were mature at 87 mm. Beginning in 2001, in addition to the size limit, an annual quota was introduced in the fishery based on the annual biomass survey and a harvest rate of 12.3% (2/3 of a 1994 estimate of F_{MSY}).

ECONOMICS OF CALIFORNIA'S DUNGENESS CRAB (*CANCER MAGISTER*) INDUSTRY, PRELIMINARY RESULTS.

Matthew J. Krachey, Department of Fisheries Biology, Humboldt State University, Arcata, CA 95521 and **Steven C. Hackett,** School of Business and Economics, Humboldt State University, Arcata, CA 95521.

The current management regime for California's Dungeness crab fishery has led to a derby, with the vast majority of the catch occurring with the first six weeks of the six-month long season. Questions have been raised about the impacts of the derby on industry structure, prices, and product quality. One thrust of our work is to identify baseline economic characteristics of the industry under current management. These include value added, product mix, employment, and capital investment in the processing sector, as well as value added by fishermen. Our preliminary findings indicate that, unlike former derby fisheries for finfish, the product forms that economically dominate are not suppressed by derby conditions. Moreover the derby fishery promotes large-scale processing facilities that create important jobs and processing capability for other fish species in economically less robust coastal communities. Ongoing research will focus on fishery participant's opinions on management alternatives, number of traps deployed, and marginal fishing cost.

THE MOLLUSCAN BROODSTOCK PROGRAM: FAMILY-BASED SELECTION IMPROVES YIELDS OF PACIFIC OYSTERS, *CRASSOSTREA GIGAS*.

Chris Langdon, Sean Matson, John Brake and **Ford Evans,** Coastal Oregon Marine Experiment Station and Dept. Fisheries and Wildlife, Oregon State University, Newport, OR 97365.

The Molluscan Broodstock Program (MBP) was established in 1995 to improve yields of Pacific oysters on the West coast, U.S., by family-based genetic selection. Parental families (P1) in three cohorts of about 60 families each were selected based on superior live weight and meat yields at harvest. Live weight yields of progeny (F1) from crossing P1 selected families were significantly greater than those of non-selected control families in four out of seven trials (ANOVA, p<0.001), resulting in an average gain of 9.5% after one generation of selection. The response to selection was greatest if F1 families were tested at the same site as that used for their parents' selection rather than at a different site. There were weak (p = 0.06; p = 0.04) positive correlations between the yields of families planted at both inter-tidal and sub-tidal sites, indicating strong genotype by environment interaction effects on

yield. Nonetheless, it was possible to identify four to six "generalist" families that were among the top ten families at both sites. Further evaluation of families across a wider range of environments is needed to determine if the best strategy to improve oyster yields will be to select "generalist" families that perform well along the whole Pacific coast, or whether it will be more effective to develop site-specific lines instead.

THE ROLE OF CULTURE PRACTICES IN STRUCTURING INTERACTIONS BETWEEN CULTURED OYSTERS AND NATIVE EELGRASS. Heather M. Macrellis, Jennifer L. Ruesink, Zoology Department, Box 351800, University of Washington, Seattle, WA 98195; and Brett Dumbauld, Washington State Department of Fish and Wildlife, Willapa Bay Field Station, P.O. Box 190, Ocean Park, WA 98640.

The potential for positive interactions between aquaculture species and native eelgrass (*Zostera marina*) is the subject of growing interest in the Pacific Northwest. We conducted surveys of cultured oysters (*Crassostrea gigas*) and *Z. marina* density to determine the nature of the relationship between these two species, and to determine whether this relationship changes under different culture practices used in Willapa Bay, Washington. Culture practices assessed included ground culture harvested by dredging, ground culture harvested by hand, and off-bottom line culture. The role of planting density was also assessed in two separate experiments where small plots were planted with several densities of oyster seed and two year old oysters respectively. Eelgrass production and density were measured throughout the experiments. Results of the survey and experiments will be discussed.

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, 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 20, 5, and 1μm 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).

BIOTIC RESISTANCE TO EUROPEAN GREEN CRAB, *CARCINUS MAENAS*, BY NATIVE ANALOGS IN THE NORTHEASTERN PACIFIC. P. Sean McDonald, Gregory C. Jensen and David A. Armstrong, School of Aquatic and Fishery Sciences, University of Washington, Seattle, WA, 98195.

The notion of 'biotic resistance', which holds that characteristics of native biota act to prevent establishment and persistence of nonindigenous species, remains a dominant component of invasion biology theory. Yet, studies of nonindigenous marine species have often focused on impacts to the recipient community while ignoring effects of the latter on the former. The case of the European green crab, *Carcinus maenas*, provides one such example; the species has successfully colonized temperate coastal embayments throughout the world and its attendant adverse consequences to native biotic communities have been well-documented. However, the distribution and habitat use of *C. maenas* in the northeastern Pacific is more limited than would be expected based on Atlantic populations, and peak abundances occur only in isolated, backmarsh or high intertidal locations. We conducted a limited survey of crab populations in Bodega Bay Harbor (BBH), California, in 1998, and subsequent intensive sampling was undertaken in 2001 in BBH and other central California estuaries. Results from snorkel surveys and trapping data suggest that *C. maenas* are largely absent from areas occupied by native ecological analogs (*Cancer* spp.). Incidence of limb autotomy in *C. maenas* is significantly higher at BBH sites shared with *Cancer* spp. than in isolated areas uninhabited by the latter or in Atlantic populations. A series of tethering experiments similarly supports the assertion that predation/aggression by *Cancer* spp. affects the distribution and habitat utilization of *C. maenas*. The significance of these interactions to the eventual distribution of *C. maenas* in the northeastern Pacific is discussed, as well as implications for monitoring and control efforts.

EFFECT OF DIET ON SOMATIC GROWTH OF JUVE-NILE GREEN SEA URCHINS (*STRONGYLOCENTROTUS DROEBACHIENSIS*). C. Pearce, Fisheries and Oceans Canada, Pacific Biological Station, 3190 Hammond Bay Road, Nanaimo, BC V9T 6N7; **T. Daggett,** Ross Island Salmon Ltd., P.O. Box 1304, Grand Manan, NB E5G 4M9; **T. Chopin,** Centre for Coastal Studies and Aquaculture, Centre for Environmental and Molecular Algal Research, Department of Biology, University of New Brunswick Saint John, P.O. Box 5050, Saint John, NB E2L 4L5; **K. MacKeigan, V. Zitkos** and **S. Robinson,** Fisheries and Oceans Canada, St. Andrews Biological Station, 531 Brandy Cove Road, St. Andrews, NB E5B 2L9.

Populations of sea urchins, harvested for their gonads, are in decline worldwide and so research is now focusing on full life-cycle grow out. The objective of this study was to compare the somatic growth rates of juvenile green sea urchins (*Strongylocentrotus droebachiensis*) fed one of seven diets. Sea urchins (test diameter: 4.5 – 10.7 mm) were collected from the wild, held in laboratory tanks supplied with flow-through seawater, and fed *ad libitum* one of seven diets: (1) a prepared diet, (2) *Porphyra purpurea*, (3) *Palmaria palmata*, (4) *Enteromorpha linza*, (5) a mixture of *Ulvaria obscura* and *Ulva lactuca*, (6) *Laminaria longicruris* collected from an Atlantic salmon culture site, and (7) *Laminaria longicruris* collected from a site uninfluenced by salmon culture. Each diet was randomly assigned to three separate tanks with each tank containing 19 individually housed urchins. Test diameter and whole wet weight measurements from each urchin were initially taken at the start of the experiment and then again once per month for a period of 12 months. Feed type significantly affected growth rate in terms of both diameter and wet weight. *Porphyra purpurea* and the prepared diet supported the best growth while *Laminaria longicruris* collected from a site uninfluenced by salmon culture was the least effective diet.

TRENDS IN PINTO ABALONE (*HALIOTIS KAMTSCHAT-KANA*) ABUNDANCE AT TEN SITES IN THE SAN JUAN ISLANDS AND MANAGEMENT OF THE SPECIES IN WASHINGTON STATE. Don P. Rothaus, R. E. Sizemore, M. J. Ulrich, Washington Department of Fish and Wildlife, Fish Management Program, Central Shellfish Unit, Olympia WA 98501-1091; and **Carolyn S. Friedman,** University of Washington, School of Aquatic and Fishery Sciences, Seattle WA 98195-5680.

As a result of concerns regarding the stability of pinto abalone (*Haliotis kamtschatkana*) populations in Washington and the closure of the abalone fishery in neighboring British Columbia, Canada, the Washington Department of Fish and Wildlife (WDFW) established index stations at ten sites in the San Juan Islands. These stations varied in size from 50 m^2 to 380 m^2, averaging about 220 m^2. WDFW divers systematically surveyed each of these stations in 1992, 1994, and 1996. A decrease in total

abalone abundance at these ten index stations from 1992 to 1994 (n = 351 to n = 288), along with anecdotal information of population decline by University of Washington (UW) researchers and WDFW Enforcement personnel, resulted in the closure of the Washington pinto abalone fishery in 1994. Following the closure, a 1996 survey by WDFW resulted in a combined n = 297. Research in other regions indicate that sedentary invertebrates, such as abalone, must be within 1.0–2.0 m of one another (d ≥ 0.337ndash;0.15 abalone/m^2) for successful fertilization. The average abalone density (d) from one half of the sites surveyed in 1996 contained d ≤ 0.15 abalone/m^2.

Based on survey data, and information from abalone fisheries around the world, it is clear that additional stock assessment is needed to analyze the trend in Washington abalone stocks. Additional index sites, early juvenile life history, population genetics, and the potential for enhancement have been proposed for study.

ECOLOGICAL ROLE AND POTENTIAL IMPACTS OF MOLLUSCAN SHELLFISH CULTURE IN THE ESTUA-RINE ENVIRONMENT OF HUMBOLDT BAY, CA. Steven S. Rumrill and **Victoria K. Poulton.** Estuarine and Coastal Sciences Laboratory, South Slough National Estuarine Research Reserve, Charleston, OR 97420.

The intertidal mudflats of Humboldt Bay, CA, provide habitat for eelgrass (*Zostera marina*), invertebrates, shellfish, finfish, and birds. Humboldt Bay is also the leading producer of Pacific oysters (*Crassostrea gigas*) in California. We have completed the first year of a 3-year project to identify and quantify the effects of commercial oyster mariculture in tideflat habitats, eelgrass beds, and invertebrate communities. Experimental oyster long-line spacing plots were established for comparison to a ground culture site and 6 reference sites (no oysters). We sampled study plots quarterly between Aug 2001-Aug 2002 for presence of eelgrass, oysters, and other cover types. We collected infaunal cores, deployed fish traps, and measured water quality, sedimentation, light intensity, and oyster growth characteristics. Eelgrass shoot density and percent cover were consistently highest in an eelgrass bed control site, lowest at the 1.5-ft. long-line spacing plot, and most variable at the ground culture site. Eelgrass metrics in the other long-line spacing plots were generally lower but within the range of variation exhibited by the reference sites. Preliminary analysis of invertebrate cores has produced a species list of over 70 taxa, many of which are known prey items for estuarine fish. Sedimentation measurements showed no consistent patterns among experimental long-line plots. Oyster growth measurements did not differ substantially between long-line plots; oysters grew 20-35 mm in length and 16-22 mm in width between May and Aug 2002. Light intensity was lower beneath oyster long-lines, but did not differ substantially between the 1.5 and 5 ft. spacing plots.

THE EFFECTS OF THE HERBICIDE RODEO® ON PA-
CIFIC OYSTER GAMETOGENESIS AND TISSUE ACCU-
MULATION. **B. C. Smith, C. E. Grue,** University of Washing-
ton, School of Aquatic and Fishery Sciences, Seattle, WA; **N. P.
Kohn,** Battelle Marine Sciences Laboratory, Sequim, WA; and
J. P. Davis, Taylor Shellfish Company, Quilcene, WA.

In Willapa Bay, WA, Rodeo® (Monsanto Agricultural Co., St.
Louis, MO) is being used to control Spartina (*Spartina alterni-
flora*), an invasive cordgrass native to the Atlantic Coast. Spartina
alters the tideland habitat by trapping sediment and raising the
elevation of the mudflats, thus reducing the available habitat for
oyster culture. Rodeo tank mixes include a surfactant to reduce the
surface tension of the spray. R-11 is currently the surfactant used
in the Bay. R-11 belongs to a class of non-ionic surfactants com-
prised of alkylphenol ethoxylates (APEO). Breakdown products of
APEOs have been implicated as endocrine disruptors in fish and
observed to cause delays in development of oyster veligers. The
objectives of our study were to assess whether applications of
Rodeo tank mixes 1) result in tissue concentrations of glyphosate
in oysters that exceed the established tolerance of 3 ppm wet
weight edible tissue and 2) impair oyster gametogenesis. To de-
termine this, Pacific oysters (*Crassostrea gigas*) were subjected to
five treatments: Rodeo; Rodeo tank mix (with R-11 surfactant);
two concentrations of R-11; and a control. The oysters were ex-
posed for 12 h once a week for 4 wks. Tissue samples were
collected for residue analysis of glyphosate, AMPA and APEO and
cross-sections of gonadal tissue were collected for histological
examination. Initial results indicate that exposure to Rodeo without
the surfactant results in concentrations of glyphosate below the
established human health criteria. Tissue resides of APEO and an
assessment of treatment effects on gametogenesis will be deter-
mined this fall.

MUSSEL GROWTH AND FOOD UTILIZATION IN RELA-
TION TO WATER QUALITY ON A RAFT SYSTEM IN
PUGET SOUND, WASHINGTON. **Andrew D. Suhrbier,
Aimee E. Christy, Hector S. Beltran, Daniel P. Cheney,** Pacific
Shellfish Institute, Olympia, WA 98501; **Jonathan P. Davis,** Tay-
lor Shellfish Farms, Shelton, WA 98584; **Kenneth M. Brooks,**
Aquatic Environmental Science Lab, Port Townsend, WA 98368;
and **Frank J. Smith,** Northwest Research Associates, Inc., Belle-
vue, WA 98007.

With an annual production of approximately 3 million pounds
live weight on the U.S. west coast, suspended mussel and oyster
culture is predicted to increase significantly in coming years. De-
scription of the changes associated with the culture of these crops
is essential for the siting and evaluation of new culture facilities
and in improving yield and production of existing facilities. This
research has three general objectives: (1) to assess mussel shell
growth and meat yield against measured physical, chemical and
biological variables; (2) to compare a suite of variables with mea-

surements of mussel feeding and biodeposit production; and (3) to
collaborate with an on-going nutrient modeling study to estimate
potential mussel carrying capacity in an entire farming area. Dur-
ing the first year (2001–02) multiple observations were made of
water currents, water chemistry, phytoplankton, mussel growth,
seston removal and absorption, fouling, and fish utilization at a
commercial mussel raft culture site in Totten Inlet, Washington.
Certain parameters, such as phytoplankton abundance varied
markedly inside and outside the raft units and under differing tidal
conditions, although these preliminary data suggest feeding effects
on phytoplankton are highly localized and largely contained in the
immediate raft system. The second project year (2002–03) will
continue the Totten Inlet experiments and add a study site at a
commercial mussel farm in Penn Cove, Washington. This research
is supported by the Sea Grant Program Office National Marine
Aquaculture Initiative grant no. NA16RG1591.

RESULTS FROM THE OLYMPIC REGION HARMFUL
ALGAL BLOOM (ORHAB) PROJECT ON THE WASHING-
TON STATE COAST. THE VALUE OF A COLLABORA-
TIVE PROJECT. **Vera L. Trainer,** NMFS, Northwest Fisheries
Science Center, 2725 Montlake Blvd. E., Seattle WA 98112; **Bar-
bara M. Hickey,** University of Washington, School of Oceanog-
raphy, Seattle WA 98195-7940; **Ervin J. Schumacker,** Quinault
Department of Natural Resources, PO Box 189, Taholah, WA
98587.

Harmful Algal Blooms (HABs) became a serious problem to
the coast of Washington state in 1991 when blooms of pennate
diatoms of the genus *Pseudo-nitzschia* produced the potent neu-
rotoxin, domoic acid. Pacific razor clams, (*Siliqua patula*), and
Dungeness crab, (*Cancer magister*), bio-accumulated toxic levels
of domoic acid and recreational and commercial fisheries were
shut down in many areas. Since 1991 *Pseudo-nitzschia* blooms
have recurred many times along the Washington coast causing
suspensions of fisheries with associated economic and cultural
losses for coastal residents. Federal funding for HAB monitoring
projects since 1991 have been contingent on collaborative efforts
that include local stakeholders. The Olympic Region Harmful Al-
gal Bloom (ORHAB) project secured federal funding in year 2000
to investigate and monitor HABs along the Olympic peninsula.
Participants include state and federal agencies, the University of
Washington, non-profit research institutions, commercial shellfish
growers, coastal tribes and shellfish managers. The ORHAB proj-
ect has made significant findings regarding the physical and
chemical processes which create and transport HABs to the Wash-
ington coast. An initiation site for *Pseudo-nitzschia* blooms has
been found in the Juan de Fuca eddy region adjacent to Washing-
ton state and Vancouver island. Blooms from this area may be
transported by storm events to the coast where they are ingested by
shellfish. Monitoring and the use of new technologies by ORHAB
participants have better protected the public health and paved the

way for better understanding of west coast HABs and more efficient means of early detection and monitoring.

GENETIC DIFFERENTIATION AMONG GEODUCK CLAM (*PANOPEA ABRUPTA*) POPULATIONS REVEALED BY ALLOZYME AND MICROSATELLITE ANALYSES.

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The genetic population structure of geoduck clams (*Panopea abrupta*) in inland waters of Washington may affect fishery management and aquacultural practices involving this species. To investigate genetic differentiation in geoduck clams, samples were collected from 16 Washington State sites located in the five Puget Sound subbasins, southern Georgia Strait, and the Strait of Juan de Fuca. A collection from Clarence Strait in SE Alaska was included as an outgroup. Individuals were genotyped at 11 allozyme and 7 microsatellite loci. To investigate the level of isolation by distance, we analyzed correlations between pairwise geographic distances and multilocus Fst values. The Freshwater Bay collection in the Strait of Juan de Fuca was differentiated from others at both microsatellite and allozyme loci. For both marker classes, there was no evidence of significant correlation between genetic and geographic distance measures. In contrast to the microsatellite loci, the allozyme loci were in Hardy-Weinburg Equilibrium (HWE). Deviations from HWE expectations at microsatellite loci were interpreted as being primarily due to primer site sequence variation rather than population level processes such as inbreeding. These results may be due to stochastic variation in reproductive success and recruitment, and warrant further investigation into temporal genetic differentiation.

TRIAL USE OF THE US NAVY REMOTELY OPERATED VEHICLE (ROV) SORD IV FOR SAMPLING DEEP WATER GEODUCK CLAMS (*PANOPEA ABRUPTA*).

B. Vadopalas, School of Aquatic and Fishery Sciences, University of Washington, Seattle WA 98195; and **Don P. Rothaus,** Washington Department of Fish and Wildlife, Fish Management Program, Central Shellfish Unit, Olympia WA 98501-1091.

The existence of geoduck clams (*Panopea abrupta*) below the legally fishable depth of 21 m in Puget Sound, Washington has been surmised from video camera drops in one embayment, but the study of population dynamics and genetic relationships has been hampered by the lack of practical deep water sampling methodology for macrobenthic infauna. We initiated a deepwater geoduck sampling trial using the U.S. Navy ROV SORD IV (Submerged Ordnance Recovery Device). The ROV suction dredge and video system were modified to enhance geoduck excavation and retrieval. The trial was conducted along a depth gradient seaward of a commercial geoduck bed in central Hood Canal. In four hours of ROV bottom time between 35 and 80 m depth we positively identified and attempted sampling of three geoducks. We obtained two specimens approximately 15 meters apart that share many characteristics, including small size, thin valves, and poor viscerosomatic condition. The two animals were of the same age, a result unlikely to arise by chance based on age frequencies in a proximate shallow collection (p<0.01). Genetic analyses indicated that the two clams are most likely full siblings (p<0.001). These findings suggest large variation in year class strength and bias in reproductive success among spawners, and underscore the need for further investigation into population dynamics and recruitment processes in deep water geoduck.

SHELL CONDITION TESTING OF DUNGENESS CRAB IN PUGET SOUND, WASHINGTON.

Donald E. Velasquez, S.F. Burton, Washington Department of Fish and Wildlife, 16018 Mill Creek Blvd., Mill Creek, WA 98012-1296; **D. A. Sterritt** and **B. McLaughlin,** Washington Department of Fish and Wildlife, 1000 Point Whitney Road, Brinnon, WA 98320-9899.

Since 1997 the State of Washington and the Treaty Tribes have been conducting cooperative shell condition testing of Dungeness crab over a large portion of Puget Sound. The purpose of testing has been to determine when it is best to conduct fisheries during the year and limit the problems associated with handling softshell crab.

A number of conclusions have been made regarding the data collected since the program began. The peak molting season for legal male crab differs between subareas within Puget Sound. A pattern where legal-sized crabs finish molting earlier in the year in Central Puget Sound and later in the year for areas adjacent to the Canadian border is apparent. Data also indicate the schedule for the peak softshell period in any given subarea can differ somewhat from year to year. In a few subareas, it is difficult to assign a single softshell period because either the softshell crab are not easily detected or multiple softshell events appear to occur. Possible explanations for the variation of the peak softshell period within Puget Sound will be discussed. Additional observations of synchrony and asynchrony in the life cycle of Dungeness crab were made during shell condition sampling and will be covered.

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COVER PHOTO: Sea urchins (*Strongylocentrotus droebachiensis, S. franciscanus,* and *S. purpuratus*) being used in a gonad enhancement experiment at the Pacific Biological Station (Fisheries and Oceans Canada, Nanaimo, British Columbia, Canada) to test the efficacy of various prepared feeds to produce suitable gonad color, taste, firmness, and texture. Photo: Chris Pearce.
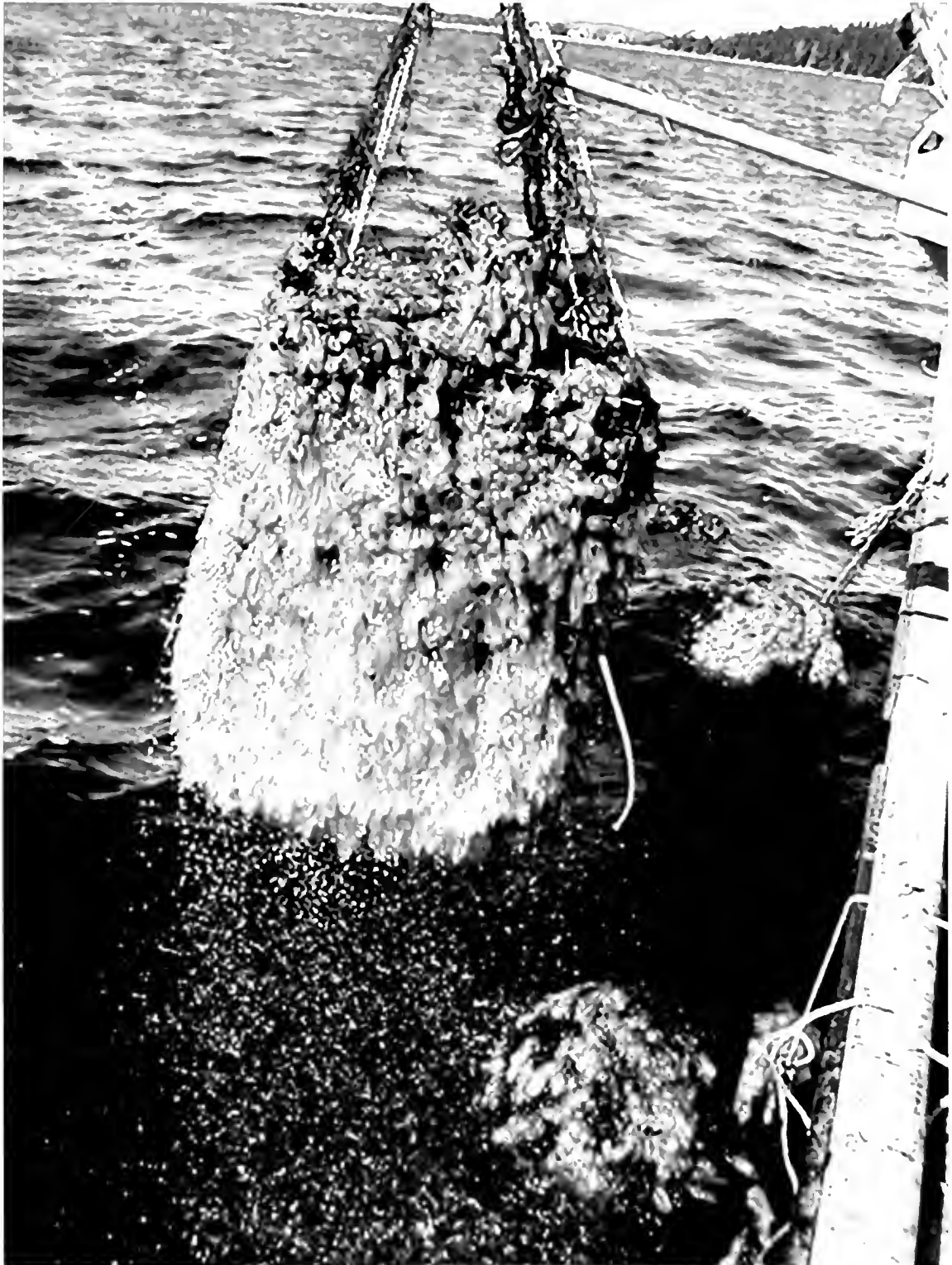
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JOURNAL OF SHELLFISH RESEARCH

Melbourne Romaine Carriker
Honored Life Member

Melbourne Carriker, or "Mel" as he is known to his many students, colleagues, and friends is a world recognized student of Malacology, and an authority on marine subjects as diverse as functional morphology, biomineralization, larval ecology, and predator-prey interactions. Mel's interest in shellfisheries extends from his intense interest in molluscs, their ecology, biology, and morphology. Scientist, scholar, husband, father, and friend—his career and his life have been punctuated by transition and achievement.

Mel's fascinating story began on February 25th, 1915 when he was born in Santa Marta, Colombia. For the first twelve years of his life, Mel lived on a coffee plantation (called Vista Nieve) with his American parents. His parents, Myrtle Carmela Carriker de Flye and Melbourne Armstrong Carriker, Jr., developed and managed the coffee plantation in the Sierra Nevada de Santa Marta Mountains. During his early years, Mel lived in an agrarian community among crops of coffee and sugarcane. Immersed in rugged surroundings, he and his siblings happily lived on the edge of a tropical paradise. When he was ten, Mel began accompanying his father, an accomplished amateur naturalist and ornithologist, on short field trips to collect birds, birds' eggs, and small mammals. Undoubtably, these experiences sparked his interest in the natural world and the seemingly secret lives that animals lead.

In 1927, Mel's parents sold the coffee plantation and moved the family to southern New Jersey, taking up residence in Beachwood. His father took a position at the Academy of Natural Sciences of Philadelphia as Associate Curator of Ornithology, and Mel was enrolled in Toms River grade school. After struggling through the depression years with his family, Mel graduated from Toms River High School in 1934. Immediately after graduation, he accompanied his father on an ornithological expedition into Bolivia, South America. This "enviable, exhilarating experience" (p. 273, Carriker 2000), reinforced Mel's desire to further his education in the field of zoology, in particular ornithology. In the fall of 1935 Mel entered Rutgers University, New Jersey, majoring in agricultural research and minoring in zoology. During several summers, he worked as the director of aquatic and recreation programs at the Boy Scout Camp, Burton-at-Allaire in southern New Jersey to earn money for college. It was at Rutgers that Mel met Thurlow C. Nelson, his undergraduate adviser and mentor, who offered him an opportunity that shaped his scientific career. Through Nelson's urging, Mel began working on Rutgers' College of Agriculture's houseboat in Barnegat Bay, New Jersey, in the summer of 1938, studying the life history of oyster larvae. In subsequent summers of 1939 to 1941, he continued this pursuit on the "Cynthia," broadening his studies to include the general biology and ecology of oysters.

In the fall of 1939, Mel traveled to the University of Wisconsin where he began graduate work with Lowell E. Noland. For his graduate work, he studied the biology of the pond snail, *Lymnaea stagnalis*, a host of the trematode worm that causes swimmer's itch. It was here that Mel began honing his skills as a scientist, studying invertebrate anatomy and physiology, and prepared his first paper on the boring mechanisms of the oyster drill snail. Wisconsin also introduced Mel to one other love, his future wife Scottie McAllister. In 1942, he participated in his first NSA annual meeting, presenting his first scientific paper on oyster-drill boring mechanisms! Mel graduated (in June of 1943) with a doctoral degree in invertebrate zoology and physiological chemistry, and with the rank of ensign in the U.S. Naval Reserve.

Immediately after graduating from the University of Wisconsin, he entered the Naval Training School, Harvard University, where he was trained in naval communications. During World War II, he served on a PC 780 ship in the Aleutian and Hawaiian Islands as communications officer. Although naval duty interrupted Mel's career, his love for malacology continued; rumor has it that during his time off Mel would explore the coast around Adak (Aleutian Islands) collecting marine molluscs and their hemolymph to mail to Rutgers

University for ongoing systematic studies. After the War, he returned to the east coast and accepted a position as instructor in the Department of Zoology at Rutgers in 1946.

Mel worked at Rutgers for eight years, being promoted to Assistant Professor before leaving in 1954. During his time as a faculty member at Rutgers, he developed courses (e.g., estuarine ecology graduate course), taught, and, during the summers, worked with T.C. Nelson and Harold Haskin (see Kraeuter and Ford 1999), investigating the biology of the quahog. In the summers of 1947 to 1949, he returned to the houseboat "Cynthia" in Little Egg Harbor, New Jersey, establishing a research program in shellfish biology that would span his career. From a small laboratory in the stern of the houseboat, he studied quahog ecology and continued researching the shell-boring mechanisms of predatory gastropods. This research was the foundation for several classic published works including, "Critical Review of Biology and Control of Oyster Drills *Urosalpinx* and *Eupleura*" (Carriker 1955), and "Interrelation of Functional Morphology, Behavior, and Autecology in Early Stages of the Bivalve *Mercenaria mercenaria*" (Carriker 1961). Mel lived on the boat with his wife Scottie and two children, Eric and Bruce; a happy but nonetheless crowded existence.

In 1954, Mel was offered, and accepted, a position as Associate Professor at the University of North Carolina (UNC), Chapel Hill. He taught marine ecology and conducted marine-related research in the Department of Zoology. During the summers of 1953 to 1955 he also conducted research on pond culture of oysters and clams on Gardiner's Island, New York. This work was sponsored by the J. & J.W. Elsworth Oyster Company and the U.S. Fish & Wildlife Service. Mel's work on Gardiner's Island was productive and brought him in contact with shellfish biologist Victor Loosanoff. In 1956, his research on clam larvae was shifted to the UNC Institute of Fisheries Research in Morehead City. Over the next five years Mel interacted with scientists at the Institute and at Duke University Marine Laboratory a few miles away, focusing his research on larval biology and the predatory drilling snails of oysters. In 1961, due to unfriendly politics that can be encountered in academia, Mel left UNC and took a position with the U.S. Bureau of Commercial Fisheries Biological Laboratory, Oxford, Maryland. At the Oxford Laboratory he began working on an emerging disease of oysters known as MSX, and this research consumed all of his time. The move to Oxford, however, was to be short lived. In 1962, Mel was enticed by an offer to head a new systematics and ecology program at the Marine Biological Laboratory in Woods Hole, Massachusetts.

The Carriker family moved to Falmouth, Massachusetts, in the fall of 1962, where Mel assumed the position as Director of the Systematics-Ecology Program. The long-term goal of this program was to spearhead research and training in marine systematics and ecology, and enhance the scientific knowledge of organisms in the Cape Cod region. This Program turned out to be "highly successful and functioned productively for ten years" (p. 281, Carriker 2000). One of the most recognized accomplishments of the Program was the publication of a set of keys and check lists of the common invertebrates of, essentially, the waters of southeastern New England. First published in 1964, the "Keys to Marine Invertebrates of the Woods Hole Region" (edited by Ralph I. Smith) provided nonsystematists a useful guide for the identification of many common invertebrates in the region, and were invaluable to students and scientists alike. The first complete revision of these keys in 35 y began in 1999, and the first revised sections can be viewed on the Marine Biological Laboratory's web site. Unfortunately, due to a shortage of funds, the Program was closed in 1972. By then, Mel's reputation as an outstanding marine scientist proceeded him, and he was offered a full professorship at the new College of Marine Studies (CMS), University of Delaware, in Lewes.

In the fall of 1972, Mel and his wife Scottie moved to Delaware where he taught, conducted research, and helped shape the CMS graduate program for thirteen years. During this time he studied oyster shell ultrastructure and chemistry as related to shell penetration by oyster borers, taught a course in malacology, and supervised the research efforts of many graduate students (including some from Central and South America). Mel officially retired in February 1985 at the age of 70, receiving the title of Professor Emeritus. After retiring, he served as president of the Delaware-Panama Partners of the Americas; he continues his scholarly contributions through his writings about his family and the science he loves. In 2000, Mel published a book concerning the fascinating history of his family and their coffee plantation titled "Vista Nieve," from which much of this biography has been gleaned.

Mel is an accomplished scientist, publishing over 45 abstracts and 160 scientific papers and reports, and coining well-known malacological terms such as the "accessory boring organ" (ABO) of muricids, and the "pediveliger" stage of bivalve molluscs. He has presented technical papers at meetings and chaired scientific session over 255 times. From 1965 to 1977, Mel served as editor for the manuals on the Marine Flora and Fauna series produced by the National Marine Fisheries Service. His dedication to the scientific community is evidenced by the many positions he has held including chairman of the Division of Invertebrate Zoology, American Society of Zoology (now the Society of Integrative and Comparative Biology); vice-president of the Association of Marine Laboratories of the Caribbean; and president of the Institute of Malacology, the American Malacological Society, and the Atlantic Estuarine Research Society.

For almost a 50 y period, Mel has served NSA in various capacities, including: Secretary-Treasurer from 1953 to 1954, Vice President between 1955 to 1957, President from 1957 to 1959, and as a source of trusted advice for many an Executive Committee ever since. As Secretary-Treasurer, he was instrumental in formalizing the regular publication of the Association's meeting notes as the "Proceedings of the National Shellfisheries Association (PNSA)," serving as its first Editor from 1954 to 1957. Mel also served several times on the Publications Committee, including during 1979 to 1980 when the name of the NSA publication was changed from the PNSA to the Journal of Shellfish Research. In 1978, Mel was presented with the Honored Life Member award by NSA, and in 1998 was recognized for his years of dedication and scientific achievement in shellfish research when the first NSA student research award was named in his honor. Presently, Mel serves as Historian of the Association, recently completing an historical account of NSA as it emerged from earlier oyster meetings and groups, titled "Taming of the Oyster" (in press).

Throughout his career Mel has been a teacher, researcher, editor, and mentor. He has supervised 35 graduate students (17 Ph.D., 18 M.S.) and has served on numerous graduate student committees. Those of us who have had the pleasure of being a student of Mel's know his objective, quiet approach to seemingly unsurmountable problems, and his deft ability to hone a piece of writing—with comments neatly scripted in pencil on just about every page of many a proposal or paper (often to the immediate displeasure of his students)—so

that it was clear and concise. Mel is a source of knowledge and encouragement, and continues to mentor, albeit informally, young students, former graduate students, and colleagues at yearly scientific meetings and events. The scientific fields of malacology, shellfish biology, and marine ecology have prospered from his life's work, and all of us who have had the pleasure of interacting with him have benefitted by Mel's wisdom, poise, and grace.

J. Evan Ward
Groton, Connecticut

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Michael Castagna
Honored Life Member

Michael Castagna, known to almost everyone in NSA as Mike, was born in Janesville, Wisconsin on October 21, 1927. His parents immigrated to this country from Sicily; his father worked in a General Motors factory in Janesville and his mother worked in the home and for a time in a woolen mill. Following graduation from Janesville High School in 1945, Mike joined the Navy, received his initial training in the Great Lakes, and first viewed the ocean when he shipped out for the Pacific. Mike was stationed in Honolulu where he served as a Pharmacist Mate 2nd Class from 1945 until 1949.

After leaving active duty in the Navy, he enrolled at Florida State University as an undergraduate where he participated in intercollegiate sports, swimming on the all-Navy swim team. In 1951, with only one semester of study remaining at FSU, Mike was recalled to active duty for the Korean conflict as a Hospital Corpsman 2nd Class. Mike's swimming talents were quickly put to use as he became one of the first Navy divers to use SCUBA, taking part in many of the initial dives that led to the development of the now familiar dive tables. When his tour of duty was over in 1953, he returned to FSU to complete work on his Bachelor of Science degree. While enrolled in school, Mike supported himself by working in the Women's Department of Physical Education. After receiving his B.S. degree in 1953, he was admitted to the graduate program at FSU where he worked on a Master's degree. He completed this degree in 1955 with a study of the distribution and ecology of the hogchoker (*Trinectes maculatus*) in the Wakulla River under the guidance of Dr. Ralph Yerger.

In his first job out of graduate school, many of Mike's talents—swimming, fisheries, biology, and a keen love of the ocean—were used as an Assistant Curator at Marine Studios of Marineland, located just south of St. Augustine, FL. He literally swam with dolphins and was in charge of caring for and treating any of the animals that became ill. At this time Mike and his wife of 48 y, Mary Sperry, got married. Mary worked for many years as a nurse and she and Mike have four children.

In 1956, Mike was hired by the Bureau of Commercial Fisheries (BCF) in Boothbay Harbor, ME, to work on the herring investigations under Les Scattergood. This job put him back out on the ocean with frequent sampling trips offshore. During the two years Mike spent in Boothbay Harbor, he served in the Naval Reserves and on several occasions was sent to Key West, FL, for Underwater Demolition Training. There, as the oldest member of the team at nearly 30, he was called "Grandpa" by the younger team members, but he went on to graduate with highest honors.

In 1958 he left Boothbay Harbor and began work at a small BCF laboratory in Franklin City, VA. This laboratory was placed at the end of a long causeway on a former railroad spur, which extended into Chincoteague Bay. The sheet metal building was built next to the former railroad pier. It was a perfect place for Mike who has both the ability to develop new techniques and a hands-on work ethic. Mike has always had a firm commitment to understanding the fundamental ecology of the area where he was working. This included what was present, where it could be found, general observations on abundance, and life history biology. To that end, he helped to design and fabricate the gear needed to investigate the marine life of the bay.

After a short time in Franklin City, he was asked by a fellow Florida State graduate, Bill Hargis, to become the Scientist-in-Charge of the Virginia Institute of Marine Science (VIMS), College of William and Mary laboratory in Wachapreague, VA (At the time it was only known as the "Eastern Shore Laboratory", and it was not until much later that the formal connection to William and Mary was

established). This new position included moving into a newly constructed, single floor building housing offices, wet and dry laboratories, and two dormitory rooms. This was an inspired choice, because it allowed Mike's skills, of leadership, mentoring, innovation, and hard work to flourish. Mike began this position in 1962, remained at the Eastern Shore Laboratory moving up the ranks at VIMS until he retired as a Professor and Division Director in 1992. He continues to work at the laboratory as a Professor Emeritus.

Mike instituted a program to gather basic biological information on the flora and fauna of the local area. He became intimately involved in seeking information from, and giving information to, the local fishing community and encouraged others to take field trips to the Wachapreague area.

In addition to the basic science efforts, Mike coordinated the Eastern Shore components of many oyster trials that were being conducted at VIMS in Gloucester Point, VA. Efforts to control oyster drills with the pesticides Polystream and Sevin, numerous studies on oyster disease and the effectiveness of disease resistant stocks in the higher salinity waters were among the research projects that kept Mike and many others busy with field work.

Although his diving skills were not used heavily at Wachapreague, he often helped members of the community find lost gear or clear fouled propellers. The love of diving and natural history were combined when Mike was invited to spend nine days in the Underwater Laboratory Helgoland in the Baltic Sea in 1974. He returned there as a scientific coordinator for a 14 day underwater mission in 1978. In between (1976) he spent five days in a Hydro Laboratory off Freeport, Bahamas.

In 1962, Mike hired Paul Chanley and they began a series of investigations into bivalve natural history. This included providing information on spawning times, salinity tolerance, larval development, and other aspects for over 60 species. By the end of Mike's tenure as head of the laboratory, 55 species had been reared to setting and 26 species had been reared through their entire life cycle. Much of this work was done in large garbage cans. Water was exchanged by siphons, but was, from time to time, carried in buckets across the road by hand. When temperatures in the wet laboratory were not high enough to rear larvae, the "culture containers" were placed on wheeled carts and aligned down the hall between the offices. This Spartan setting was certainly indicative of funding limitations, but it also reflected Mike's frugal, get-the-job-done approach.

As a direct result of the efforts to document the various life history parameters of bivalves, Mike developed expertise in hatchery technology and aquaculture. This led to the development of a greenhouse for culturing large quantities of algae via the Wells-Glancy technique and later, in a converted oyster shucking house, to a fairly large nursery for the hatchery output of bay scallops and hard clams. Here again, Mike's ability to design and engineer simple, cost-effective solutions was critical. One of the most enduring images from this hatchery was a heat exchanger crafted from an old whiskey barrel and salvaged tubing. Mike often said, "There's no reason to spend $2 on a valve if pinching a hose will work just as well."

Already involved with maintaining a large number of oysters in trays, scattered throughout several bays, Mike was well aware of the difficulties with field studies. This reality and the lack of seed caused his early focus on hatchery and nursery work with clams and scallops. The success of this program provided burgeoning numbers of clams and scallops and he began to develop experimental field plantings. Unless they were heavily protected in trays, the early clam plantings were nearly all consumed by crabs, and even modest size grow-out experiments required tremendous effort. As an example of Mike's inventiveness, one fall, with a substantial number of clam seed on hand, and the necessity of having to close down the seawater pumps for the winter, Mike happened to glance out the window. Within the past week, the road had been tarred and covered with gravel. Most of the gravel had been pushed to the side of the road. Mike decided that, because of the well-documented association of clams with shell beds, gravel might be a good shell substitute. The gravel was swept from the road, loaded into a scow and placed on an intertidal mud flat of a marsh creek. Clams were planted in this gravel and survival was excellent! Unfortunately, subsequent years' plantings did not survive as well. It took Mike, the Eastern Shore Laboratory staff, input from various watermen, many clams, a number of years and a lot of trial and error to develop the knowledge of planting size and protective mechanisms to assure consistent results with seed planting. This effort, as with the innovative descriptive work of Chanley and Castagna a decade earlier, established the Eastern Shore Laboratory as a premier place to do research on bivalve shellfish. This reputation was enhanced by the development of a course to teach basic techniques in clam aquaculture, including how to make the gear, to a cadre of individuals. Many of these individuals became leaders in the hard clam aquaculture industry that has spread throughout the east and gulf coasts, now employs hundreds of individuals and is worth tens of millions of dollars annually.

Mike has authored or co-authored >75 peer reviewed publications, many abstracts, served as editor for two books and was a co-author on a host of reports—including one that has probably been read by more individuals than any work published in the peer-reviewed literature, "A manual for growing the hard clam *Mercenaria mercenaria*".

Field Trips

Because of his interest in natural history and his gregarious nature, Mike was always ready to lead a field trip. These were of two types, those for fellow scientists visiting the Eastern Shore Laboratory and those for students.

Always the raconteur *par excellence* Mike had many tales to tell about visits from scientists. One that left a distinct impression was a visit by a distinguished senior scientist from Europe. Mike was impressed by the scientist's world reputation and wanted to provide a grand tour, which included visiting the habitats on a nearby barrier island. The island had a few cabins that were used primarily on summer weekends, and in the winter for hunting. Mike anchored the boat and indicated they would have to wade ashore. The senior scientist had already figured this out and proceeded to disrobe—completely. Though there were seldom people on the island, passing sport or commercial fishing boats were not uncommon. Mike, thinking that someone might pass by, and wanting to keep the situation as decorous as possible for the laboratory's reputation, handed the individual a towel. The scientist thanked him and proceeded to wrap the towel around his head as a turban and walked ashore.

A significant part of the program at the VIMS Eastern Shore Laboratory was the hosting of field trips for students from other

institutions. This program, which Mike enthusiastically instituted and formalized, required the maintenance and use of small boats. Laboratory staff ran the boats and depending on the group size, availability of various personnel, Mike, or senior staff members were often responsible for conducting the tour. During Mike's tenure, thousands of students from dozens of institutions of higher learning were housed at the Eastern Shore Laboratory and given a first class "hands on" introduction to local habitats.

As might be expected at such a small laboratory, everyone on Mike's staff was expected to do a little of everything and to be on hand to help everyone else. This expectation included a weekly, Friday afternoon general clean up of the laboratory and offices. Everyone was expected to grab a broom or mop, haul out the trash and perform other janitorial duties. While this might seem like a waste of "valuable staff" time to some, the system worked well because Mike participated regularly. It also made everyone aware that if the laboratory was kept clean all week, there was less to do on Friday afternoon. In addition, the "janitor" for the laboratory and dorm was also an individual who helped run the hatchery, ran the nursery and helped in the field when needed. The entire laboratory staff also participated in building the new shop, installing bulkheads, and refurbishing the seawater system and the "new dorm". In the more sophisticated environments of today's laboratories, such a system might help reestablish the "hands on" and "everyone is responsible for the entire laboratory" attitude that is so often lacking, but to do so requires commitment and leadership. This Friday clean up continued as Mike expanded the laboratory's footprint by purchasing a complex of buildings including a former oyster shucking business and a house with a large lot next door (a future dormitory). Also included in the laboratory administration and staff duties, with a few hired local hands during the winter, was building the greenhouse mentioned above, the construction of a new shop/storage complex, refurbishing of the bulkheads along the entire property, and converting the shucking house to a wet-laboratory/bivalve nursery.

Society Work

Leadership is a hallmark of Mike Castagna. While Mike's "aw shucks" demeanor might not lead one to conclude that he was leading, he did so by example. This leadership quality has always been clearly evident to all who worked with him, and was recognized by his peers. Evidence of this is his enormous efforts on the part of the Atlantic Estuarine Research Society, Estuarine Research Federation and his beloved National Shellfisheries Association. In all three organizations he served as Secretary, Treasurer (or Secretary-Treasurer) and President, and has been active on numerous committees and subcommittees, often for many years. Mike spent 16 y as Chair of the NSA Publications Committee and almost single handedly rescued the *Journal of Shellfish Research* from near oblivion. For this and his continued efforts on behalf of the NSA, he was recognized with an honorary award and a student endowment was established in his name accompanying this honor. It was Mike who recruited Sandy Shumway as Editor, and thus he is directly responsible for the expansion of the journal quality and quantity.

He has received honorary awards from the Atlantic Estuarine Research Society (1983) and the Estuarine Research Federation (1985). He also became an Honorary Life Member of the Virginia Shellfish Growers Association (1992). The National Shellfisheries Association honored him with the Wallace Award (1983), the Honored Life Member Award (1990), a special recognition in 1992, and lastly the Society rewarded him for 16 y of service to the Publications Committee and the NSA with the establishment of the Castagna Student Endowment, noting specifically that the award was to go to a student carrying out applied research.

In addition, Mike was an early enthusiastic supporter of the then fledgling Nature Conservancy. He particularly liked the fact that they didn't spend a lot of time litigating or trying to infringe on others land use, but simply bought the land and then tried to develop appropriate management plans. Again this is a "hands on" approach and it earned Mike the Oak Leaf Award from the Nature Conservancy as the Conservationist of the Year in 1974 for his efforts to preserve portions of the Eastern Shore for future generations.

In addition to these formal society activities, Mike also enjoyed the evening meeting socials, particularly if there was good music for dancing. If there were music and willing partners, Mike would be on the dance floor until the music stopped, and then he would often organize a group to go out and find a spot to continue the dancing. Somehow he always seemed to be ready for the first paper of the meeting the next day.

A true love for the natural world and its mysteries, leadership coupled with humbleness, a "can do" spirit and interest in seeing these combined and applied are the mark of someone who cares and makes a difference. These are the hallmarks of Mike's efforts for NSA, Virginia, shellfish culturists, and science. We can all be Mike's students in this regard.

John N. Kraeuter
Haskin Shellfish Laboratory
IMCS
Rutgers University
Port Norris, NJ

Mark W. Luckenbach
Virginia Institute of Marine Science
Wachapreague, VA

Dexter Stearns Haven
Honored Life Member

Dexter Stearns Haven may have officially "retired" from the Virginia Institute of Marine Science (VIMS) in 1984, but a quick look at his publication list or curriculum vitae will illustrate that Dexter has far from actually retired. He has published and or coauthored over 11 papers in the intervening years. In addition, he has been involved as a Director of the York Chapter of the Chesapeake Bay Foundation, and can be seen selling brooms to raise funds for the York Lions Club, of which he is a Charter Member. He volunteers regularly as a docent at the Watermen's Museum in Yorktown, where he shares his knowledge of the Chesapeake Bay, its resources, and the men who harvest them with thousands of visitors. He assists the archaeologists of Jamestown by examining and dating old oyster shells. In truth, Dexter is far from retired.

Fortunately for VIMS, Dexter is continuing his research by working with Bill Hargis, Helen E. Woods, and others on the ecology of oyster bars (reefs) of the Chesapeake Bay. This research, aided by new technology, has resulted in a number of three-dimensional posters on the reefs of the James, York, Rappahannock, and Potomac subestuaries of the Chesapeake system. Formal papers are expected to follow.

Dexter Haven was born in Lake Forrest, Illinois, on November 2, 1918. In 1942, he received a Bachelor of Science degree in premedical subjects at the University of Rhode Island. After receiving his degree, he served his country in the U.S. Army Air Corps as a weatherman in the 9th Weather Squadron.

At the end of his military service in 1946, he again enrolled at the University of Rhode Island, receiving a Master of Science degree in marine biology in 1948. After completing the masters degree, Dexter joined the U.S. Fish and Wildlife Service in 1948. In 1949, he joined the staff of the Virginia Fisheries Laboratory at Yorktown, Virginia (predecessor of the VIMS of the College of William and Mary, and now located at Gloucester Point, Virginia, just across the York River from Yorktown).

In addition to his research and teaching duties before retiring, Dexter served in several capacities at VIMS: as Senior Marine Scientist and Head of the Department of Applied Biology; and as Professor of Marine Science of the School of Marine Science. Upon officially retiring in June of 1984, he became Professor Emeritus of the College of William and Mary.

During his 35 years of service at the VIMS (and the School of Marine Science), Dexter worked primarily on the physiology and life history of molluscs, and on the natural history and sedimentology of oyster bars, or reefs.

His work resulted in over 50 formally published papers, including one paper on the precarious state of the Chesapeake public oyster resource in 1995, and another on the oyster reefs of the Chesapeake, their destruction and possible restoration period. He also authored a number (about 60) of research contract reports, some 20 to 30 VIMS papers, and over 30 VIMS data reports.

Dexter, an excellent field and laboratory scientist and teacher, worked with numerous graduate students while at the School of Marine Science and with other members of the VIMS scientific staff, including Dr. J. D. Andrews, Curtis Leigh, and Reinaldo Morales-Alamo.

In addition to the National Shellfisheries Association (NSA), of which he continues to be a member, Dexter has belonged to the American Society of Limnology and Oceanography, the Atlantic Estuarine Research Society, the Ecological Society of America, and the Malacological Society. He is also a member of the Society of Cincinnati, a group whose members trace ancestry to persons who served in the American Revolutionary War. During his membership in NSA, Dexter was President-Elect from 1974 to 1975, and President from 1975 to 1976.

Dexter and his wife, Doris Mills Haven, live in Yorktown, Virginia. They have been married since 1951 and have a daughter, Penny.

William J. Hargis, Jr.
Gloucester Point, Virginia

STRATEGIES TO MITIGATE THE IMPACT OF *CIONA INTESTINALIS* (L.) BIOFOULING ON SHELLFISH PRODUCTION

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ABSTRACT A sudden increase in the population of the solitary ascidian *Ciona intestinalis* (L.) is causing serious biofouling problems for shellfish growers on the Atlantic coast of Nova Scotia, Canada. The objective of the present study was to document the growth, spawning, and recruitment patterns of this species, and to develop strategies to minimize its impact on the culture of European oysters at two locations in Lunenburg Bay, Nova Scotia. Profiles of condition index, which may be indicative of spawning activity, suggested that the *C. intestinalis* population at the Bayport site spawned from mid-May through June, whereas the population at Mason's Beach spawned from mid-July to mid-August. Histological assessment of reproductive status indicated a period of gametogenesis in March–April (>3°C) followed by spawning from mid-May to mid-August (>8°C). Although mature eggs were observed in the ovary in July–August, spawning trials suggested a decline in the fecundity of the Bayport population during this period. Two main recruitment events were observed at Mason's Beach (June and August), but only one at Bayport (June). From the data on fecundity and settlement rates, it was estimated that a 100-mm long *C. intestinalis* (0.6 g dry weight) may produce 12,000 eggs in a season and that recruitment intensity may reach 3,000 individuals m^{-2}. Laboratory predation trials indicated that rock crabs (*Cancer irroratus*) consumed significantly more *C. intestinalis* than did green crabs (*Carcinus maenas*). A maximum predation rate of 11 individuals per day per rock crab (80 mm carapace width) was recorded at peak water temperatures of 18°C. In a series of chemical width eradication trials, exposure to 5% acetic acid was found to be a more effective strategy for eliminating *C. intestinalis* than hydrated lime, saturated brine, or hypochlorite solution. Total mortality was observed following exposure to 5% acetic acid for 15 to 30 s, with no corresponding mortality in the control mussels or oysters. Initial field trials indicated that spraying with acetic acid might prove to be an effective means of eliminating *C. intestinalis* under commercial conditions.

KEY WORDS: *Ciona intestinalis*, tunicates, biofouling, shellfish production, predation

INTRODUCTION

Ciona intestinalis is a solitary phleobranchiate ascidian, or tunicate, which occurs on natural substrates such as rocky bottoms and eelgrass beds, or on artificial structures such as aquaculture gear, marker buoys, dock pilings, and boat hulls (Petersen & Riisgard 1992, Connell 2000, Mazouni et al. 2001). Although native to the northern Atlantic Ocean (Van Name 1945, Plough 1978), this species is now distributed worldwide, most likely as a result of dispersion by shipping activities (Monniot & Monniot 1994, Lambert & Lambert 1998). Published accounts indicate that *C. intestinalis* has recently become a serious biofouling problem for many shellfish culture operations including those in Scotland (Karayucel 1997), South Africa (Hecht & Heasman 1999), and Chili (Uribe & Etchepare 2002). In eastern Canada, the severe impact of *C. intestinalis* biofouling was first documented in 1997 at a mussel farm in Lunenburg Bay, Nova Scotia (Cayer et al. 1999). In an unprecedented recruitment event, this tunicate species heavily colonized the mussel sleeves, causing a substantial reduction in growth and the eventual loss of the crop. Subsequent reports of significant *C. intestinalis* recruitment at several other shellfish growing sites in Nova Scotia suggest that this species has become a widespread biofouling problem. In a similar scenario, the nonindigenous club tunicate *Styela clava* has recently infested several mussel farms on the eastern coast of Prince Edward Island and is now recognized as a serious threat to the viability of the mussel industry (Boothroyd et al. 2002).

Information on the basic life-history traits of *C. intestinalis* originates primarily from natural populations in northern European waters (Gulliksen 1972, Svane 1983, Petersen et al. 1995, Petersen et al. 1997). Under these conditions, the life cycle of *C. intestinalis* is reportedly 12 to 18 mo, with growth and longevity varying in response to temperature and food levels (Millar 1952, Petersen et al. 1995). Growth rates in terms of length are estimated at 1 to 3% day^{-1} or 10 to 20 mm mo^{-1} (Dybern 1965, Petersen et al. 1995). In contrast, reports from Japan indicate that *C. intestinalis* has a life span of 3 mo in the summer at temperatures of 20 to 26°C, and 6 mo in the winter at 14°C (Yamaguchi 1975). The timing of reproductive activity also varies depending on temperature. In more northerly regions, such as in Sweden, reproductive activity peaks in May and June, whereas in warmer zones, such as Britain, gamete release may occur throughout the year (Dybern 1965, Gulliksen 1972). Given the various life-history strategies of this species, it is important to document this basic information for *C. intestinalis* populations in Atlantic Canada.

The primary objective of this study was to develop a strategy to mitigate the impact of *C. intestinalis* on an oyster culture operation in Lunenburg, Nova Scotia. In contrast to mussel culture, oysters are contained in a cage from which the tunicates can be removed without losing the inventory. Heavy infestations, however, have the potential to depress shellfish growth, and to increase mortality due to competition for food (Lesser et al. 1992) and obstruction of water flow (Uribe & Etchepare 2002). The removal of these tunicates from the grow-out structures and oyster inventory is labor intensive, and, in some cases, disposal of the waste biomass can be costly. A series of field and laboratory experimental trials were undertaken from November 1999 to November 2000 for the following purposes: (1) to document the local distribution of *C. intestinalis*; (2) to investigate the growth, spawning, and recruitment patterns of this species; and (3) to evaluate possible biological and chemical strategies for eliminating this species from the culture equipment and the oyster inventory.

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MATERIALS AND METHODS

Field Ecology

Distribution, Growth, and Condition

Several exploratory dives aimed at documenting the local distribution of *C. intestinalis* were carried out at the two field sites in Lunenburg Bay, Bayport, and Mason's Beach, in the fall of 1999 and the fall of 2000 (Fig. 1).

Two experimental oyster tables with oyster bags containing adult *C. intestinalis* (year 1999 class) were set up at each of the two grow-out sites (i.e., Mason's Beach and Bayport) on October 30, 1999 (Fig. 2). Temperature recorders were attached to the tables at each site. The two experimental groups were sampled monthly from November 1999 to May 2000 and then every 3 wk until September 2000. On each occasion, a random sample of 10 individuals was collected from each site to evaluate their condition index. Each individual was measured and dissected to obtain estimates of wet tunic and wet body weight, and then they were dried overnight at 60 C for 24 h and reweighed. The condition index was calculated as dry body weight divided by total dry weight.

In early June 2000, oyster bags with recently recruited individuals were transferred to the experimental tables. Growth in terms of length, whole wet weight, and whole dry weight were estimated for the newly settled year 2000 cohort. Ten individuals from each site were measured, weighed, and then dried. Due to the difficulty in obtaining measurements from individuals in a fully extended position, a relationship was derived between body diameter when contracted and body length when alive and fully extended. This was used to estimate the mean body length of the
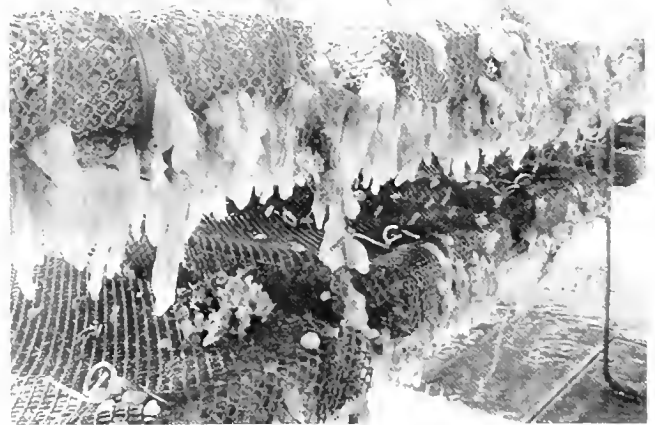
Figure 2. Photograph of an oyster table with oyster bags. Tunicates are apparent on the lower side of the oyster bags.

cohort over time. A final sample was collected in November 2000 to document the development of the year 2000 class.

Reproductive Status

Five individuals from each year class at each site were dissected and weighed, and the body was fixed in 1% glutaraldehyde and 4% formaldehyde. The samples were then sent to the Diagnostics Laboratory at the Atlantic Veterinary School (Prince Edward Island) for histological processing. The tissues were embed-
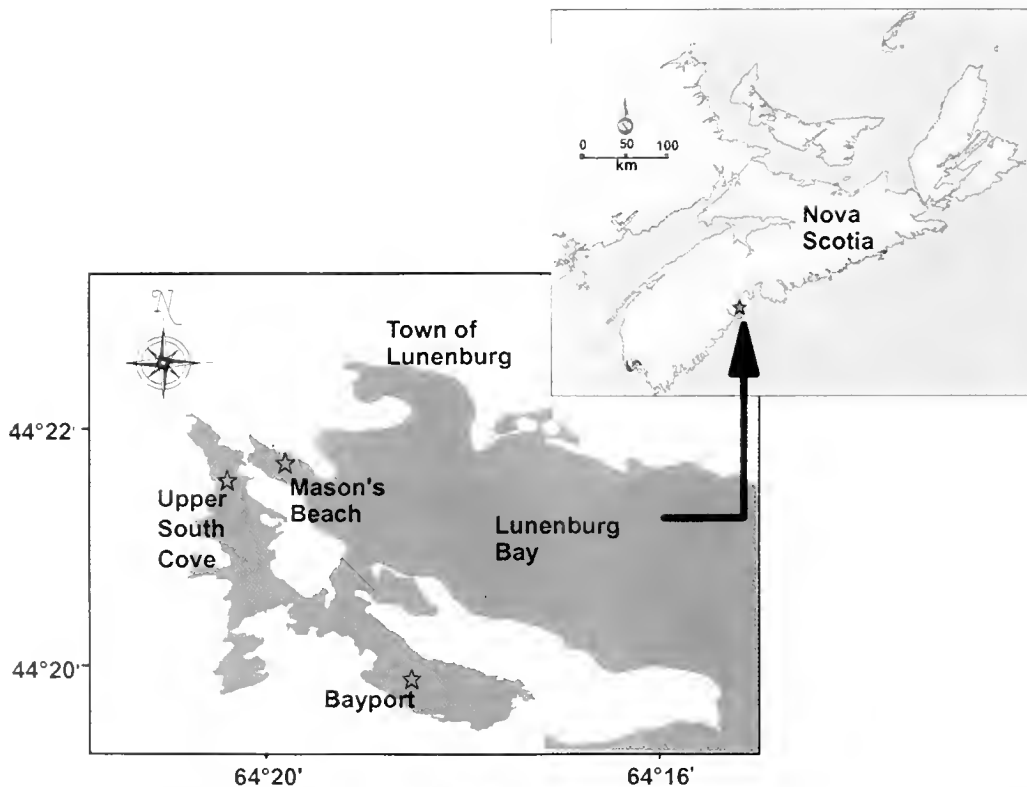
Figure 1. Map of Lunenburg Bay showing the location of the mussel farm in Upper South Cove, the site of the initial *C. intestinalis* infestation, and the two experimental sites, Bayport and Mason's Beach.

ded in paraffin and sectioned (6-μm thick), and the sections were stained with hematoxylin and eosin. The histology sections were assessed for reproductive status using a Weibel graticule (two fields per slide). The contents of each field were assigned to five categories: empty of follicle tissue; eggs in early development stage; eggs in late development stage; mature eggs; and regressing eggs. Mature eggs are surrounded by a thick layer called the vitelline coat, which clearly distinguishes them from immature or developing eggs. These data were used to estimate the proportion of the ovary that contained follicle tissue and the proportion of that area occupied by eggs in various stages of development. The cross-sectional area of the ovary was also measured using an image analyzer system.

Recruitment

Four recruitment plates (~200 cm^2) cut from clean but used oyster bags (4-mm mesh) were attached to the lower side of each oyster table on each sampling occasion. Plates deployed on the previous sampling trip were retrieved and were placed in separate plastic containers filled with seawater for transfer to the laboratory. The plates were examined with a stereomicroscope to detect the presence of newly recruited juvenile *C. intestinalis*. The plates were then placed in flowing filtered seawater (50 μm) for 2 to 3 wk to allow for the development of very small individuals that may not have been counted initially. The plates were then reassessed, and the maximum of the two counts was retained. The final counts for both sides of each plate were tallied and divided by the available solid area to estimate the intensity of settlement over the previous sampling period. The data were plotted such that any settlement that was observed at the end of a particular interval was assigned to the midpoint of that interval.

Laboratory Trials

Larval Development

The objective of the first series of trials (January–May 2000) was to induce natural spawning in the laboratory, to document the various phases of larval development, and to devise a protocol for rearing juveniles. Adult *C. intestinalis* from the 1999 cohort were collected from the field populations at each sampling event and were transferred to a flow-through system running at ambient temperature with unfiltered water. Spawning trials were undertaken on January 19, February 16, February 28, March 13, March 30, April 18, and May 3. To determine whether the adults possessed competent gametes, attempts were made to trigger spontaneous spawning by exposing individuals to a natural-light regimen for 24 h. When this proved unsuccessful, adults were strip-spawned and cross-fertilized to determine whether the eggs were competent. Fertilization trials were conducted at ambient water temperatures (0–6°C).

Fecundity

A series of five spawning trials were conducted in the quarantine unit at the Bedford Institute of Oceanography from May 15 to August 25, 2000, to estimate the fecundity of individuals obtained from the 1999 *C. intestinalis* cohort at both sites. Several individuals from the newly recruited 2000 cohort were included in July and August in an attempt to determine the minimum size at which spawning was initiated. The first four trials each lasted from 14 to18 d (May 15–June 2, June 8–26, July 4–21, and July 25–August

10), but the fifth trial (August 14–25) was discontinued after 10 days because of technical problems with the water supply system.

Five individuals of various sizes from each site were placed in separate 500-mL Mason jars in a tank of ambient flowing seawater. The water was prefiltered through a 40-μm mesh to remove any risk of contamination from eggs originating outside the system. The water level in the main reservoir was adjusted such that the flowing water just cleared the top of each jar; the objective was to allow sufficient flow for gas exchange and particle renewal but not enough to entrain the eggs. Control jars were placed downstream in the tank to estimate whether eggs were being lost. No eggs were retrieved from the control jars, and observations of fecal deposition suggested that negatively buoyant particles, including eggs, were retained inside their respective jars.

The experimental tank was set up approximately 3 m from an east-facing window such that the dawning light each morning would induce normal spawning behavior (Lambert & Brandt 1967). Every second or third day, the individual tunicates were transferred to new jars, and the contents of each old jar were screened through a 60-μm mesh to retain any eggs (150 μm size) produced over the previous 48 to 72 h. The jar and the screen were well rinsed with filtered seawater to remove any eggs stuck to the surface and were then flushed with hot freshwater to avoid contamination between samples. The eggs from each jar were collected in a petri dish and were counted using a stereomicroscope. Fecundity was estimated in terms of eggs produced per individual per day over the duration of the trial. At the end of each trial, the surviving individuals were dissected for assessment of dry body weight.

Methods of Control

Natural Predation

A series of predation experiments were set up in flowing seawater tanks in the quarantine unit at the Bedford Institute of Oceanography, Nova Scotia. Various sizes of *C. intestinalis* attached to weighted pieces of oyster bag were offered to a range of potential predators including starfish (*Asterias vulgaris*), green crabs (*Carcinus maenas*), rock crabs (*Cancer irroratus*), and hermit crabs (*Pagurus acadianus*). The first three trials were conducted in late January 2000 at water temperatures of 2 to 4°C. The second series of five trials focused on assessing the predation activity of rock crabs versus green crabs at a range of temperatures. Trials were undertaken on February 4 to 14 (2°C), April 13 to May 3 (5°C), July 27 to 31 (15°C), August 8 to 10 (18°C), and August 14 to 15 (18°C). The crabs ranged in carapace width (CW) from 40 to 100 mm, and the tunicate prey ranged in length from 15 to 125 mm. The duration of the experiments had to be reduced in the later trials to ensure that the supply of prey was not exhausted prior to the end of the trial. Predation rates were calculated in terms of individual tunicates consumed per crab per day.

Chemical Treatment

A series of physical/chemical eradication trials were undertaken in the laboratory from February to August 2000. The chemicals tested included sodium hypochlorite (10–60 parts per million), hydrated lime (1–4%), saturated brine, freshwater, and acetic acid (1–5%). The effectiveness of heated freshwater (40°C and 60°C) for eradicating *C. intestinalis* was also investigated. Various sizes of tunicates were used in each trial to determine whether younge

stages might be eliminated more easily than older stages. Mussels and oysters were also included in the trials to ascertain whether the treatment could potentially be used to remove tunicates from shell surfaces or from gear containing shellfish.

RESULTS

Local Distribution and Conditions

Diving surveys carried out at both sites in the fall of 1999 and the fall of 2000 did not identify any *C. intestinalis* attached to natural substrates, including rocks or eelgrass. None were observed on local wharf pilings at Bayport, but there was a substantial population attached to the bottom of a floating dock at Mason's Beach. Otherwise, *C. intestinalis* was only observed attached to oyster tables or suspended culture gear such as mussel sleeves and longlines. Both experimental sites typically have a lower incidence of *C. intestinalis* than the more sheltered Upper South Cove, the site of the original 1997 infestation, where the conditions tend to be warmer and more productive (Mallet & Carver 1993). Temperature profiles for the two experimental sites were virtually identical (Fig. 3).

C. intestinalis: 1999 Year Class

Growth and Condition Index

Estimates of body length and total wet weight per individual for the 1999 year class showed low variation over time or location (mean values: November 1999 69 mm and 5.9 g, respectively; September 2000 76 mm and 6.7 g, respectively). The mean dry weight per individual remained at 0.3 to 0.4 g (range 0.1–0.9 g) for the duration of the study, and the overall relationship between whole dry weight (g) and body length (mm) was estimated as y = $0.0000106x^{2.38}$ (r^2 = 0.91). Note that both primary tissues, the outer tunic and the body, are composed of approximately 95% water. Although the smaller individuals did grow from April to September 2000, the mortality of the larger individuals during the summer obscured any population growth trend.

During the colder months, the condition index (dry body weight/total dry weight) declined slightly from 44% in November 1999 (6°C) to 40% in late February 2000 (0°C) (Fig. 4). The condition index then increased sharply at both sites to a maximum of 60% at Bayport in late April, and 55% at Mason's Beach in
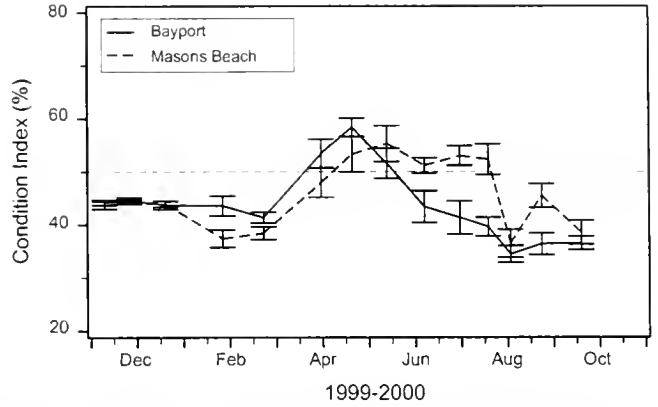
Figure 4. Condition index (dry body weight/total dry weight) for the year 1999 class of *C. intestinalis* at Bayport and Mason's Beach.

mid-May. At that time, the ambient water temperature at both sites was in the 6 to 9°C range (Fig. 3). The condition index of *C. intestinalis* at the Bayport site declined steadily from late April to early August, stabilizing at 35%. This profile would suggest that spawning started between April 20 and May 13, and continued through June and July. In contrast, the 1999 cohort at Mason's Beach exhibited a slight drop in condition in May but then maintained a condition index of >50% until mid-July, at which time values declined sharply. If the condition index is related to reproductive status, this profile suggests that the major spawning event at Mason's Beach occurred after mid-July or later than at Bayport.

Reproductive Status

Data on the reproductive status of *C. intestinalis* were pooled over the two sampling sites. The mean cross-sectional area of the adult ovary increased from 10 mm² in November 1999 to 25 mm² in late January 2000, declined slightly in February-March, and then rebounded in April-May to 24 mm². Between May 13 and June 7, the mean size of the ovary fell to approximately 10 mm², where it remained until September. Estimates of the proportion of the ovary occupied by follicle tissue ranged from 55 to 70% from November 1999 to March 2000, increased to 90% in April-May, and then declined to 70% in July (Fig. 5). The follicle area occu-
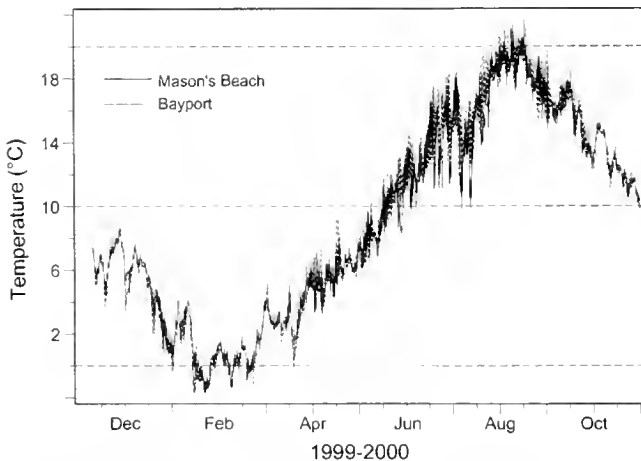
Figure 3. Temperature profiles for Bayport and Mason's Beach from November 1999 to November 2000.
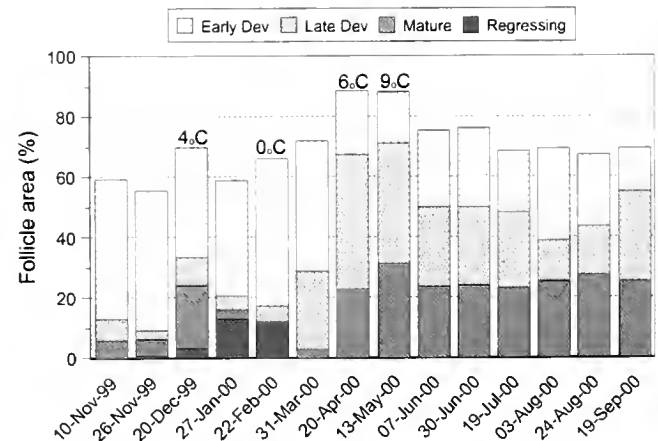
Figure 5. Reproductive status of the year 1999 class of *C. intestinalis*: proportion of the ovary that contained follicle tissue with early development, late development, mature, or regressing eggs.

pied by mature eggs increased from 10% in November to 35% in December 1999, but then declined from January to late March 2000. By mid-May, the incidence of mature eggs had returned to 30% and remained relatively stable until mid-September.

In summary, it would appear that egg development proceeded during the late fall 1999 when water temperatures exceeded 2°C but ceased when temperatures fell below 2°C in January-February 2000. During this latter period, there was an abundance of hemocytes in the ovary, which suggested that resorption or regression may have been occurring. By late March (4°C), the follicle area was starting to increase, as was the incidence of late development and mature egg stages. The major period of gametogenesis occurred in April through to mid-May (4–9°C), followed by the initiation of spawning in mid-to-late May. This event was coincident with a significant decline in the size of the ovary as well as the proportion of follicle tissue. From early June onward, the production of mature eggs continued, but the size of the ovary remained smaller than in April–May.

Fecundity

A total of five 2-wk spawning trials (May–July 2000) were carried out at ambient temperature in the laboratory using individuals collected from the two field sites. The number of eggs produced per individual varied widely from day to day, but there was no consistent decline in the rate of egg production over time within a trial. The maximum daily production estimated for a single individual was 1998 eggs day^{-1}, or a total of 5994 eggs over 3 days (May 19–22). (Table 1). The maximum fecundity for a single individual averaged over one trial period was 533 eggs d^{-1}.

Fecundity was positively correlated with whole dry weight (Fig. 6). The results indicated that individuals with dry weights as low as 0.1 g (40-mm long) could produce up to 200 eggs day^{-1}, whereas individuals with dry weights of 0.9 g (120-mm long) could produce as many as 500 eggs day^{-1} (averaged over 10–18 days). In general, fecundity was higher for the individuals from Mason's Beach than for those from Bayport. Estimates of mean fecundity for Bayport individuals (Table 2) showed a steady decline in egg production from May 15 onward. This was consistent with the profile of condition index (Fig. 4). The data for Mason's Beach suggest that the 1999 year class was producing >250 eggs ind^{-1} day^{-1} in May-June. However, unlike the Bayport population, the individuals at Mason's Beach continued to produce >100 eggs

TABLE 2.

Egg production rates for year 1999 class *C. intestinalis* (eggs ind^{-1} day^{-1}) from the two experimental sites over time.

Trial Duration	Bayport (eggs ind^{-1} d^{-1})	Mason's Beach (eggs ind^{-1} d^{-1})
May 15–June 2	183 ± 80	221 ± 98
June 8–June 26	172 ± 71	257 ± 70
July 4–July 21	97 ± 34	160 ± 40
July 25–August 10	35 ± 15	150 ± 30

Values given as mean ± SE.

day^{-1} through July-August. This was consistent with the higher condition index for this population.

Larval/Juvenile Development

From January 19 to March 30 2000, eggs were obtained by dissection because of failures to trigger spontaneous spawnings. Very few mature eggs were obtained from January through March, and the sperm rapidly lost motility. In the few instances in which mature eggs were obtained, fertilization was generally poor (<10%), and development did not proceed to the larval stage. In the April 18 and May 3 trials, however, larvae were successfully produced both by spontaneous spawning and dissection. As in the earlier trials, the eggs were fertilized at ambient temperature (6–9°C in April-May) and then were allowed to gradually warm up to 15°C in the dark.

The development of *C. intestinalis* eggs at 15°C typically took 24 to 36 h, hatching and growth of the tadpole larvae lasted 24 h, followed by settlement and metamorphosis over another 12 h for an approximate total of 3 days to the juvenile stage (see also Berrill 1947). Larvae were successfully settled on plastic petri dishes, where they metamorphosed into juveniles. The dishes were submerged in a 10-L tank, and the water was changed every 2 to 3 days. The juveniles proved to be remarkably resilient and survived for weeks with minimal handling/feeding. A series of photos were taken to document the development of *C. intestinalis* from the egg to the juvenile phase (Fig. 7a, b, c, d, e, and f). It should be noted that the species identity of *C. intestinalis* was confirmed by the presence of single refringent bodies in the halo of follicle cells that surround the egg (Byrd & Lambert 2000).

TABLE 1.

Results of first spawning trial (May 15–June 2) indicating the individual variability in daily egg production rate over time. *C. intestinalis* individuals were brought in from the two field sites on May 13 and were held in flowing seawater until Jun 2.

Ind No.	May 15–17	May 17–19	May 19–22	May 22–24	May 24–26	May 26–29	May 29–31	May 31–Jun 2	Mean Eggs day^{-1}	Length (mm)	Whole Dry Weight (g)
M1	7	292	91	378	615	206	717	378	317	89	0.41
M2	2	1032	66	120	165	178	72	224	223	65	0.47
M3	0	0	0	0	0	0	54	0	6	56	0.27
M4	0	798	1998	363	0	398	0	44	533	89	0.91
M5	0	228	0	6	0	6	5	0	28	79	0.37
B1	0	0	0	105	74	126	60	102	59	65	0.16
B2	0	339	18	1158	453	416	140	1674	491	89	0.53
B3	117	344	45	135	281	164	71	135	155	74	0.36
B4	42	77	43	0	174	78	80	0	62	61	0.27
B5	225	180	111	0	392	0	371	0	148	70	0.29
Mean	44	329	237	226	215	157	157	256	202	74	0.40

Abbreviations: M = Mason's Beach; B = Bayport.
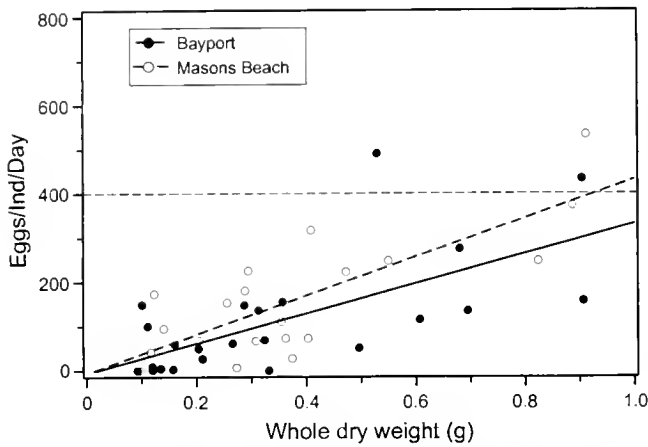
Figure 6. Fecundity (eggs ind⁻¹ day⁻¹) versus whole dry weight of the 1999 *C. intestinalis* from Mason's Beach and Bayport from May through August 2000. Data from individuals that died during the trials were not included.

C. intestinalis: 2000 Year Class

Recruitment Patterns

The observed recruitment profiles suggest one settlement event at Bayport and two at Mason's Beach (Fig. 8). Estimates of settlement intensity ranged as high as 47 per 100 cm² of solid collector area, but levels often varied substantially among replicate plates. The timing of the settlement peak at Bayport (May 13–June 29) is consistent with the condition index/spawning profile for the year 1999 class (Fig. 4). In the case of Mason's Beach, the timing of the second recruitment peak (Aug 3–24) closely followed the decline in condition observed at that site (Fig. 4). The absence of a decline in condition in May-June may indicate that the first recruitment event at Mason's Beach was related to an influx of larvae from other areas such as Bayport or Upper South Cove. However, the fecundity trials confirmed that Mason's Beach adults were capable of producing eggs from mid-May onward. Recruitment plates deployed from August 24 to September 19 exhibited some new settlement at Mason's Beach, but no juveniles were observed on the plates deployed from September 19 to November 29, 2000.

Growth Rate

The growth rate of the first year 2000 cohort in terms of body length was relatively steady from mid-July through to mid-September and then decreased, possibly due to declining water temperature or the onset of maturity (Fig. 9). Continued growth in terms of whole dry weight through October-November was apparently related to an increase in body weight as opposed to length (Fig. 10). Whereas profiles of mean body length were similar for the two sites, estimates of whole dry weight were consistently higher at Mason's Beach than at Bayport. At Mason's Beach in November 2000, the mean body length was 96 mm, the whole wet weight was 11 g, and the whole dry weight was 0.7 g. These values were consistently higher than those for the year 1999 class the previous November. Individuals from the second year 2000 cohort at Mason's Beach had a body length of 36 mm, a mean wet weight of 0.5 g, and a mean dry weight of 0.05 g on November 29, 2000. The overall relationship between whole dry weight (g) and body length (mm) for the year 2000 class was estimated as y = 0.00000801x^{2.4} (r² = 0.93).

Reproductive Status

Profiles of the percentage of follicle area as well as the proportion of the follicle area occupied by mature eggs increased rapidly between July 19 and August 3, 2000 (Fig. 11). At that time, the first year 2000 cohort had a mean length of 47 mm and a mean whole dry weight of approximately 0.1 g. Although dry weight continued to increase through the fall, gonad area increased only slightly to approximately 10 mm², and follicle area remained at 70%. Over the same period, the proportion of mature eggs declined from 40 to 20% in late November. Final values for all three reproductive indices were slightly higher than those for the 1999 year class recorded 1 y previously.

Fecundity

A few individuals from the year 2000 class did produce eggs in the July-August fecundity assessment trials. Estimates were typically <10 eggs day⁻¹ in the July 27 trial but increased to as high as 460 eggs day⁻¹ for the largest individual in the August 14 trial. The mean daily egg production was higher for the Mason's Beach recruits (245 eggs day⁻¹) than for the Bayport recruits (25 eggs day⁻¹), which was consistent with the greater dry weight of the former group (Fig. 10). The presence of mature eggs in the ovary from early August onward (Fig. 11) suggested that individuals from the first year 2000 cohort were likely spawning during this late summer period. However, given that there were 1999 individuals still spawning in August, the relative contribution of the first 2000 cohort to the second year 2000 recruitment peak cannot be ascertained.

Methods of Control

Natural Predators

A series of predation experiments were set up in flow-through seawater tanks at the Bedford Institute of Oceanography. Various sizes of *C. intestinalis* attached to weighted pieces of oyster bag were offered to a range of potential predators including starfish (*A. vulgaris*), green crabs (*C. maenas*),* rock crabs (*C. irroratus*), and hermit crabs (*P. acadianus*). In the first three trials, conducted in late January 2000 at water temperatures of 2 to 4°C, only the green crabs and, in particular, the rock crabs showed any feeding activity. The rock crabs were observed to use two different feeding strategies, depending on the size of the prey. Small *C. intestinalis* individuals (15–35 mm CW) were generally consumed whole, although after extracting the body tissues the tunic was rejected. Larger individuals (35–125 mm CW) were cut open with the

TABLE 3.

Effectiveness of various chemicals for the elimination of *C. intestinalis* (% mortality) under laboratory conditions.

Chemical Treatment	Duration (min)	Mortality (%)
Sodium hypochlorite 60 ppm	20	0
Salt brine (saturated)	8	25
Hydrated lime (4%)	6	50
Fresh water (15°C)	1	10
Fresh water (40°C)	1	66
Acetic acid (5%)	0.5	95
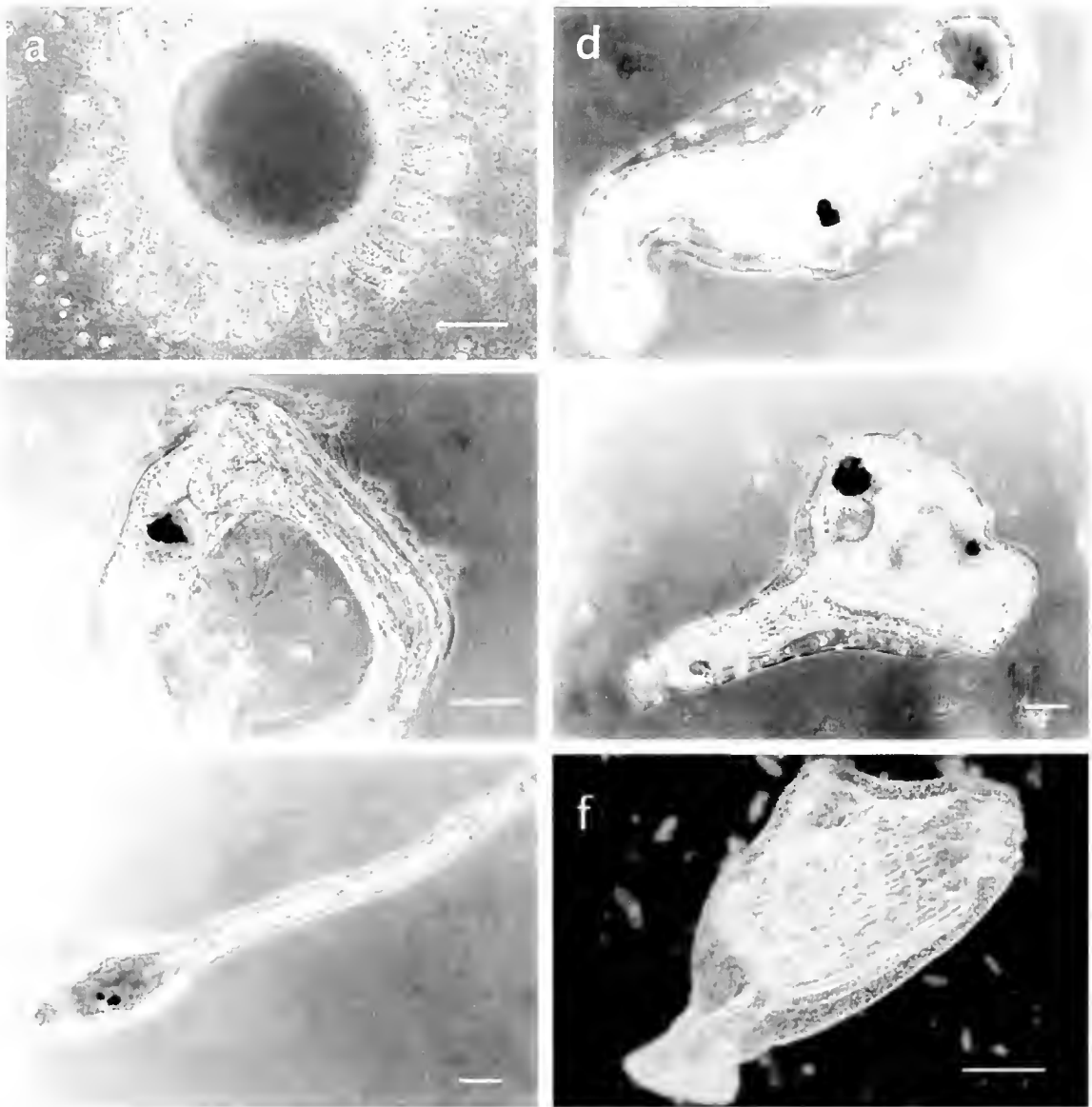
Figure 7. Photographs of the various stages of development of *C. intestinalis* reared under laboratory conditions: (a) egg (150 µm) with vitelline coat and follicle cells (protrusions) with distinctive refringent bodies (bright spots); (b) tadpole larvae with otolith (dark spot) and notochord hatching from egg (250 µm); (c) tadpole larvae with notochord and adhesive papillae on head (800 µm long); (d) metamorphosing larvae (360 × 120 µm) developing peduncle for attachment and resorbing tail; (e) juvenile (525 µm) with developing siphons; and (f) juvenile (1.3 mm) with stigmata or slits evident in the branchial chamber (photo (f) courtesy of Dr. Dan Jackson, (Department of Fisheries and Oceans). Scale bars for (a) to (e) are 50 µm; scale bar for (f) is 500 µm.

claws, and the body tissues were dragged out and consumed, leaving the empty tunic attached to the original substrate.

The predation trials undertaken at a range of temperatures indicated that rock crabs (50–90 mm CW) may consume as many as 11 *C. intestinalis* ind day^{-1} (35–80 mm long) at 18°C (Fig. 12). Predation rates were substantially lower at <6°C, but activity was steady. The trials also suggested that the small to medium rock crabs (<80 mm CW) tended to consume greater numbers of the <35-mm tunicates than did the larger crabs (Fig. 13). In general, the green crabs showed less interest than the rock crabs in preying on tunicates. There was also a tendency among the smaller green crabs (50 mm CW) to consume the <80-mm tunicates and ignore the larger individuals.

Physical/Chemical Eradication Trials

The results of the various eradication trials indicated that exposure to 5% acetic acid was by far the most effective strategy (Table 3). After the first trial, which indicated that a 1-min exposure to 5% acetic acid was sufficient to cause 100% mortality, further trials were carried out using shorter intervals. Exposure times of 5 to 10 sec were found to be insufficient, but 30 sec was generally 95% effective. The application of this chemical treatment by spraying or by immersing the tunicates proved equally useful. Rinsing post-treatment was included in the protocol to mimic conditions in the field where the acetic acid would be rapidly diluted by seawater. Oysters and mussels >20 mm in she
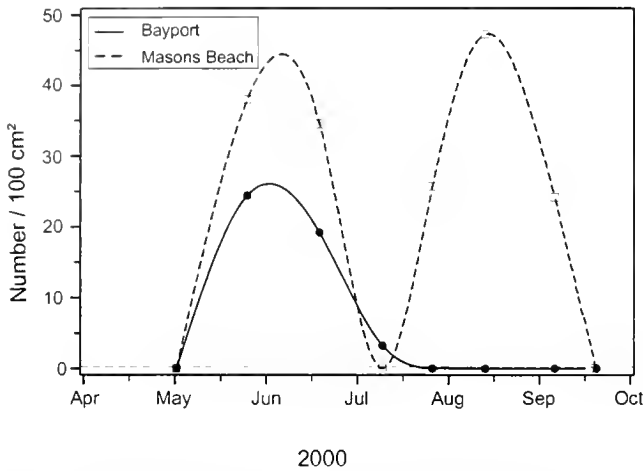
Figure 8. Projected recruitment profiles for the two year 2000 cohorts of *C. intestinalis* at Bayport and Mason's Beach.

length (SL) were typically unaffected by the acetic acid spray/dip, but control mussels <10 mm SL died in one comparative trial.

Other chemical methods were consistently less effective at eradicating tunicates. Exposure to hydrated lime for 8 min was 70% effective, whereas saturated brine was only 20% effective over the same exposure time. Solutions of sodium hypochlorite at concentrations up to 60 parts per million for as long as 20 min had no impact on tunicate survival. Exposure to freshwater for 1 min resulted in only 10% mortality. Longer exposure times may be more effective, but under field conditions this may not be practical. A 1-min exposure to 40°C freshwater was 100% effective at eradicating *C. intestinalis*, but the European oyster (40 mm SL) and one of the two mussels (50 mm SL) also died.

The second phase of the eradication trials was to test the effectiveness of acetic acid treatment on *C. intestinalis* attached to oyster grow-out bags in the field. It should be noted that these trials were preliminary and were only assessed at a qualitative level. To administer the treatment, the acetic acid solution was placed in a garden-spraying unit. Goggles, gloves, and appropriate clothing were used, and care was taken to ensure that the bag being sprayed was located downwind. Although there was a slight smell, the fumes were rapidly dispersed in the open air. The treatment protocol followed was similar to that developed in the laboratory trials: 5% acetic acid spray for 30 s followed by air exposure for
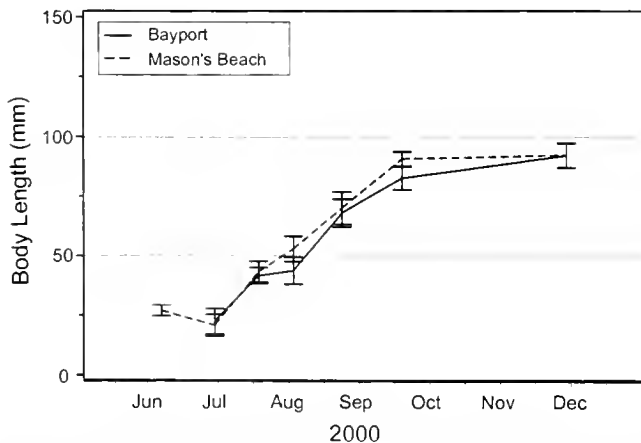
Figure 9. Increase in body length (mm) over time for the year 2000 cohort of *C. intestinalis* at Bayport and Mason's Beach.
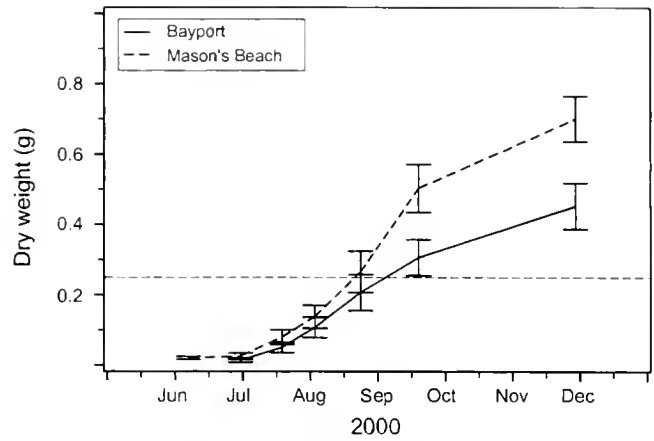
Figure 10. Increase in whole dry weight (g) over time for the year 2000 cohort of *C. intestinalis* at Bayport and Mason's Beach.

30 s. Note that the *C. intestinalis* individuals had ample time to fully contract before being exposed to the treatment.

The preliminary trials were carried out on August 24 and September 19, 2000. The oyster bags were covered with a heavy settlement of 70 to 80-mm-long year 2000 individuals. In each case, individuals on one third of the bay were covered to act as controls. It should be noted that the health of these control individuals was apparently not affected by either the nearby application of the acetic acid or the subsequent mortality of their immediate neighbors.

The effectiveness of the treatment varied from 60 to 100%, depending largely on the density of the settlement (qualitative assessment). In the second field trial (September 19), 10 European oysters (40 mm SL) were placed in the bags while the acetic acid treatment was administered. On average, 80% of the oysters were alive when the bag was examined on November 29. Although these small-scale experiments were in no way conclusive, the results were sufficiently promising to warrant further trials.

DISCUSSION

Natural Distribution

In northern Europe, substantial natural populations of *C. intestinalis* are found in eelgrass beds or attached to rocky substrates (Dybern 1965, Gulliksen 1973, Petersen & Riisgard 1992). Diving surveys in 1999-2000 aimed at documenting the local distribution of *C. intestinalis* at the two study sites in Lunenburg found no individuals attached to natural substrates. Two independent surveys of the Bayport area carried out in August 2000 and August 2001 also failed to locate any *C. intestinalis* on eelgrass or rocky bottom areas (Barry MacDonald, Department of Fisheries and Oceans, pers. comm.). It would appear that the distribution of this species is generally restricted to man-made structures, such as floating docks and aquaculture gear.

The sudden proliferation of the *C. intestinalis* population in Lunenburg is a classic example of the potential impact of man-made structures on the settlement and survival of sessile invertebrate species (Connell & Glasby 1999). Various Australian studies comparing the species assemblages found on pier pilings and pontoons versus adjacent natural substrates have suggested that the introduction of artificial structures may effectively increase local species abundance and diversity (Butler & Connolly 1996, Glasby
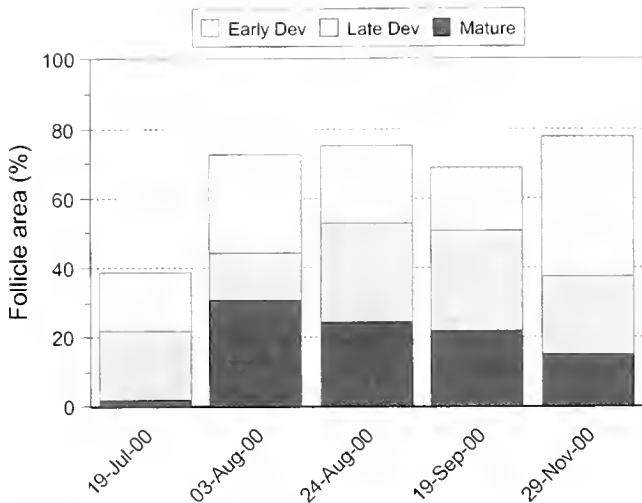
Figure 11. Reproductive status of the first year 2000 cohort of *C. intestinalis:* proportion of the ovary that contained follicle tissue with early development, late development, or mature eggs.

1999. Connell & Glasby 1999, Connell 2000). In particular, solitary ascidians such as *C. intestinalis* were typically more abundant at marinas than at reference locations (Glasby 1997). Ascidians in general have been recognized as the dominant biofouling organism on oysters grown in rope culture in L'Etang de Thau (France) (Mazouni et al. 2001).

The conspicuous absence of *C. intestinalis* from natural substrates suggests that manmade structures may function as a refuge from predation. Field studies in Denmark and Norway have reported that variations in spatial abundance of this species are linked to predation by sea stars (*Asterias rubens*), plaice (*Pleuronectes platessa*), and cod (*Gadus morhua*) (Gulliksen 1972, Gulliksen & Skjaeveland 1973). Natural predators include jellyfish, sea stars, rock crabs, hermit crabs, dog whelks, and surface-feeding fish (Gulliksen 1972, Yamaguchi 1975, Svane 1983, Olesen et al.1994). Recently settled juvenile stages may also be susceptible to dislodgment by surface grazers such as gastropods and sea urchins (Svane 1983). Predation trials conducted in the present study demonstrated that the rock crab, *C. irroratus*, can rapidly excise the body tissues of *C. intestinalis* from the heavy tunic and may consume as many as 11 ind day^{-1} during the summer months.
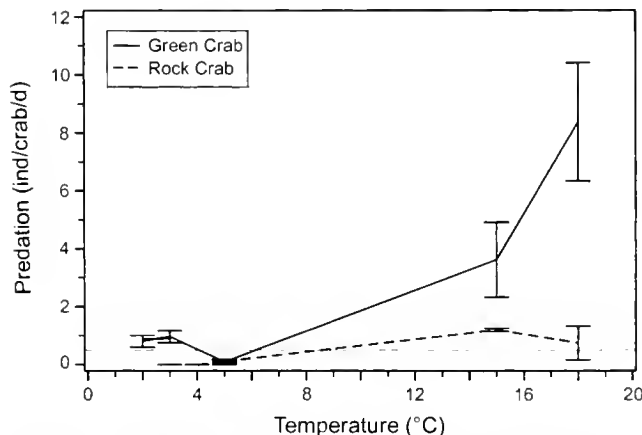
Field observations also suggested that predators, in particular crabs, were actively reducing the abundance of *C. intestinalis* on the upper surface of the oyster bags but were apparently unable to access individuals attached to the underside of the bags (Fig. 2). Other surface-feeding predators such as sea stars may also play a role in controlling the distribution of small individuals, but, apart from the crab activity, there was no indication of any significant predation pressure on the larger tunicates.

Life History Traits

Field observations suggested that most of the individuals from the 1999 year class died prior to November 2000. This pattern of mortality, apparently as a result of natural senescence, was consistent with life span estimates of 12 to 18 mo for *C. intestinalis* in Scandinavian waters (Petersen et al. 1995). Similar to Lunenburg, reports from Sweden indicate that *C. intestinalis*, which settles in the summer, spawns the following spring and dies during the winter (Dybern 1965). Reports from Japan suggest the life span of this species is apparently determined by cumulative environmental temperature (Nomaguchi 1974). Thus, individuals that settle early in the summer, such as those in the first year 2000 cohort at Lunenburg, may die at a younger age than those that settle in late summer.

Estimates of growth rate in terms of body length for the year 2000 cohort were approximately 20 mm mo^{-1} from July through September, which is similar to estimates from Swedish (Petersen et al. 1995) and Chilean waters at 12 to 21 mm mo^{-1} (Uribe & Etchepare 2002). Observations on maximum size in terms of body length (100–140 mm) were higher than the 60 mm reported for Japanese waters (Yamaguchi 1975). Perhaps this species can attain a larger body size under colder conditions.

The results of the histological assessment and the spawning trials indicated that individuals that settled in May-June were capable of initiating egg production and spawning by August of the same year. This was consistent with observations from Sweden where two breeding generations of *C. intestinalis* have been found to co-occur in populations living close to the surface (Dybern 1965).

Figure 12. Predation rates of two crab species (ind crab^{-1} day^{-1}) on *C. intestinalis* (35–80 mm long) for a range of temperatures.
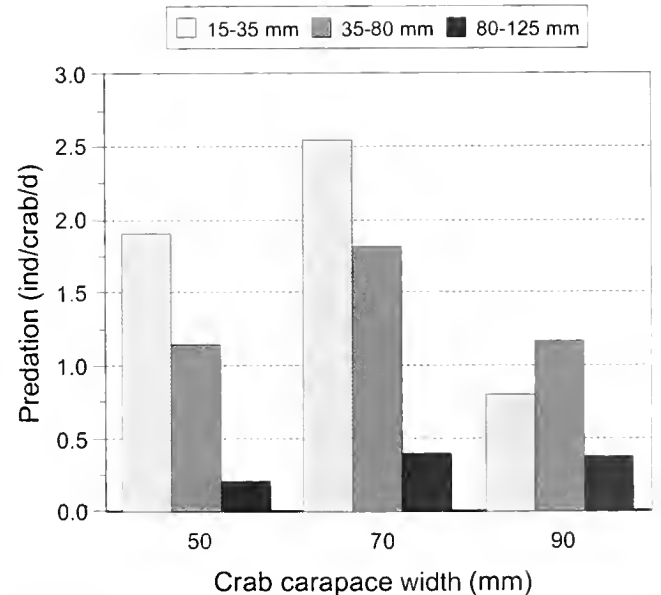
Figure 13. Predation rates of various sizes of rock crabs (ind crab^{-1} day^{-1}) on a range of sizes of *C. intestinalis.*

Yamaguchi (1975) also reported that *C. intestinalis* reached sexual maturity within 2 mo of settlement in winter, and within 1 mo at higher summer temperatures. This variability confirms that reproductive capability is size-dependent rather than age-dependent (Petersen et al. 1995).

Gulliksen (1972) concluded that the lowest temperature for the production of cionid larvae in Norwegian populations was in the range of 6 to 8°C, or comparable to their deep water winter temperatures. This was generally consistent with observations from the present study, which indicated possible gonad regression in January-February at <3°C, gametogenesis in March-April-May at 4 to 8°C, and production of competent gametes from mid-May onward when ambient temperatures exceeded 8°C.

The juvenile settlement data indicated that *C. intestinalis* populations in adjacent inlets may differ in their spawning and recruitment patterns. In Bayport, the recruitment peak was observed in May-June, whereas at Mason's Beach recruitment peaks were recorded in May-June and again in early August. The timing of the first peak was consistent with the histological data, indicating the presence of mature eggs in both populations in early May, and the spawning trials, suggesting that these two populations were capable of releasing eggs. However, unlike the Bayport population, the condition index for the Mason's Beach population remained relatively high until mid-July, suggesting that they did not spawn in May-June. It should be noted that the use of the condition index (body dry weight/total dry weight) as an index of spawning may be misleading. Petersen et al. (1995) found that this index reflected the level of growth but did not link it to spawning activity. It is possible that the relatively high condition index values for the Mason's Beach population in June-July were related to higher food levels at that site rather than to a delay in the onset of spawning activity. It remains unclear whether the first recruitment event at Mason's Beach originated from larvae produced by the local population or from other spawning populations such as those in Bayport and Upper South Cove.

Unlike many shellfish species that spawn over an interval of weeks, *C. intestinalis* can apparently spawn continuously over a 3-mo period (mid-May through mid-August). Information on reproductive status combined with estimates of fecundity illustrated the duration and intensity of the spawning events at the two sites. On the basis of these data it was estimated that one adult tunicate (100 mm long, 0.6 g dry weight) can produce on average 150 eggs day^{-1} for 60 days for a total of 12,000 eggs per year. This was consistent with the estimate of Petersen and Svane (1995), who suggested a conservative figure of 10,000 eggs ind^{-1} over a season. In contrast, Yamaguchi (1975) reported that adult *C. intestinalis* in Japanese populations released 2000 to 3000 eggs per night, every other night, and that the total fecundity of a single specimen was estimated conservatively at 100,000 eggs. At this stage, it is impossible to estimate egg to juvenile survival rates, but observations of dense aggregations of tunicates on any floating surface, up to 3000 ind m^{-2}, suggest that the population has considerable potential to expand.

Management Strategies

There are few published reports on strategies for controlling the proliferation of tunicates on shellfish culture gear. In general, growers who use nets recommend husbandry procedures such as changing the gear more often, using power sprayers, or treating bags with antifouling compounds. Other suggestions include ex-

posing tunicates to air, fresh water, lime, or saturated brine dips (90 parts per thousand) followed by air (Shearer & MacKenzie 1997). Among mussel growers who use sleeving material rather than nets, there are few cost-effective solutions. Suggestions from anecdotal reports include air-drying lines overnight, *in situ* pressure washing with a bleach solution, and stabbing individual tunicates. Although feasible in the case of small farms or low-level infestations, these options are logistically impossible in the case of large-scale operations.

An alternative strategy is to develop a site-specific management plan for minimizing the level of settlement; for example, growers in South Africa re-sleeve their mussels immediately following the recruitment of *C. intestinalis* (Hecht & Heasman 1999). Based on the recommendations of the present study, the Nova Scotian company involved in oyster culture adjusted its work schedule to mitigate the impact of *C. intestinalis* on its operation. In particular, the major annual cleaning/changing of the shellfish and the culture gear was postponed from May to September when the heaviest settlement had passed; this strategy has since proved to be a reasonably cost-effective option for the company. Because *C. intestinalis* tends to occur in highly aggregated distributional patterns (Havenhand & Svane 1991, Svane & Havenhand 1993, Petersen & Svane 1995), the annual eradication of the broodstock population from the culture gear may reduce future recruitment levels.

Encouraging natural predation is always a preferred strategy for pest management in aquaculture (e.g., Enright et al. 1983) but may only have a limited application in this instance. At an estimated recruitment level of 25 ind 100 cm^{-2}, 3000 tunicates may settle on one oyster bag; even at a consumption rate of 11 tunicates day^{-1} at peak water temperatures, it would take one crab 273 days to clean one bag. Moreover, tunicates that have settled directly on the shellfish inventory are not accessible. Field observations suggest, however, that natural predation, possibly by rock crabs, may play an important role in reducing the abundance of tunicates during the winter. Another potential control method that has yet to be investigated is based on the hypothesis that recently recruited *C. intestinalis* may be vulnerable to dislodgement by surface grazers such as periwinkles (*Littorina littorea*). Enright et al. (1983) reported that the addition of periwinkles to lantern nets containing European oysters resulted in a significant reduction in biofouling levels.

Chemical treatment protocols including lime and brine immersion have been developed for the purpose of eliminating the fouling tunicate *Molgula* sp. from oyster spat collector units (MacNair & Smith 1998). Laboratory trials undertaken in the present study indicated that acetic acid was considerably more effective than more traditional methods for eliminating *C. intestinalis*. However, it should be noted that the use of acetic acid dips or sprays under field conditions should be carefully evaluated to ensure personal safety as well as containment and/or neutralization of the chemical so as to minimize any environmental impact.

This study has shown that the ascidian *C. intestinalis* is well adapted to conditions on the Atlantic coast of Nova Scotia, being capable of developing mature eggs and spawning at temperatures upward of 8°C. It can apparently tolerate a wide range of environmental conditions and has the potential to rapidly establish substantial populations on floating structures. The presence of suspended or off-bottom shellfish culture operations that offer refuge from natural predators may inadvertently promote its survival. Given the tendency of *C. intestinalis* to attach to the hulls of ships,

local maritime traffic will likely facilitate its dispersal to other sites in Atlantic Canada over the next few years

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FOULING ORGANISMS OF THE BLUE MUSSEL *MYTILUS EDULIS*: THEIR EFFECT ON NUTRIENT UPTAKE AND RELEASE

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ABSTRACT The effects of fouling organisms are a cause for concern among mussel growers. On Prince Edward Island, Canada, most of the foulers are sedentary filter feeders, and are, therefore, potential competitors with cultured mussels for available resources. This could translate into a reduction in meat yield in mussels. Laboratory experiments were carried out in July, September, and December 2001 to determine the relative impact of fouling organisms on the uptake and release of nutrients. The uptake of chlorophyll *a*, and the production of ammonium, phosphate, nitrate, nitrite, and organic matter were investigated. There were some significant differences in chlorophyll *a* uptake between mussel/fouler units and mussels alone. The mean (±SE) chlorophyll *a* uptake by mussel/fouler units was 5.05 ± 1.48, 8.53 ± 0.94, and 12.87 ± 1.03 L/h, respectively, for July, September, and December. The mean consumption by mussels only was 5.37 ± 1.19, 8.72 ± 0.83, and 9.64 ± 0.97 L/h for each of the experiments. Foulers increased ammonia production before water temperatures dropped in the fall (end of September-early October). Mussel/fouler units released mean amounts of ammonia of 58.42 ± 10.01 and 667.54 ± 252.69 L/h, respectively, in July and September, while mussels alone did not produce ammonia in July, and in September they released 103.10 ± 13.25 L/h. There was a significant production of phosphate by mussels in July (6.67 ± 2.96 L/h) and in December (46.11 ± 3.02 L/h), and by the mussel/fouler units in December (27.95 ± 1.8 /h). In the presence of foulers, the nitrite production was 16.01 ± 6.53 L/h. In its absence, however, nitrite consumption was 17.09 ± 5.63 L/h. Mussel/fouler units consumed nitrate (4.25 ± 1.47 L/h), however, there was no significant difference when foulers were absent (0.86 ± 1.44 L/h). There was a significant consumption of organic matter by foulers in summer only (6.22 ± 1.38 L/h). Foulers have the potential to prolong phytoplankton blooms by increasing the production of inorganic nutrients, especially ammonia. This study shows that the effect of foulers on mussels may not be as great as previously thought, and it may not be profitable to invest time and money in trying to reduce them.

KEY WORDS: *Mytilus edulis*, blue mussel, epifauna, fouling organisms, chlorophyll *a*, ammonia, phosphate, nitrate, nitrite, feces, Tracadie Bay

INTRODUCTION

Culture of the blue mussel, *Mytilus edulis* Linneaus 1758, on Prince Edward Island (PEI), Canada, began in the 1970s and grew into a $25 million per year industry by 2002. This expansion can be attributed to an increase in the number of mussel grow-out sites accompanied by the rapid development of husbandry practices. Presently, however, no new grow-out sites that can support mussel culture are available on PEI, and it seems that any further development of a sustainable industry relies on the optimization of productivity at the farm level (Thomas Landry, pers. comm.).

Mussel socks are fouled by different species of marine invertebrates. Some of these foulers are filter feeders that compete with mussels for resources and therefore represent an additional strain on a system. In this context, the fouling of culture units by various epifaunal species is an issue that warrants further attention.

It is well-documented for molluscs such as scallops and oysters grown in nets or cages that the settlement of fouling organisms can restrict water flow to a point where both food availability and growth are negatively affected (Claereboudt et al. 1994, Lodeiros & Himmelman 1996, Taylor et al. 1997). PEI mussels, however, are cultivated on longline systems, which consist of subsurface buoyed backlines permanently anchored at each end. Mussels are suspended in socks along the backlines. Little is known about the effects of the fouling community on the growth of mussels on longline systems. In theory, since the fouling organisms tend to settle directly on mussel shells, they may obstruct the opening of the valves, thereby interfering with feeding (Lesser et al. 1992, Lodeiros & Himmelman 1996). Moreover, because most foulers are sedentary filter feeders (Arakawa 1990, Lesser et al. 1992, Lodeiros & Himmelmann 1996, Taylor et al. 1997, Mazouni et al. 1998a, Mazouni et al. 1998b, Cayer et al. 1999, MacNair & Smith 1999, Uribe & Etchepare 1999), it is possible that they contribute to the depletion of the phytoplankton biomass at culture sites. Despite these possibilities, evidence suggests that foulers do not significantly limit the yield of mussels cultured in suspension on backlines (Beristain & Malouf 1988, Lesser et al. 1992). Moreover, it has been suggested that the fouling community may in fact enhance seasonal phytoplankton blooms by altering nutrient fluxes (Mazouni et al. 1998a, Mazouni et al. 1998b) in a favorable way. Such an effect is plausible, given that the metabolic wastes released by fouling organisms may introduce nutrients into the water column that would otherwise not be available to the phytoplankton community (Kaspar et al. 1985, Dame et al. 1991, Prins & Smaal 1994, Smaal & Zurburg 1997, Mazouni et al. 1998a, Mazouni et al. 1998b, Landry 2002).

The goal of this study was to investigate the relative uptake of food and the release of nutrients by foulers commonly found on cultured mussels in Tracadie Bay, PEI. Our experimental approach was based on measuring food (i.e., seston and chlorophyll *a*) intake and nutrient (i.e., ammonia, phosphate, and nitrate) release in two study groups (mussels and mussel/fouler units) during the ice-free period. Results are compared with previous work on mussels, and an attempt is made to relate laboratory findings to applied methods used in mussel culture.

MATERIAL AND METHODS

Experimental Design

Mussel socks were collected from Tracadie Bay, PEI, Canada in July, September, and November 2001. During each trip, fou

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socks were collected and transported to the Ellerslie Hatchery, PEI. At the hatchery, a small quantity [mean quantity (±SE) 30 ± 9 to 902 ± 82 mg ash-free dry weight] of foulers and 30 mussels of approximately the same size were carefully removed by hand from each of the four socks. Foulers and mussels were placed in individual mesh (window screening) bags (four mussel/fouler units) and were maintained alive using running water from the Bideford Estuary. Water temperatures were 23°C in July, 16°C in September, and 3°C in December, and salinity was 28 parts per thousand for all experiments. After a short acclimation period (<1 wk), the experimental animals were transferred to 12-L flow-through containers. Four containers with no animals were used as controls. All containers were connected to a single supply tub that was continuously fed sand-filtered seawater. Water flow was set at about 300 mL/min.

Immediately after the introduction of animals into the containers, 1-L water samples were taken from the supply tub (input) and also from the output spout of all containers. Thereafter, additional 1-L samples were taken every hour during a 7-h period. At the end of this time, feces were collected from the bottom of the containers. Foulers were separated from mussels and were frozen for subsequent determination of weights (i.e., ash-free dry weight AFDW). Mussels (the same individuals as in the mussel/fouler units), on the other hand, were subjected to a short reacclimatization period (<1 wk) and then were subjected to the same protocol as the mussel/fouler units.

Laboratory Analyses

At the hatchery, suspended solids (500-mL water subsamples) were filtered onto preashed and preweighed Whatman (Clifton, NJ) GF/C 47-mm filters and were rinsed with 4% ammonium formiate. Identical methods were used to filter feces samples. All filters were stored frozen. Filters were dried at 70°C for 24 h, weighed, combusted at 500°C for 12 h, and reweighed. The results are reported as total seston (i.e., organic + inorganic), organic seston, total feces, and organic feces. Other water subsamples (250 mL) were filtered through Whatman GF/F 25-mm filter for chlorophyll a determination. The filters were immersed in 90% acetone, frozen, and later analyzed using a Turner Design (Sunnyvale, CA) fluorometer, as suggested by Parsons et al. (1984).

Ammonium concentrations were determined from frozen water samples (20 mL) using the phenol method (Parsons et al. 1984) and a spectrophotometer with a flow-through 5-cm path length cell at 640 nm. Phosphate concentrations were measured from frozen water samples (10 mL) using either a spectrophotometer (5-cm path length flow-through cell at 885 nm; July 26 and December experiments) or a YSI (Yellow Springs, OH) 9100 photometer (July 30 and September 29/October 2 experiments). Concentrations were determined by using commercial Palintest kits (YSI, Yellow Springs, OH) with the YSI photometer. All nitrate and nitrite concentration values were derived from the YSI 9100 photometer (Palintest kits).

Nutrient Uptake and Release

Nutrient budgets were calculated by subtracting the output nutrient concentration (container spout) from the input nutrient concentration (supply tub) at a corresponding time then dividing by the input concentration. It was then multiplied by the water flow. A positive value indicated an uptake of nutrients by the study animals, while a negative value indicated nutrient release. Uptake

and release values were corrected for processes unrelated to animal filtration (e.g., phytoplankton reproduction or evaporation) using data from the control containers.

Statistical Analyses

Paired sample t tests were used to compare the use of nutrients by the mussel/fouler units and by the mussels. Each pair of experiments was analyzed separately. The pairs were as follows: July 26 and 30, September 28 and October 2, and December 5 and 13. All probability levels were fixed at 0.05. Statistical tests were conducted with SPSS 10.0 for Windows (SPSS, Chicago, IL).

RESULTS

The ash-free weight proportion of foulers in the experiments ranged from 0.4 to 9.0%. The experimental conditions were representative of the naturally occurring changes in Tracadie Bay. However, the proportion of foulers varied between 0.4 to 5.5% in the bay. Therefore, the effect of foulers may have been overestimated in our experiment in September when the foulers represented 9.0% of the experimental animals. Table 1 shows the mean ash-free weight and composition of the animals present in each experiment.

Food and inorganic nutrient concentrations varied throughout the study (see Table 2). There were notable changes in food uptake over the course of this study (Fig. 1a). Under stressful summer conditions (temperature 23°C), the mussels consumed no organic matter. However, when an epifaunal community, although low in abundance (0.4%), was added to the experimental containers, the uptake of organic matter became noticeable. In September, as the water cooled to 16°C, mussels began assimilating organic matter, and the effect of foulers on organic matter became insignificant. In December, water temperatures dropped to near-freezing values,

TABLE 1.

Mean ash-free weights in mg ($n = 4$) of organisms in the four experimental containers.

	July	September	December
Temperature (°C)	23	16	3
Organisms			
M. edulis	7000 (300)	10,000 (700)	17,000 (400)
Foulers	30 (9)	906 (82)	548 (26)
Fouling community			
Annelida			
Scale worms	29	53 (13)	150 (26)
Arthropoda			
Balanus crenatus	AB	3 (4)	3 (0.4)
Caprellids	AB	45 (15)	AB
Gammarus sp.	AB	17 (7)	AB
Isopods	AB	3	AB
Bryozoa			
Bugula turrita	AB	11	AB
Chordata			
Molgula sp.	AB	452 (88)	AB
Mollusca			
Anomia sp	AB	AB	4 (3)
Crepidula fornicata	AB	90 (13)	191 (22)
Mussel (_M. edulis_) spat	AB	40 (16)	48 (12)
Snails	AB	9 (2)	AB
Plants			
Red algae	23 (12)	225 (60)	147 (40)

Abbreviation: AB = absent. Values in parentheses are the SE.

TABLE 2.

Nutrient concentrations in input container for all experiments ($n = 8$).

Date	Chlorophyll a (µg/L)	Ammonium (µg N/L)	Phosphate (µg P/L)	Nitrite (µg N/L)	Nitrate (mg N/L)
July 26	5.66 ± 0.14	0.30 ± 0.03	1.07 ± 0.19	n/a	n/a
July 30	2.96 ± 0.77	2.36 ± 0.18	37.50 ± 8.57	n/a	n/a
September 29	7.42 ± 0.31	0.32 ± 0.06	19.00 ± 4.90	0.8 ± 0.4	0.07 ± 0.01
October 2	11.01 ± 0.80	0.18 ± 0.04	33.40 ± 13.40	2.0 ± 0.5	0.11 ± 0.02
December 5	8.09 ± 0.38	0.51 ± 0.14	0.20 ± 0.05	0.8 ± 0.4	0.13 ± 0.02
December 13	6.27 ± 0.18	0.26 ± 0.07	0.17 ± 0.02	5.0 ± 3.0	0.11 ± 0.01

Values given as mean ± SE. N/A = not available. N/L = µg of nitrogen per litre. P/L = µg of phosphorus per litre.

and all organisms ceased consuming organic matter. Therefore, with respect to organic matter, the epifaunal effect was limited to the summer period. However, the mussels consumed phytoplankton continuously throughout the study (Fig. 1b). It was at its highest in winter conditions. It is also only at this period that the effect of foulers was visible.

Ammonium was released by mussels except under summer conditions (Fig. 2a). Ammonium release was increased by almost

100% when foulers were added. In September, ammonia release was doubled by the presence of foulers. In cold water conditions, the effect of foulers on ammonia release was insignificant.

Mussels did not consume or release phosphate except in December when they released it (Fig. 2b). When foulers where added in summer conditions, there was a net release of phosphate, but it remained significantly equal to mussels. In winter, when foulers were more abundant than in summer (3.2% compared with 0.4%), there was a release of phosphate, but it was smaller than when
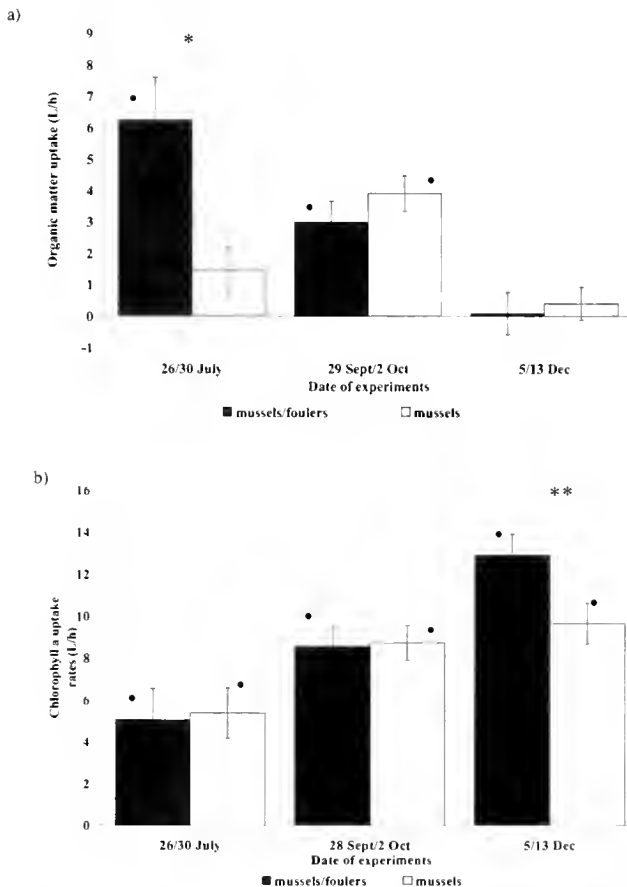
Figure 1. Food uptake by the mussel/fouler units and mussels from experiments carried out in summer and fall 2001. (a) Organic matter (L/h) and (b) chlorophyll a (L/h). Means are presented with SE as error bars ($n = 32$) (b) 26/30 July ($n = 13$). *$0.01 < P < 0.05$; **$0.001 < P < 0.01$; ***$P < 0.001$ in t test comparing mussel/fouler units and mussels; •significant positive and negative values represent an uptake and a release, respectively.
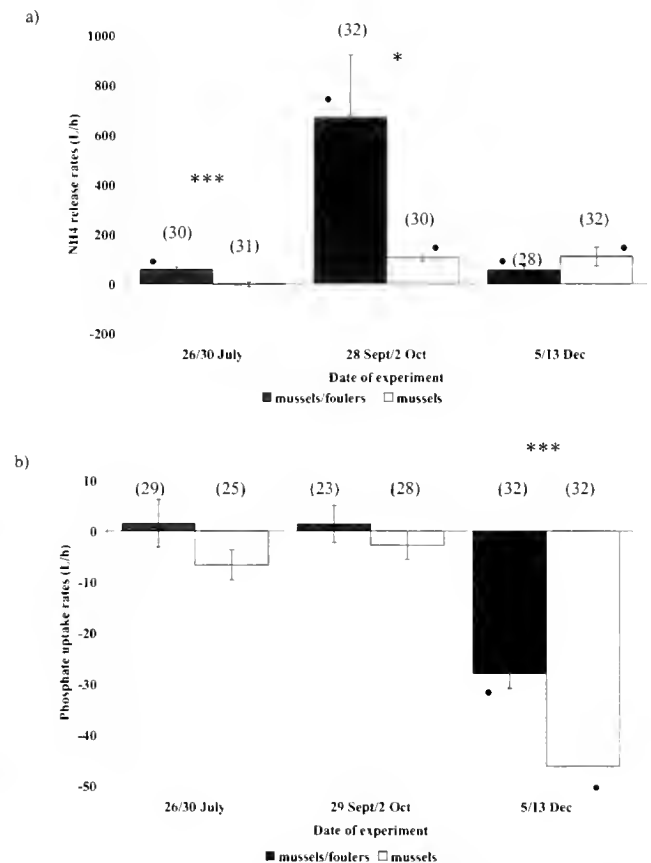
Figure 2. Inorganic nutrient fluxes by the mussel/fouler units and mussels from experiments carried out in summer and fall 2001. (a) Ammonium (L/h) and (b) phosphate (L/h). Means are presented with SE as error bars. Values in parentheses are the number of replicates. *$0.01 < P < 0.05$; **$0.001 < P < 0.01$; ***$P < 0.001$ in t test comparing mussel/fouler units and mussels; •significant positive and negative values represent an uptake and a release, respectively.

foulers were absent. In September, when foulers were most abundant (9.0%), phosphate fluxes remained insignificant.

Mussels consumed nitrite in fall conditions (Fig. 3a). The addition of foulers resulted in a net release of nitrite. In winter, neither mussels nor foulers had a significant effect on nitrite fluxes. Mussels had no effect on nitrate fluxes. There was significant consumption of nitrate (Fig. 3b) when foulers were added in winter

Foulers increased total feces production in summer despite their low abundance. They had no effect when their abundance was higher. They did, however, reduce organic feces production in the fall when they were the most abundant (Fig. 4).

DISCUSSION

Mussels are selective feeders. This selectivity is influenced by the quality and quantity of the seston (Thompson & Bayne 1972, Riisgård & Randlov 1981, Newell et al. 1989, Asmus & Asmus 1993, Bayne 1993, Prins et al. 1994, Hawkins et al. 1997), which is composed of phytoplankton and other sources of organic and inorganic matter. They choose food that is higher in nutrient content and that will, therefore, maximize growth. Phytoplankton is the preferred food because of its higher nutritional value, therefore
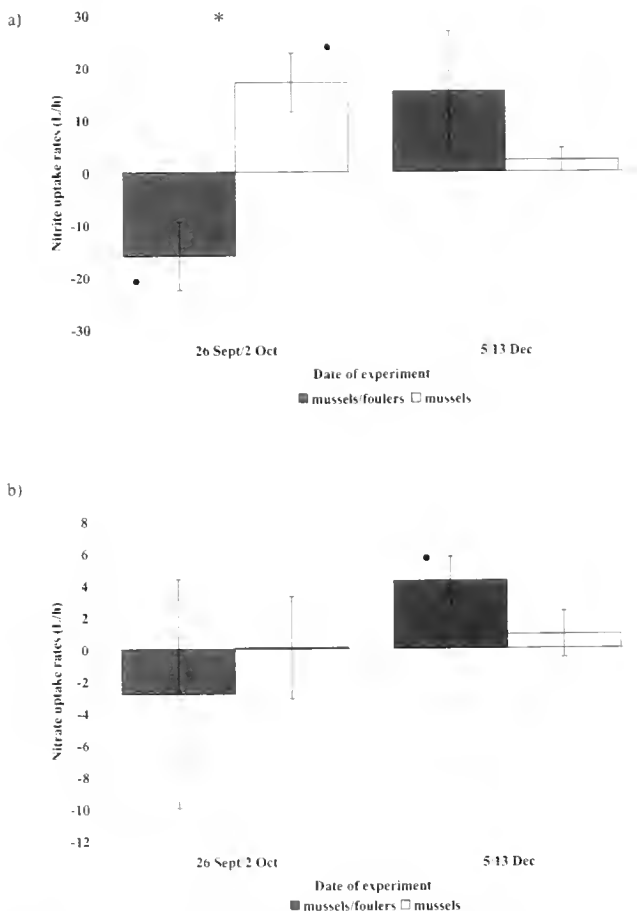
Figure 3. Inorganic nutrient fluxes by the mussel/fouler units and mussels from experiments carried out in summer and fall 2001. (a) Nitrite (L/h) and (b) nitrate (L/h). Means are presented with SE as error bars ($n = 32$). *$0.01 < P < 0.05$; **$0.001 < P < 0.01$; ***$P < 0.001$ in t test comparing mussel/fouler units and mussels; •significant positive and negative values represent an uptake and a release, respectively.
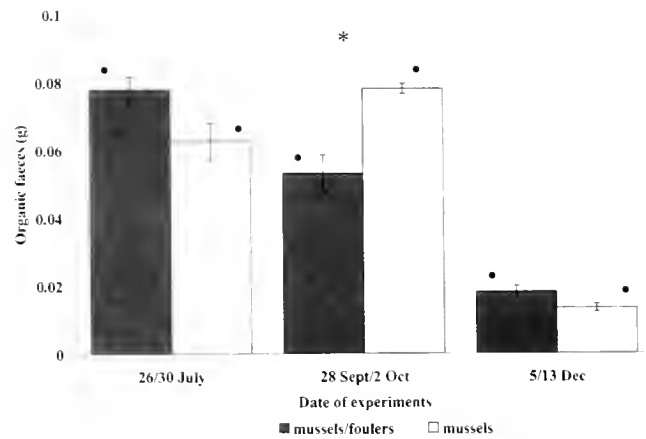
Figure 4. Organic feces production (g) by the mussel/fouler units and mussels from experiments carried out in summer-fall 2001. Means are presented with SE as error bars ($n = 4$). *$0.01 < P < 0.05$; **$0.001 < P < 0.01$; ***$P < 0.001$ in t test comparing mussel/fouler units and mussels; •significant positive and negative values represent an uptake and a release, respectively.

when food is abundant and varied, mussels will consume more phytoplankton than other types of organic matter. Foulers are composed of filter feeders, predators (carnivores), and herbivores. Their nutrition depends on the species composing the community. In July, scale worms and red algae were the only fouling species present. Neither one of these species consumes phytoplankton. Scale worms, however, consume organic pseudofeces, thereby increasing the available organic matter uptake by mussel/fouler units. In fall conditions, when the abundance of filter feeders is maximal, the consumption of phytoplankton and organic matter is not increased by the presence of foulers. However, both food sources are consumed. When the organic content of seston is high, mussels become less selective (Bayne 1993, Dame 1996), which may explain why both types of food sources are exploited. Filtration by ascidians is also influenced by seston concentrations (Millar 1971, Holmes 1973, Fiala-Médioni 1979, Robbins 1984, Riisgård 1988, Petersen & Riisgård 1992, Petersen et al. 1995). When food is abundant and varied, competition between species is insignificant (Zajac et al. 1989, Lesser et al. 1992). In winter, mussel spat form part of the fouling community, thereby increasing phytoplankton consumption.

There were problems during the spectrophotometric analyses for phosphate in December and for ammonium. Blanks (deionized water) were too high, and negative concentrations were obtained. To obtain positive values, the lowest concentration of an experiment was added to all concentrations of the same experiment, and calculations were made from the adjusted concentrations. Foulers increased ammonium release. This is consistent with the findings of Mazouni et al. (1998a), who found that foulers increased ammonium concentrations around oyster beds. Therefore, foulers may have a beneficial effect on primary production by prolonging blooms. Ammonium released by mussels is immediately available to primary production, while other sources of nitrogen (e.g., denitrification of sediments) are not as readily available (Kaspar et al. 1985, Dame & Dankers 1988, Mazouni et al. 1998a, Landry 2002).

The effect that foulers have on cultured mussels seems to be limited to food uptake and ammonium production. Competition for food could be reduced when sources are diversified and abundant. Many studies suggest that the main source of inorganic nutrients,

excluding ammonia, is through the remineralization of material from sediments and biodeposition (Kaspar et al. 1985, Dame et al. 1991, Prins & Smaal 1994, Smaal & Zurburg 1997, Mazouni et al. 1998a, Mazouni et al. 1998b). However, the presence of a diversified community of foulers results in reduced levels of fecal production. Certain species of foulers (e.g., scale worms or caprellids) consume organic fecal matter. They could, therefore, potentially reduce the input of inorganic nutrients (other than ammonia) by reducing the biodeposition of mussels.

This study suggests that foulers may not be as detrimental to mussel aquaculture as previously thought. While they could increase phytoplankton consumption, they could also contribute to a rise in ammonia levels, thereby counteracting the positive effect they have on phytoplankton. In contrast, they can increase diversity, which can prevent or reduce invasions (McGrady-Steed et al. 1997, Osman & Whitlatch 1999, Stachowicz et al. 1999) or population explosions by specific species that can be potentially harmful to mussel operations. In diverse environments, most ecological niches are occupied, therefore, there is less potential for a new species to invade.

ACKNOWLEDGMENTS

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AGE AND GROWTH OF THE MEDITERRANEAN SCALLOP *PECTEN JACOBAEUS* (LINNAEUS 1758) IN THE NORTHERN ADRIATIC SEA

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ABSTRACT   Age and growth of the scallop *Pecten jacobaeus* (Linnaeus 1758) were investigated on specimens collected from a commercial catch in the northern Adriatic during January and February 2003. Shells were aged based on ligament scars and formation of growth rings on the external shell surface, and data were fitted to the von Bertalanffy growth function $L_t = L_\infty (1-e^{-k(t-t0)})$. In addition, age and growth were estimated from growth increments using Gulland-Holt plot and relative growth function was constructed. Length of analyzed *Pecten jacobaeus* ranged from 75.1 to 142.0 mm, while estimated age ranged from 2 to 13 y. With respect to two methods applied, obtained von Bertalanffy equations for height were: $H_t = 108.79 (1-e^{-0.47t+0.16})$ and $H_t = 110.08 (1-e^{-0.53(t-t0)})$. The asymptotic shell lengths were estimated to be 127.93 and 127.35 mm, respectively.

KEY WORDS: age, growth, scallop, *Pecten jacobaeus*, Adriatic

INTRODUCTION

The scallop *Pecten jacobaeus* (Linnaeus 1758) lives on sand, mud, and gravel bottoms between 25 and 250 m depth (Poppe & Goto 2000) and can grow up to 162 mm in diameter (Onofri & Marguš 1995). Although present throughout Mediterranean coastal waters, *P. jacobaeus* occurs in commercial quantities only in the Northern Adriatic, where it is highly prized at local markets and restaurants (Mattei & Pelizzato 1996, Relini et al. 1999).

However, recent publications point out that population of *P. jacobaeus* in the western (Italian) part of the northern Adriatic show obvious signs of overexploitation (Relini et al. 1999). This species also is exploited in the eastern (Croatian) part of the northern Adriatic, but no signs of overexploitation of *P. jacobaeus* have been recorded in that area (Cetinić & Soldo 1999). However, studies from that area have not been based on the age composition or population structure of *P. jacobaeus*.

Previous studies in the region include bilateral research program, between Italy and Croatia, conducted in 1980s in almost entire Adriatic Sea, which only monitored and assessed the abundance of *P. jacobaeus* (Piccinetti et al. 1986). Other studies in the Croatian part of the Adriatic investigated age composition, distribution, reproduction, and larval settlement in the Krka river estuary–middle Adriatic (Marguš 1990, 1991, 1994, Marguš et al. 1992, 1993), biometry and age composition in Mljet lakes–south Adriatic (Onofri & Marguš 1995), and dredge selectivity and some biologic characteristics of *P. jacobaeus* in the northern Adriatic (Cetinić & Soldo 1999).

Scallops are traditionally aged using growth rings on the external shell surface and ligament scars (Tang 1941, Mason 1957, Merrill et al. 1961, Minchin & Mathers 1982, Richardson 2001). Furthermore, a recent study has shown that distribution striae, located on the external shell surface, can be used as a proxy for the onset of winter growth and for distinguishing between disturbance and truly annual surface rings (Owen et al. 2002).

The aim of this work is to present estimates of the age composition of the commercial catch and growth parameters of *P. jacobaeus* in the Croatian part of the Northern Adriatic by using the analysis of the external shell surface, ligament scars, and growth increment data.

MATERIALS AND METHODS

This study was conducted on western coast of the Istrian peninsula, which is, according to Croatian fishery legislation, the only area where fishing of *P. jacobaeus* is allowed in Croatia (Fig. 1). Commercial vessels with maximum breadth of dredges ranging from 2.0 to 3.0 m have been used for fishing *P. jacobaeus*. Towing speed varied from 3.0 to 3.8 knots, whereas sampling depth ranged from 23 to 35 m. The study was conducted in January and February of 2003, when lowest temperatures (~8°C) occur in northern Adriatic Sea (Soldo pers. comm.). According to literature (Gibson 1953, Mason 1957) ring formation in a related species, *P. maximus* (Linnaeus 1758), occurs in U.K. waters at the beginning of spring, following a period of coldest sea temperatures. Therefore, the edge of the shell was treated as the last ring and growth increment between previous ring and edge of the shell was treated as annual growth increment. Shells of 292 specimens were set aside and analyzed in the laboratory, including shells smaller than 100 mm ($n = 70$) that are usually returned back into the sea.

The length (anterior-posterior axis) and height (dorso-ventral axis) of each specimen was measured, using vernier callipers, to the 0.1 mm. Shells were aged based on ligament scars and the number of growth rings on the external shell surface, taking into account that first scars and rings are often missing (Tang 1941, Mason 1957, Merrill et al. 1961, Minchin & Mathers 1982, Richardson 2001). For observation of ligament scars, rubbery ligament was first softened in water so that it could be easily rubbed away and that the underlying growth lines could be reveled. Disturbance rings on the external shell surface were distinguished from annual rings based on distribution of striae: disturbance rings do no have small crowded striae and striae on either side of disturbance ring are equally spaced (Mason 1957, Owen et al. 2002). Length at age data were fitted to the von Bertalanffy growth function $L_t = L_\infty (1-e^{-k(t-t0)})$, where L_t is shell length t, L_∞ is asymptotic shell length, k is curvature parameter and t_0 is theoretical age at zero length (Beverton & Holt 1957).

Because of difficulties in determining marks on the shell related to the first year of life, a second method was applied to confirm the shell growth rates. Distances between clearly visible growth rings on the external shell surface were measured along th

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Figure 1. Map of study area.

Figure 2. Histogram of *P. jacobaeus* age frequencies, as determined from external growth rings.

main growth axis of each shell. These data were used for construction of a Gulland-Holt plot, where growth rates are plotted against the mean height (Gulland & Holt 1959). Growth parameters were estimated from a numerical value of the slope (k) and x-intercept (L_∞). Because it is not possible to calculate a value of t_0 using this method, this constant was omitted from the von Bertalanffy equation, and a relative growth curve was constructed (Sparre & Venema 1998).

RESULTS

The length of the analyzed *Pecten jacobaeus* ranged from 75.1 to 142.0 mm (x = 108.73 ± 13.19 mm). Smallest measured specimen had a height of 66.8 mm, while the height of the largest one was 124.5 mm (x = 94.27 ± 10.96 mm). The relationship between shell length and shell height can be described by the following equation L = -2.54 + 1.18 H ($n = 292$, $r^2 = 0.76$, $P < 0.001$).

The first ring on the external shell surface was not clearly visible in over 80% of the inspected shells. With respect to the analyses of the ligament, it was also noticed that the first scar was missing and that the ligament was less firm and flexible in animals older than 5 y. The estimated age of the analyzed specimens ranged from 2 to 13 y (x = 4.71 ± 2.27). Shells in age class three, four and five constituted 22.6%, 26.7% and 19.9% of the total sample, respectively (Fig. 2). Based on the observed shell height at each scar and ring, asymptotic shell height (H_∞) of *P. jacobaeus* was estimated at 108.79 mm, while the calculated curvature parameter (k) was 0.473 y^{-1} ($r^2 = 0.803$). With respect to length, asymptotic value (L_∞) was estimated at 127.93 mm and calculated curvature parameter (k) was 0.420 y^{-1} ($r^2 = 0.804$). According to the obtained von Bertalanffy growth equation, *P. jacobaeus*

growth is intensive during the first four years of its life and growth rate slows down considerably after the shell reaches 5 years of age (Fig. 3). Shell reaches the legal catch length of over 100 mm after its third year.

Results of the shell growth increment analysis using a Gulland-Holt plot are similar to those obtained by the analyses of number of rings and scars. The equation obtained in the plot was y = -0.53 x + 58.62 ($n = 680$, $r^2 = 0.768$; Fig. 4A). The calculated value of r^2 indicates the degree of variation in shell growth. Asymptotic shell height (H_∞) and curvature parameter (k), obtained from this equation, were 110.08 mm and 0.53 y^{-1}, respectively. Based on these data a relative growth curve was constructed (Fig. 4B). From the previously established relationship between shell length and shell height, the asymptotic shell length was estimated to be 127.35 mm.

DISCUSSION

Previous researchers have shown that bivalve species in temperate waters form annual surface rings and prismatic shell growth lines as a result of reduced growth in winter caused by declining seawater temperatures and decreased food availability (Richardson 2001). According to recent genetic work, *Pecten jacobaeus* and *Pecten maximus* are closely related, and may even be the same species (Wilding et al. 1999). Taking into account Wilding et al.'s
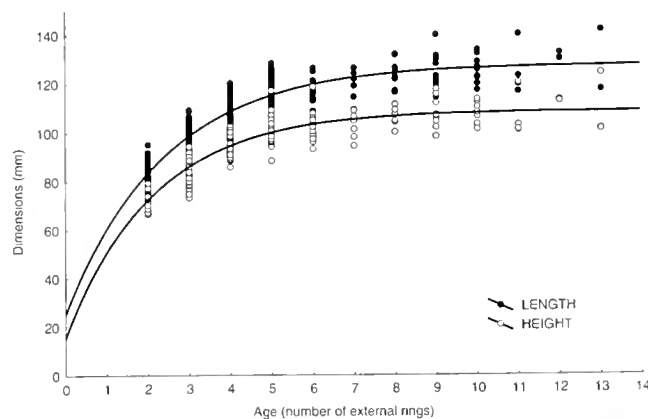
Figure 3. Growth curve for *P. jacobaeus* fitted using the von Bertalanffy growth equation for height $H_t = 108.79 (1-e^{-0.47t+0.16})$ and length $L_t = 127.93 (1-e^{-0.42t+0.22})$.

A.

B.
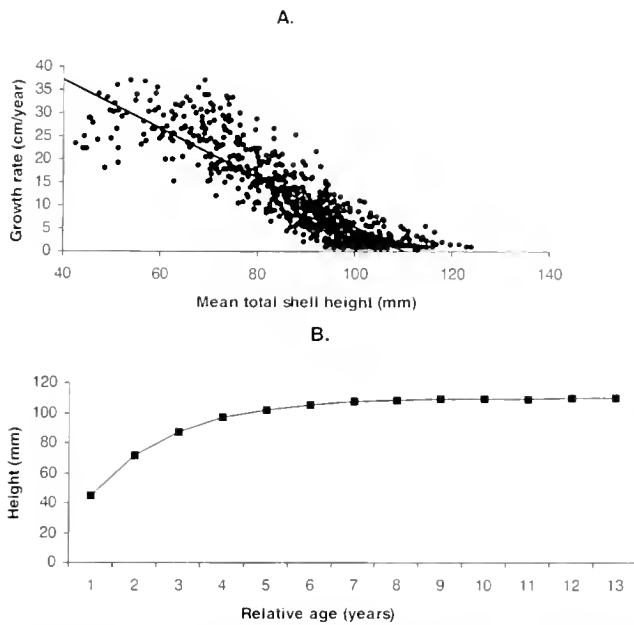
Figure 4. A. Gulland – Holt plot for *Pecten jacobaeus* y = -0.53x + 58.62 (*n* = 680, *r²* = 0.768). B. Von Bertalanffy growth equation of *Pecten jacobaeus* height based on results of the Gullland-Hold plot: $H_t = 110.08 \ (1-e^{-0.53(t-10)})$.

study (1999) and the lack of growth data for *P. jacobaeus*, we compared the results of this study with results previously published for both, the Mediterranean scallop *P. jacobaeus* and Atlantic scallop *P. maximus*.

Shell growth of the Atlantic scallop *Pecten maximus*, in Menai Strait (Wales, U.K.) exhibits the slowest growth rates during January and February, when shell growth effectively ceases at water temperatures below 8–9°C (Owen et al. 2002). According to Mason (1957), rings in *P. maximus* are formed during a period when the temperature starts rising and growth resumes. Sampling of *P. jacobaeus* in the northern Adriatic was conducted in January and February, during a period of the lowest sea water temperatures (Soldo pers. comm.), and thus the shell margin was treated as the beginning of a growth ring.

The largest *P. jacobaeus* recorded in this study was 142 mm in length, whereas previous studies in the Croatian Adriatic recorded specimens of 150 mm in the northern Adriatic (Cetinić & Soldo 1999), 146 mm in Krka river estuary (Marguš et al. 1992), and 162 mm in Veliko jezero, Island of Mljet (Onofri & Marguš 1995). The oldest shell in this study had 13 growth rings, whereas in samples from Krka river estuary scallops attained an age up to 11+ years (Marguš et al. 1992) and in Mljet lakes up to 17 y (Onofri & Marguš 1995). It is interesting to note that for *P. maximus*, Tang (1941) noted that some scallop he collected had 22 growth rings, whereas Mason (1957) found a shell with 18 rings. In this study, shells in age classes three, four and five constituted 69.2% of the total sample. In the Krka river estuary, around 70% of the analyzed shells belonged to age categories from 4+ to 6+ (Marguš et al. 1992), while in the Irish Sea about 65% of *P. maximus* belonged to age categories 3, 4, and 5. Category 4+ indicates that shell has four rings, with new growth outside the fourth.

In Mljet lake, scallops attained the legal catch length of 100 mm after their fourth year (Onofri & Marguš 1995), whereas in the Krka river estuary shells reached 100 mm length after their fifth

year (Marguš et al. 1992). According to Mattei & Pelizzato (1996), *P. jacobaeus* is a fast-growing species that in the Italian part of the northern Adriatic reaches a size of 100 mm or more in about 2 years. On average, *P. maximus* attains a size of 100 mm after its third growth ring and its growth rate decreases thereafter (Mason 1957, Allison 1994). Our data indicate that *P. jacobaeus* collected in the Croatian part of the northern Adriatic attains a length of over 100 mm after its third year and therefore appears to have a similar growth rate to *P. maximus*, it is faster growing than *P. jacobaeus* from Krka and Mljet and slower than specimens found in Italian part of the northern Adriatic.

Variations in growth of bivalves, among other things, are location dependent (Wilbur & Owen 1964). For example, it has been shown for some other bivalve species, namely *Arca noae* and *Pinna nobilis*, that their growth in Mljet lakes is slower than in other regions in the Adriatic (Peharda et al. 2002, Peharda 2003). Furthermore, higher concentrations of nutrients and hence greater primary production in the northern Adriatic than in other areas in the Adriatic Sea (Poulain et al. 2001), probably promoted faster growth of this species.

Unfortunately, Marguš et al. (1992), Mattei & Pelizzato (1996), and Onofri & Marguš (1995) did not calculate growth parameters for *P. jacobaeus*, so further comparison is not possible. However, growth data obtained in this study are similar to those obtained for *P. maximus* by Mason (1957) and Allison (1994), as indicated by values of o (o = Ln (k) + 2*Ln (L_∞)), calculated according to Sparre & Venema (1992; Table 1).

According to Mason (1957) *P. maximus* has different growth rates during it's first few years, depending whether they were the spring (April–May) or fall (August–September) spawned. Spring-spawned scallops grow faster in the first 2 years, whereas growth of fall-spawned ones is greatest in the second and third annual growth periods. In the western Mediterranean, *P. jacobaeus* settled on artificial collectors from April to July, reaching a peak in June, whereas some spat settled also in November and February (Pena et al. 1996). In the Gulf of Trieste, gonads are mature from May until July and a second maturation can be observed between November and February (Renzoni et al. 1988). In the estuary of the river Krka, one settlement peak of *P. jacobaeus* was recorded in a period from April until July, with the highest values recorded in June, while settlement did not occur in the fall or winter (Marguš 1994). Based on shell length at second ring found in a present study, the results of above-mentioned studies from the Adriatic and findings of Mason (1957), majority of analyzed shells from the northern Adriatic were probably spring spawned. However, observed variations in growth during first few years suggest that a certain number of fall settlers is also present in the population.

The observed age structure of *P. jacobaeus* from the Croatian

TABLE 1.

Values of asymptotic length (L_∞, in mm), curvature parameter (k) and o for *Pecten maximus* (Mason 1957, Allison 1994) and *Pecten jacobaeus* (this study)

Study	Location	L_∞	k	o
Mason (1957)	Isle of Man, spring spawned	146.20	0.396	9.04
Mason (1957)	Isle of Man, fall spawned	146.96	0.371	8.99
Allison (1994)	Bradda, Isle of Man	133.68	0.466	9.03
Allison (1994)	SE Douglas, Isle of Man	133.92	0.329	8.68
This study	Northern Adriatic	127.93	0.420	8.8

part of the northern Adriatic and the presence of older individuals, up to 13 y, indicates that overexploitation is probably not a problem under the current fishing pressure. However, because of a relatively long life span and slow growth of the species after it reaches its fourth year, periodic monitoring of the stock age structure, rather than length frequency monitoring, should be conducted in the future. This is necessary because of variations in shell growth of individual specimens that were observed in this study and previously noted by Mason (1957), who stated that a large

range of sizes is covered by each age-group, and that it is possible to find a 4+ scallop larger than an 8+ scallop in the same sample.

ACKNOWLEDGMENTS

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GEOGRAPHICAL PATTERNS IN GROWTH ESTIMATES OF THE SCALLOP *ZYGOCHLAMYS PATAGONICA*, WITH EMPHASIS ON URUGUAYAN WATERS

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ABSTRACT Growth parameters of the scallop *Zygochlamys patagonica* were estimated in Uruguayan waters of the southwestern Atlantic Ocean. Data used to estimate growth were collected at latitudes 35°50'S (northern end of the geographical distribution of the species) and 36°40'S (southern end of Uruguayan waters). Scallop ages were estimated by counting external growth rings on the left-hand valves. The von Bertalanffy function (VBGF) successfully explained some 93% (36°40'S) and 84% (35°50'S) of the variance. A likelihood ratio test indicated that scallops grew significantly faster at latitude 36°40'S than at latitude 35°50'S, confirming previous results showing large scale variation in density and individual muscle weight. Between-latitude differences were mainly ascribed to variations in the parameter t_0, which in turn could be explained by differences in observed lengths-at-age at earlier ages, notably age 1. Information on growth parameters of *Z. patagonica*, extracted from published sources over a wide latitudinal range (35°50'S–54°30'S), showed that asymptotic height H_∞ and the index of growth performance ϕ' were inversely correlated with latitude, decreasing from north to south. The growth parameter estimates provided in this study are consistent with the pattern. Management implications of these findings are discussed, placing special emphasis on the applicability of spatially explicit management tools.

KEY WORDS: scallops, *Zygochlamys*, growth, large-scale patterns, Uruguay

INTRODUCTION

The scallop *Zygochlamys patagonica* (King and Broderip) is a species widely distributed over the Magellanic Biogeographic Province. In the Pacific it is found in the Chilean Channels, from Puerto Montt (41°25'S) to Magellan Strait (53°00'S), mainly in shallow waters ranging from 5 to 25 m (Urban & Tesch 1996, Valero 2002). In the southwestern Atlantic Ocean (SAO), it is mainly found between ca. 35°50'S and 55°00'S, with largest scallop beds occurring at a depth range of 70 to 120 m (Waloszek 1991, Defeo & Brazeiro 1994, Gutiérrez & Defeo 2003), although a few individuals have been found as deep as 960 m (Waloszek 1991). The discontinuous distribution and marked differences in life history traits among beds have been considered in relation to hydrographic features of the SAO, notably the presence of frontal zones (Orensanz et al. 2003). Density-dependence was also mentioned as a possible factor behind geographic variation (Ciocco et al. 2003).

Several surveys undertaken between 1993 and 1994 identified the stock of *Z. patagonica* from the Uruguayan shelf in this zone as a potential fisheries resource (Defeo & Brazeiro 1994). Recently, Gutiérrez & Defeo (2003) documented the spatial structure of scallop beds in Uruguayan waters of the SAO between latitudes 35°50'S and 36°50'S, of which the salient aspects are: (1) beds close to the Uruguayan southern border (36°50'S) had densities 16 times higher than the northern border (35°50'S), (2) average individual size increased southwards, and (3) muscle yield increased northwards. In this article we provide first growth estimates for the scallop *Z. patagonica* stock inhabiting Uruguayan waters of the SAO, and analyze latitudinal patterns in growth parameters of the species from a wide range of published sources. Implications for management are discussed.

MATERIAL AND METHODS

Data were collected at latitudes 35°50'S, close to the northern limit of species distribution in the SAO, and 36°40'S, close to the southern end of the Uruguayan shelf, during surveys conducted by the Uruguayan RV "Aldebarán" (Gutiérrez & Defeo 2003). Each 15' tow was carried out at a mean trawling speed of 3.2 knots/h. Sampling gear was an otter trawl directly attached to the doors (otter boards) with a net opening of 9.5 m and a mesh size of 5 cm. The total catch of scallops per tow was recorded, and a subsample retained for processing. Scallops shell height (H) was measured with 1 mm accuracy in the laboratory, from the umbo to the ventral border of the shell. Some 270 and 96 individuals were used for estimating growth at latitudes 35°50'S and 36°40'S, respectively.

Age in each scallop was estimated by counting external growth rings on the left-hand valves, assuming annual periodicity. The latter was validated for scallop beds in contiguous Argentinean waters (Waloszek & Waloszek 1986, Waloszek 1991, Lasta et al. 2001, Ciocco et al. 2003). The data was used to fit the von Bertalanffy growth function (VBGF; von Bertalanffy 1938):

$$H = H_\infty [1 - e^{-K(t-t_0)}]$$

where H_t is shell height at age t, H_∞ is the asymptotic height, K is the curvature parameter and t_0 is the estimated age at length zero. A quasi-Newton method was used to estimate the parameters (mean ± SE). Growth of scallops from both latitudes was compared by likelihood ratio tests (Kimura 1980, Cerrato 1990), under different null hypotheses (Palacios 1994). First, we compared all three parameters simultaneously under the null hypothesis H_0: $H_{\infty 1} = H_{\infty 2}$; $K_1 = K_2$; $t_{01} = t_{02}$. Afterwards, the model selection process was done by sequentially altering the number of parameters under comparison. We used raw data instead of mean length-at-age information following Haddon (2001).

Latitudinal information on growth parameters of *Z. patagonica* came from published sources, and combined with our own results. Estimates of H_∞ and K from 14 geographical sites between 35°50'S and 54°30'S were obtained: two from Uruguay (this

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TABLE 1.

Sources of data used in regression analyses between latitude and growth parameters of Z. *patagonica* in Atlantic and Pacific scallop beds of South America. The growth performance index ϕ' was estimated in this study, using information of H_∞ and K. Data used in all cases are size-at-age.

Ocean	Country	Latitude	H_∞ (mm)	K (yr^{-1})	ϕ'	N	Age Range	Source
Atlantic	Uruguay	30°50′	75.98	0.39	3.35	270	1–9	This study
Atlantic	Uruguay	36°40′	81.15	0.31	3.31	96	1–7	This study
Atlantic	Argentine	39°24′	74.70	0.42	3.37	197	1–9	Valero (2002; in Ciocco et al., 2003)
Atlantic	Argentine	39°47′	68.69	0.50	3.37	75	1–8	Waloszek & Waloszek (1986)
Atlantic	Argentine	41°50′	74.18	0.38	3.32	83	1–10	Valero (2002; in Ciocco et al. 2003)
Atlantic	Argentine	41°50′	69.93	0.37	3.25	87	1–8	Waloszek & Waloszek (1986)
Atlantic	Argentine	42°30′	59.76	0.49	3.25	152	1–7	Waloszek & Waloszek (1986)
Atlantic	Argentine	43°53′	66.32	0.50	3.34	79	1–8	Waloszek & Waloszek (1986)
Atlantic	Argentine	44°00′	75.59	0.54	3.49	124	1–8	Waloszek & Waloszek (1986)
Atlantic	Argentine	46°47′	65.67	0.63	3.43	65	1–7	Waloszek & Waloszek (1986)
Atlantic	Argentine	49°50′	62.65	0.40	3.19	89	1–8	Waloszek & Waloszek (1986)
Atlantic	Argentine	52°00′	54.66	0.58	3.24	91	1–8	Waloszek & Waloszek (1986)
Atlantic	Argentine	54°30′	54.90	0.39	3.07	90	1–7	Waloszek & Waloszek (1986)
Pacific	Chile	53°00′	66.00	0.14	2.78	95	1–11	Urban & Tesch (1996)

study), 11 from Argentina and one from Chile (Table 1). The growth index $\phi' = 2\log_{10}(L_\infty) \log_{10}K$ (Pauly & Munro 1984) was calculated and used to assess growth performance. The relationships between growth parameters and latitude (centesimal units) were modeled by linear and nonlinear fitting procedures, and the model with the best goodness of fit selected.

RESULTS

Scallops at latitude 36°40′S grew significantly faster than at latitude 35°50′S (Fig. 1). The non-linear fitting of the VBGF explained 93% (36°40′S) and 84% (35°50′S) of the variance, and all parameters were significant, except t_0 at latitude 35°50′S (Table 2). Results of likelihood ratio tests showed that the overall VBGF significantly differed between latitudes ($\chi^2 = 45.234$, df = 3, $P < 0.0001$). Testing of the remaining null hypotheses showed that the H_∞ and K parameters did not differ significantly (χ^2 test, df = 1, $P > 0.05$). Conversely, a significant difference between t_0 values was strongly indicated, either in isolation (χ^2 test, df = 1, $P < 0.01$) or in combination with the other two VBGF parameters (Table 3). Marked differences in mean-length-at age at earlier ages, notably age 1 (ANOVA test: p < 0.01; Cochran test for

homoscedasticity C = 0.68; $P = 0.49$) could explain the observed differences between curves (see Fig. 1).

The large-scale analysis showed that both H_∞ and ϕ' were inversely correlated with latitude ($r = -0.80$; $P < 0.0006$ for H_∞ and $r = -0.63$; $P < 0.015$ for ϕ'), but K was not ($r = 0.03$; $P = 0.91$) (Fig. 2). Values of H_∞ were in the range 55 to 81 mm, with the lowest value at 54°30′S and the highest at 36°40′S (this study: Table 1).

DISCUSSION

Scallops grew significantly faster at the southern limit of Uruguayan waters when compared to the northern border. Gutiérrez & Defeo (2003) also showed that muscle weight increased linearly towards the southern end of Uruguayan waters, whereas the harvestable stock (ie, individuals >55 mm H), mean individual height and maximum height increased asymptotically in the same direction. Defeo & Brazeiro (1994) found hardly any scallops north of the range considered here (between 35°50′S and 35°00′S) and, where few specimens were caught, individual height also tended to be low. This is in agreement with the higher estimate of H_∞ found at 36°40′S (81.15 mm H) when compared with that at 35°50′S (75.98 mm H). The occurrence of lower abundances and sizes at the northern distribution end has been ascribed to habitat
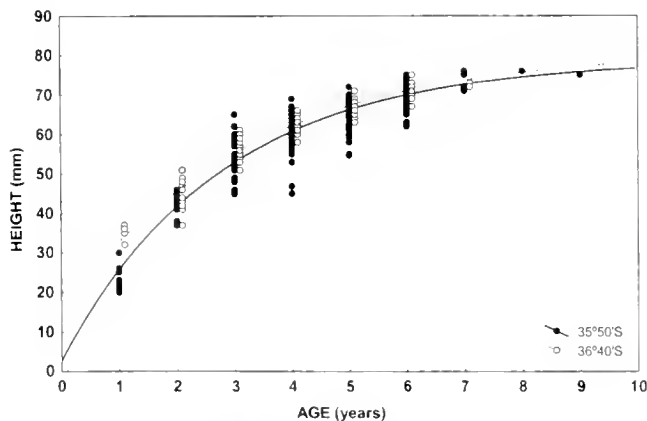
Figure 1. Growth models for the scallop Z. *patagonica* in Uruguayan waters. Details are provided in Table 2.

TABLE 2.

Z. *patagonica*. Results of the von Bertalanffy growth models fitted by nonlinear least squares for scallops of Uruguayan waters. Significant values ($P < 0.01$) are highlighted in bold and italics.

Parameters	Latitude 35°50′S		Latitude 36°40′S	
	Estimate (SE)	P	Estimate (SE)	P
H_∞ (mm)	75.98 (1.60)	*0.0000*	81.15 (2.81)	*0.0000*
K (yr^{-1})	0.39 (0.03)	*0.0000*	0.31 (0.04)	*0.0000*
t_0 (yr)	−0.01 (0.11)	0.9336	−0.65 (0.19)	*0.0011*
R^2	0.84	*0.0000*	0.93	*0.0000*

TABLE 3.

Likelihood ratio tests comparing von Bertalanffy parameter estimates for the two scallop beds in Uruguayan waters. Results of the RSS (residual sum of squares), the χ^2 test and associate statistics are shown on the basis of the seven null hypotheses tested (columns 3 to 9), assuming that the listed parameter or a combination of them do not differ between scallop beds. The second column refers to the independent fitting of the two separate curves (see also Table 2). Significant values of the likelihood ratio test are highlighted in bold and italics.

Parameter	Independent	$H_0: = H_\infty$ $= K$ $= t_0$	$H_0: = H_\infty$	$H_0: = K$	$H_0: = t_0$	$H_0: = H_\infty$ $= K$	$H_0: = H_\infty$ $= t_0$	$H_0: = K$ $= t_0$
Latitude 35 50'S								
H_∞ (mm)	75.979	76.485	77.102	76.845	77.693	77.148	77.063	76.361
K (yr^{-1})	0.387	0.374	0.368	0.371	0.351	0.369	0.358	0.364
t_0 (yr)	−0.009	−0.200	−0.062	−0.062	−0.169	−0.053	−0.170	−0.203
Latitude 35 50'S								
H_∞ (mm)	81.147	76.485	77.102	77.584	75.854	77.148	77.063	79.759
K (yr^{-1})	0.312	0.374	0.375	0.371	0.420	0.369	0.404	0.364
t_0 (yr)	−0.647	−0.200	−0.411	−0.401	−0.169	−0.454	−0.170	−0.203
RSS	5167.717	5848.974	5206.290	5190.891	5271.624	5199.697	5284.775	5379.007
χ^2		45.234	1.657	1.638	7.286	2.258	8.198	14.667
df		3	1	1	1	2	2	2
P		*0.0000*	0.1980	0.2007	*0.0069*	0.3234	*0.0166*	*0.0007*

unsuitability and scarcity of food (Gutiérrez & Defeo 2003). In spite of the above, the likelihood ratio test indicated that major between-latitude differences in the VBGF could be ascribed to parameter t_0, which jointly with H_∞ strongly determines the form of the VBGF (Haddon 2001). Differences between curves could be attributed to variations in length-at-age at ages 1 to 5, notably age 1, where individual sizes-at-age at 36°40'S were greater than at 35°50'S (see Fig. 1).

Growth parameters of *Z. patagonica* showed clear latitudinal patterns: asymptotic height H_∞ and the overall growth performance ϕ' both significantly decreased from north to south in the SAO. The results of our study are consistent with the pattern found at a large-scale, and indirectly reaffirm the annual nature of growth ring formation. These results provide additional evidence for the large-scale patterns found for Argentinean waters, where a significant decrease in H_∞ was estimated as latitude increases (Valero 2002, Ciocco et al. 2003). Between-latitude differences in growth rate have been attributed to variations in environmental parameters, such as temperature and food availability (Ciocco et al. 2003 and references therein). Valero (2002) presented new evidence on the effects of factors acting at small spatial scales (eg, intrabed scale), seasonal cycles and year-to-year variation in growth in this species, which were mainly related to variations in temperature and oceanographic regimes. These factors seem to be critical in explaining growth variations in other scallop populations (see eg MacDonald & Thompson 1985, Schick et al. 1988 and papers in Shumway 1991). Latitudinal differences in growth rate could also be explained by density-dependence operating at the scale of scallop beds, a mechanism already proposed by Orensanz et al. (2003) to explain intra and inter-cohort variations in population dynamics. Ciocco et al. (2003) showed that for high-density patches occurring in Argentine waters, individuals at high concentrations are affected by density-dependence even in small areas (Lasta & Bremec 1998). The low growth performance in high-density beds in southern waters of the SAO (as denoted by ϕ') provides additional support to the density-dependence hypothesis, which was also suggested by Gutiérrez & Defeo (2003) for Uru-

guayan scallop grounds. Finally, differences between Atlantic and Pacific estimates could be attributed to different environmental scenarios and the fact that the species occurs in relatively shallow waters in the Pacific.
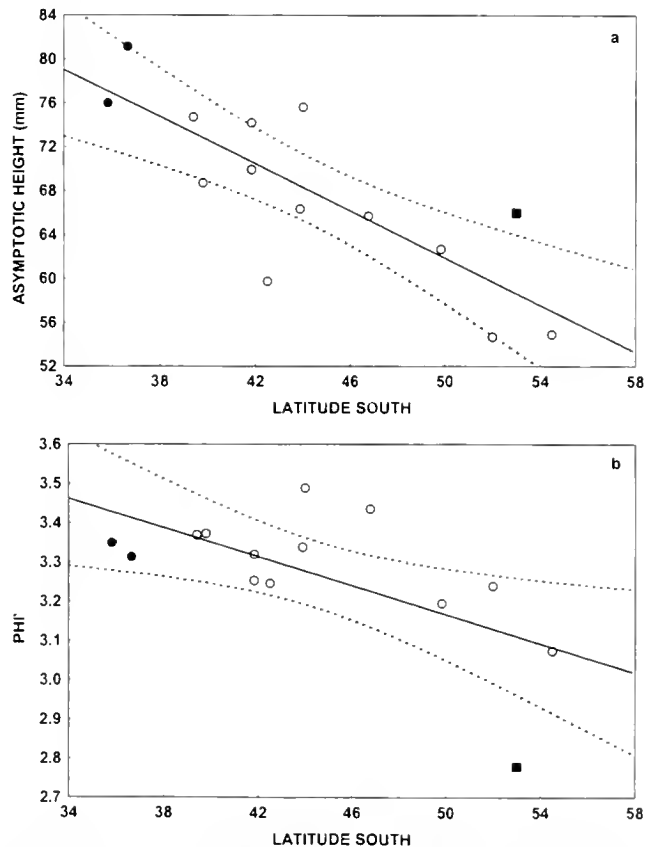
Figure 2. *Z. patagonica.* Regression lines (±95% CI) between latitude (centesimal units) and (a) asymptotic height and (b) the growth performance index ϕ'. (●) Uruguay; (○) Argentina and (■) Chile.

Management Implications

The significant latitudinal gradient in growth rate detailed here could have implications for fishery management, as spatial variation in population dynamics and life history traits are used to provide area-based estimates of potential yield (Caddy 1975) and to implement spatially explicit management measures (eg. rotation of fishing areas and reproductive refugia: Orensanz & Jamieson 1998, Catilla & Defeo 2001). This should call for a spatially discrete analysis of population dynamics and other life history traits, the surrounding environment, and the fishery. In this setting, mapping of density and related population processes is worthwhile as

a way of forecasting the spatial features of the stock, and to assess the economic potential of the fishery (Caddy 1989a, b).

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INTERMEDIATE CULTURE OF KING SCALLOP (*PECTEN MAXIMUS*) IN SUSPENSION IN CAGES: EFFECT OF STOCKING DENSITY AND DEPTH

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ABSTRACT Scallop spat settled on collectors were grown in suspended cages in O Grove, (Ría de Arousa, Galicia, northwest Spain) and in Fuengirola, (Málaga, Andalucía, southern Spain). Mean (±SD) spat heights of 20.4 ± 3.7 mm (Fuengirola, September 1998) and 26.6 ± 5.8 mm (O Grove, November 1998), were stocked at densities ranging between 25 and 200/cage^{-1} (≈200–1600 spat m^{-2}), and at depths of 6 and 10 m in O Grove, and between 13 and 25 m in Fuengirola. Even low stocking densities were found to affect scallop growth, therefore juveniles (>35 mm) were used to set up new cultures at lower stocking densities (12 and 24 juveniles/cage^{-1}) at the end of winter (Fuengirola) and at the beginning of spring (O Grove). The most rapid growth took place at Fuengirola, where the mean height reached on May 19, 1999, was 63.9 ± 4.1 mm compared with a mean height of 51.2 ± 4.5 mm for the O Grove spat on May 27, 1999.

KEY WORDS: Andalucía, density, depth, Galicia, intermediate culture, *Pecten maximus*, suspension culture

INTRODUCTION

Attempts are currently being made in several European countries to cultivate *Pecten maximus* on a commercial scale (Fleury et al. 1997), using both hatchery-produced spat (Norway and France) and spat captured by natural settlement on collectors (Ireland and Scotland). Pectinids (pectinoid form *sensu* Waller 1992) cultivated in suspended cages grow slowly after a certain size (Slater 1995), possibly because of the differences in the conditions in the cages and in the natural habitat (recessed in sediment), although waves have been observed to have a negative effect on *Euvola ziczac* and *Nodipecten nodosus* (Freites et al. 1999). Because of these difficulties, the present trend in Europe is for intermediate culture, usually in suspension, followed by the seeding of juveniles of different sizes, depending on the conditions in each area, on the sea floor. However, in certain areas the environmental conditions or the techniques result in the successful use of suspended culture to grow scallops to commercial size (Román & Fernández 1991, Gallagher 1999, Cano et al. 2000).

The desired final size of the juveniles maintained in intermediate culture will obviously depend on the method of on growing. In the case of seabed culture, the size required depends on the environmental conditions (i.e., sediment, current, and predators) and varies from region to region: 30 mm in France (Fleury et al. 1995); and 50 mm in Norway, Ireland, and Scotland (Fleury et al. 1997). The size required for ear-hanging culture is more standardized. Generally, scallops are not ear-hung until they have reached at least 55 mm shell height (Ventilla 1982, Dadswell & Parsons 1991, O'Connor et al. 1999). Gallagher (1999) considers the minimum size for ear-hanging culture to be 50 mm, whereas Cano et al. (2000) used juveniles of between 51.3 and 64.3 mm height. Román and Fernández (1991) describe ear-hanging culture in Galicia, where scallops of heights of between 60 and 70 mm are used.

In order for the scallops to reach the size required for the on-growing stage, they must undergo a period of intermediate culture in mesh trays or cages, which are usually suspended in the water, although cages are also placed on the seabed (Dao et al.

1996). Subsequently, scallops are seeded on the seabed (Fleury et al. 1997), are suspended using the ear-hanging technique (Paul 1988, Román & Fernández 1991, Gallagher 1999), or are held within lantern nets, cages, or other artifacts (Cano et al. 2000).

There are many reports in the literature on the effects on growth and survival of the use of mesh enclosures (O'Connor et al. 1999) and of the stocking density and depth at which pectinid spat are cultured (Côté et al. 1993, Duggan et al. 1973, Leighton 1979, Parsons & Dadswell 1992, Rhodes & Widman 1984, Wallace & Reisnes 1984, 1985, Román et al. 1999, Cano et al. 2000, Freites et al. 1995). Most of these studies refer to aequipectinoid pectinids (form *sensu* Waller, 1991), the natural habitat of which is similar to the conditions of suspended culture.

Scallops are commercially produced in two areas of Spain: Galicia (in northwest Spain); and in the province of Málaga (Andalucía, in southern Spain). Cultivation on the seabed is not possible for legal and social reasons, and only suspension culture is feasible. The aims of the present study were to determine, in each area, the optimum conditions (in terms of depth and stocking density) required for the intermediate culture of spat and juveniles in suspended cages to obtain scallops of a suitable size for ear-hanging culture (≈60 mm height), and to compare the growth and survival of spat cultured in the different areas. In addition, the possibility of cultivating scallops to commercial size in suspended cages in Galicia was evaluated, which is an aspect that has been studied previously in Málaga (Cano et al. 2000).

MATERIALS AND METHODS

Study Area

The study was carried out at two sites, in O Grove, Ría de Arousa, in the Atlantic Ocean (Galicia, in northwest Spain), and in Fuengirola, in the Alborán Sea (western Mediterranean, Málaga, Andalucía, in southern Spain) (Fig. 1).

Environmental Conditions

The temperature and levels of chlorophyll *a* were measured, using a conductivity-temperature-depth (CTD) recorder, every week in O Grove and every fortnight in Fuengirola. Salinity also was recorded in O Grove.

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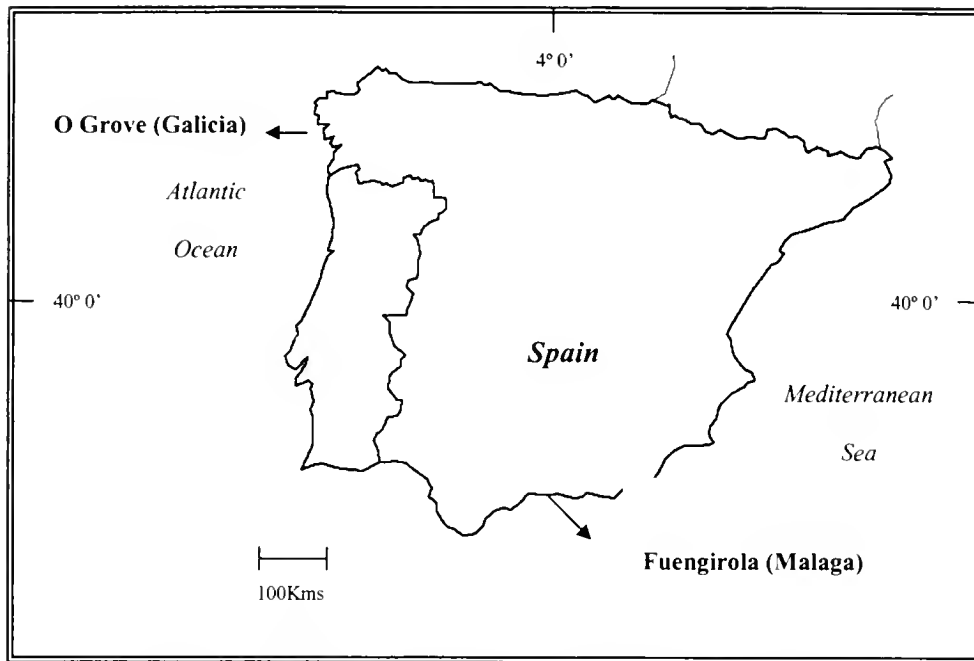
Figure 1. Locations of the experimental intermediate culture of *P. maximus*.

Animals

Scallops of up to 30 mm are considered to be spat, and between 30 and 60 mm they are considered to be juvenile. In both areas, the spat was obtained by natural settlement on collectors.

Suspended Culture

The spat were cultured in circular rigid plastic cages (40 cm diameter, 10 cm height, 10 mm mesh size). In O Grove, the cages were hung from a raft, whereas in Fuengirola they were anchored, following the scheme outlined by Cano et al. (2000). Spat growth was greatly affected by stocking density, therefore new experiments were started in the spring in both areas using juveniles at lower stocking densities of 12 and 24 scallops/cage^{-1}. The experiments were carried out in duplicate (Table 1).

Sampling

The cages were raised periodically so that the height of the scallops (measured to the nearest millimeter using calipers) and the number of dead could be recorded, and they then were resuspended.

Statistical Methods

Mean sizes (height) were compared by factorial analysis of variance (ANOVA) using stocking density and depth as factors. Normality was previously checked using the Kolmogorov-Smirnov test, and the homogeneity of variance was checked by Bartlett's test. The differences in size were compared *a posteriori* using a Newman-Keuls test ($\alpha = 0.05$), except when there was interaction between factors. Comparisons between pairs of samples were made using a Student's t test. Arcsin transformation was used to compare percentage survival.

TABLE 1.

Description of experimental intermediate culture of *P. maximus*.

Area	Date Start End	Initial Size (mm)[a] (Mean ± sd)	Density Culture n Scallops Cage^{-1}	Initial Coverage (%)[b]	Depth Culture (m)
Intermediate culture of spat					
Fuengirola	9/24/98–2/24/99	20.4 ± 3.7	25/50/100/200	6.5/13.0/26.0/52.0%	13/18/23/26
O Grove	11/17/98–4/6/99	26.6 ± 5.8	25/50/100	11.1/22.1/44.2%	6/10
Intermediate culture of juvenile					
Fuengirola	2/24/99–5/19/99	52.1 ± 5.0	12/24	20.4/40.7%	13/18/23/26
O Grove	4/6/99–5/30/99	39.9 ± 3.2 (6 m)	12/24	11.9/23.9% (6m)	6/10
		41.4 ± 3.9 (10 m)		12.9/25.7% (10m)	

[a] Presented as mean ± SD.
[b] Experimental design 2 × 2 factor.

RESULTS

Environmental Conditions

O Grove

There was a slight temperature inversion with depth in winter, however, during the rest of the year the temperature was slightly higher in the first 6 m. In general, the temperatures were very similar throughout the year, at both depths, ranging between 11°C and 18°C, with only occasional differences of >1°C (Fig. 2). Salinity ranged between 34‰ and 35.5‰ throughout most of the study period, except in May 2000, when minimum values of 32.0‰ and 33.1‰ were registered at depths of 6 and 10 m, respectively. There was a trend toward slightly higher levels of chlorophyll *a* in the surface layers of water from the end of autumn until the beginning of spring, but from May onward increasing levels were found at depth. There were large variations in the levels, with minimum values of approximately 1 μg L^{-1}, and maximum values of 2 and 3 μg L^{-1} at 6 and 10 m, respectively (Fig. 3).

Fuengirola

High temperatures (17–18.5°C) were registered at the beginning of September, followed by a maximum of 21°C at the end of September. From the end of October until May, the temperature varied between 14°C and 16°C (Fig. 2). The levels of chlorophyll *a* observed were rarely <1 μg L^{-1}, with peaks of between 2 and 5 μg L^{-1} in September, October, and February, and particularly between April and May. As there was strong vertical mixing during the period of the study, there was very little variation with

depth, with only a slight trend toward lower temperatures and chlorophyll *a* levels at lower depths (Fig. 3).

Growth

O Grove

Spat culture. On March 2, 1999, the mean heights ranged between 35.6 and 39.8 mm, depending on the culture conditions. There were significant differences in the heights achieved at the different stocking densities (25 scallops/cage^{-1} > 50/cage^{-1} > 100/cage^{-1}), but there were no significant differences associated with depth (Fig. 4). One month later, on April 6, 1999, only very small increases in size were registered, with mean heights ranging between 36.4 and 41.4 mm, depending on the culture conditions. Again, growth was affected by stocking density but not by depth. Scallops cultivated at a stocking density of 25/cage^{-1} were significantly larger than those cultivated at higher stocking densities, whereas there was no difference in the size of scallops cultured at the two higher stocking densities (50 and 100/cage^{-1}). The mean coverage was 25.6% at 25/cage^{-1}, 45.0% at 50/cage^{-1}, and 86.0% coverage at 100/cage^{-1}. The spat growth experiment finished on this date, and a new experiment, using juveniles at lower stocking densities, was started.

Juvenile culture. This experiment was begun on April 6, 1999, using scallops previously grown at stocking densities of 25/cage^{-1} (the mean heights reached by scallops cultivated at depths of 6 and 10 m were 39.9 ± 3.2 and 41.4 ± 3.9 mm, respectively). The new stocking densities were 12 and 24/cage^{-1}, at the same depths as before (i.e., 6 and 10 m). The initial coverage was 11.9% and 23.9% and 12.9% and 25.7%, respectively, at 12 and 24 spat/cage^{-1} at 6 and 10 m.
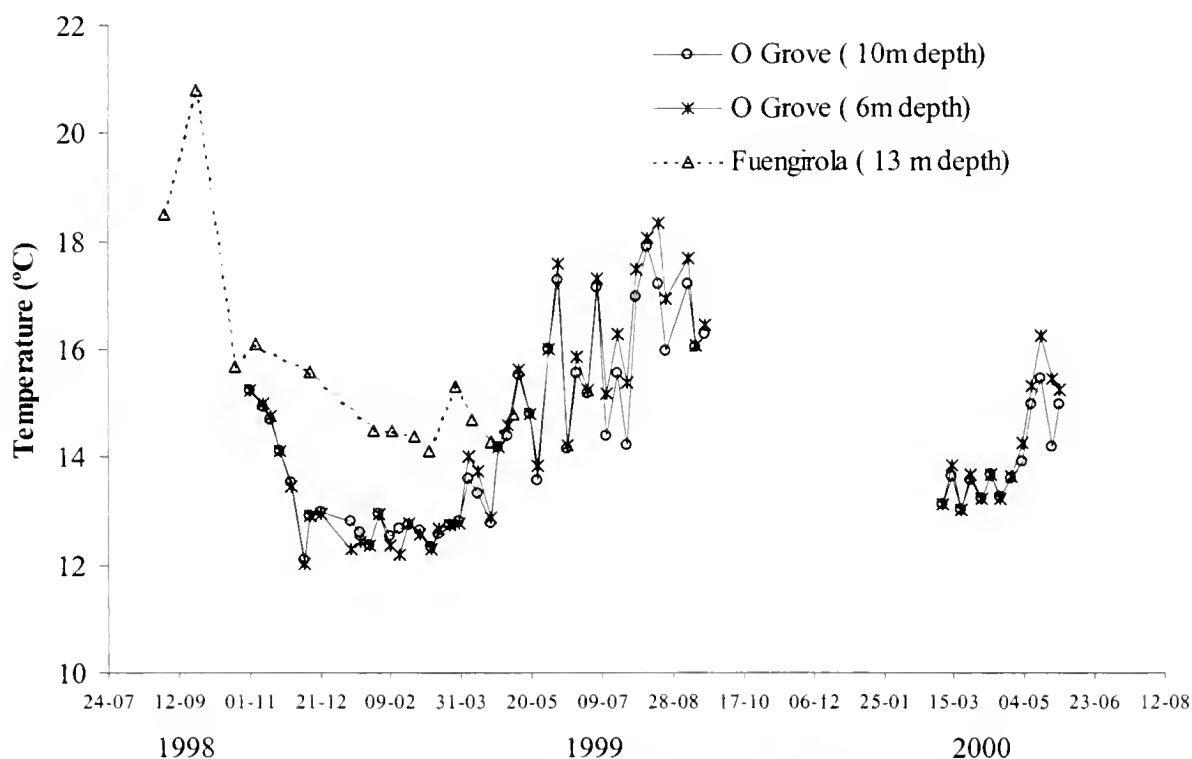
Figure 2. Interannual variation of temperature in O Grove and Fuengirola.
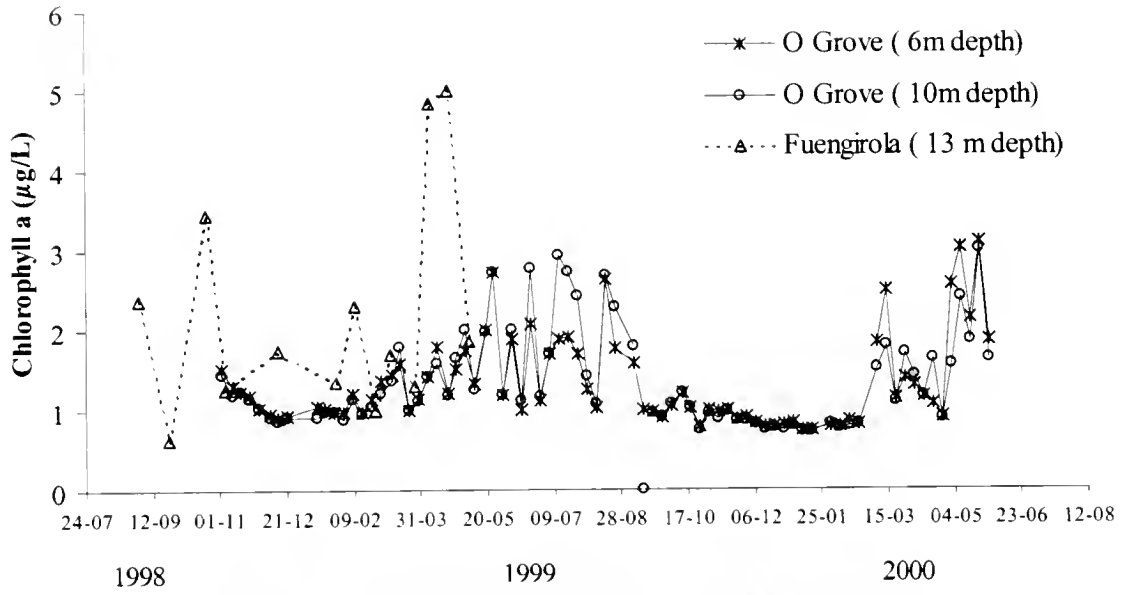
Figure 3. Interannual variation of Chlorophyll *a* in O Grove and Fuengirola.
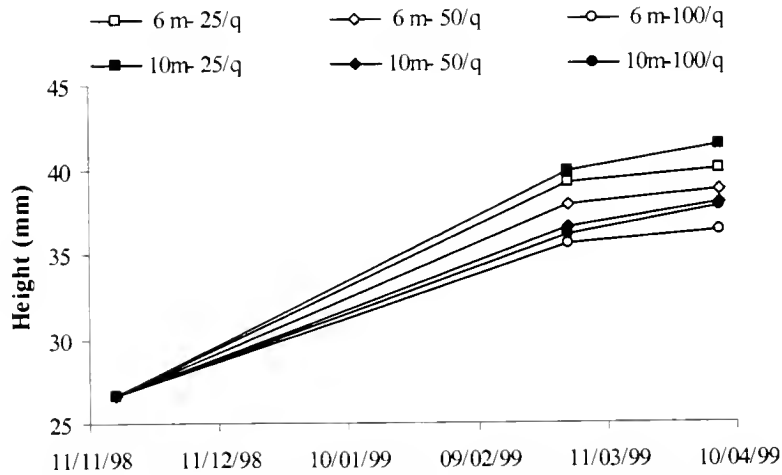
Figure 4. Growth of *P. maximus* spat on intermediate culture in O Grove.
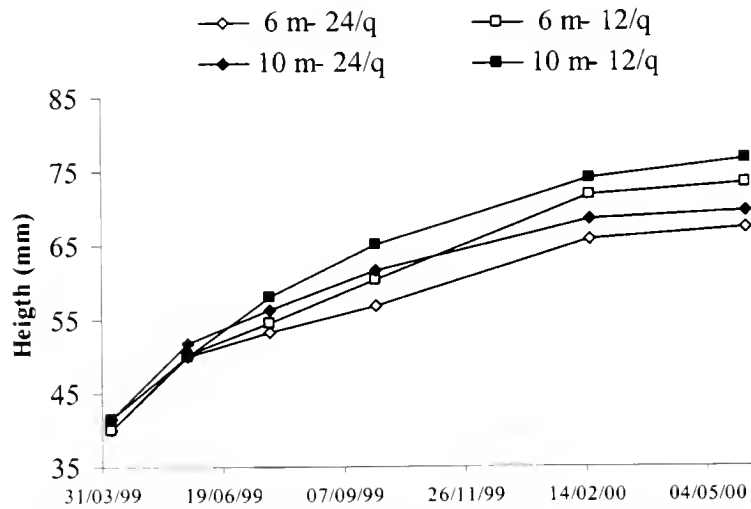
Figure 5. Growth of *P. maximus* juvenile on intermediate culture in O Grove.

Depth affected the growth of the juveniles between May 27 and September 28, 1999 (the juveniles maintained at a depth of 10 m reached a larger size than that cultivated at 6 m; Fig. 5). On February 17, 2000, there were no effects associated with depth, but on May 30, 2000, a depth effect was once again observed (ANOVA; Table 2).

The effect of stocking density on growth rate was observed from September 28, 1999, until the end of the experiment on May 30, 2000, with larger scallops being obtained at the lower stocking density (Fig. 5). Very little growth was registered between February 17 and May 30, 2000. On February 17, 2000, the mean height reached ranged between 66.1 and 73.9 mm, depending on the culture conditions, whereas on May 30, 2000, it ranged between 67.1 and 76.4 mm. In May, 9.2% of the scallops cultured under the most favorable conditions (i.e., 12 scallops/cage^{-1} at 10 m depth) had reached commercial size. (ANOVA; Table 2).

TABLE 2.

Effect of depth (6 and 10 m) and stocking density (25, 50, and 100 per cage^{-1}) on the growth of king scallop in O Grove, Ria de Arousa.

Source of Variation	Df	F Ratio	P Value	Newman-Keuls Test
March 2, 1999				
Depth	1	0.04	0.852	D 6 m = D 10 m
Stocking density	2	42.34	0.003*	SD 25 cage^{-1} > 50 cage^{-1} > 100/cage^{-1}
Density × depth	2	3.97	0.080	
April 6, 1999				
Depth	1	1.80	0.229	D 6 m = D 10 m
Stocking density	2	14.57	0.005*	SD 25cage^{-1} > 50 cage^{-1} = 100 cage^{-1}
Density × depth	2	1.75	0.252	
May 27, 1999[a]				
Depth	1	8.09	0.047*	D 6 m < D 10 m
Stocking density	1	0.43	0.547	SD 12 cage^{-1} = SD 24 cage^{-1}
Density × depth	1	0.28	0.624	
July 20, 1999				
Depth	1	12.48	0.024*	D 6 m < D 10 m
Stocking density	1	2.44	0.193	SD 12 cage^{-1} = SD 24 cage^{-1}
Density × depth	1	0.11	0.757	
September 28, 1999				
Depth	1	5.77	0.074	D 6 m = D 10 m
Stocking density	1	9.67	0.036*	SD 12 cage^{-1} > SD 24 cage^{-1}
Density × depth	1	0.00	0.996	
February 17, 2000				
Depth	1	5.26	0.084	D 6 m = D 10 m
Stocking density	1	25.69	0.007*	SD 12 cage^{-1} > SD 24 cage^{-1}
Density × depth	1	0.05	0.831	
May 30, 2000				
Depth	1	20.41	0.011*	D 6 m < D 10 m
Stocking density	1	129.58	0.000*	SD 12 cage^{-1} > SD 24 cage^{-1}
Density × depth	1	0.63	0.472	

D, depth; SD, stocking density; DF, degrees of freedom.
[a] After April 6 new stocking densities were used (12 and 24 per cage^{-1}).
* Indicates a significant result. $P < 0.05$.

Fuengirola

Spat culture. Scallops were sampled on November 23, 1998, January 27, 1999, and February 24, 1999. Faster growth rates always were observed at lower stocking densities. The effect of depth was not clear, although growth rates were generally higher at shallower depths (Fig. 6). *A posteriori* analysis of data was not carried out because there was interaction between factors each month. At the end of the experiment, on February 24, 1999, the mean heights of the spat at each stocking density (pooled for the different depths) were 36.7 ± 5.9 (200/cage^{-1}), 42.1 ± 6.6 (100/cage^{-1}), 48.2 ± 5.9 (50/cage^{-1}), and 53.0 ± 6.2 mm (25/cage^{-1}).

Juvenile culture. A new experiment was started in February, using stocking densities of 12 and 24 juveniles/cage^{-1}. The initial mean size was 52.1 ± 5.0 mm. Monthly sampling was carried out on March 23, 1999, April 21, 1999, and finally on May 19, 1999, when the scallops had reached a suitable size for ear-hanging culture (overall mean height 63.9 mm) and the experiment was finished (Fig. 7). Significant differences in size were observed from March onward that were related to stocking density. Growth was not affected by depth (ANOVA; Table 3).

Survival

O Grove

Spat culture. The survival rates between November 1998 and April 1999 were 100% at stocking densities of 25 and 50 spat/cage^{-1} at both depths, and 90% at a stocking density of 100 spat/cage^{-1} at both depths.

Juvenile culture. The survival rate ranged between 73.5% and 87.0% (Table 4). The multifactorial ANOVA revealed interaction between factors, and *a posteriori* analysis was not carried out.

Fuengirola

Spat culture. The survival rate ranged between 91% and 100%.

Juvenile culture. In May, the survival rate ranged between 83.3% and 95.8% (Table 5). There were no significant differences in mortality associated with density or depth.

DISCUSSION

Effect of Depth

In Fuengirola, the environmental conditions showed very little difference at the different depths tested. Possibly because of this,
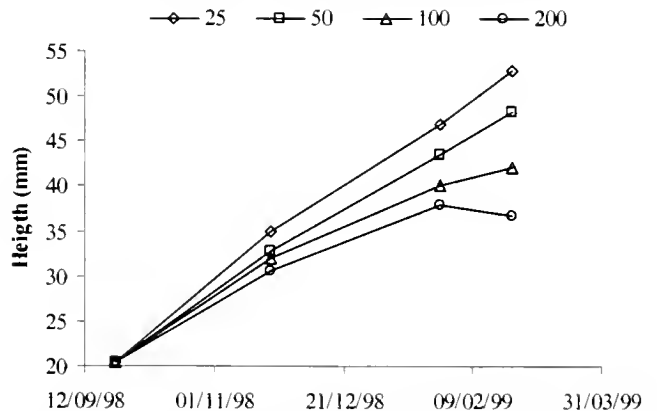
Figure 6. Growth of *P. maximus* spat at four densities, depth pooled, on intermediate culture in Fuengirola.
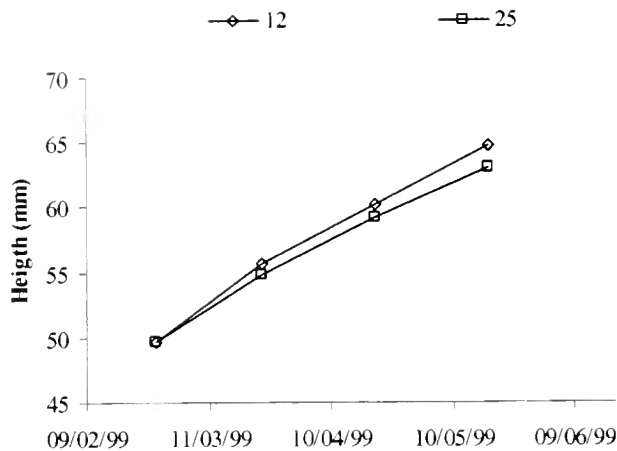
Figure 7. Growth of *P. maximus* juvenile at two densities, depth pooled, on intermediate culture in Fuengirola.

there was very little difference in growth at different depths of either spat or juveniles.

In O Grove, during the period of spat culture no differences were observed in temperature, levels of chlorophyll *a*, or growth at the different depths. However, during the period of juvenile culture in spring and summer of 1999, growth was faster at a depth of 10 m than at 6 m, possibly because of higher levels of chlorophyll *a*

TABLE 3.

Effect of depth (13, 18, 23, and 26 m) and stocking density (25, 50, 100, and 200 per cage[-1]) on the growth of king scallop in Fuengirola, Málaga.

Source of Variation	Df	F Ratio	P Value	Newman-Keuls Test
November 23, 1998				
Stocking density	3	64.06	0.000*	
Depth	3	10.11	0.000*	
Density × depth	9	7.47	0.000*	
January 27, 1999				
Stocking density	3	790.28	0.000*	
Depth	3	331.28	0.000*	
Density × depth	9	24.12	0.000*	
February 24, 1999				
Stocking density	3	1163.68	0.000*	
Depth	3	199.75	0.000*	
Density × depth	9	8.11	0.000*	
March 23, 1999[a]				
Stocking density	1	6.37	0.036*	SD 12 > SD 24
Depth	3	1.35	0.326	
Density × depth	3	0.23	0.876	
April 21, 1999				
Stocking density	1	5.43	0.048*	SD 12 > SD 24
Depth	3	1.05	0.422	
Density × depth	3	1.49	0.289	
May 19, 1999				
Stocking density	1	43.46	0.000*	SD 12 > SD 24
Depth	3	1.13	0.394	
Density × depth	3	3.41	0.074	

D, depth; SD, stocking density.

[a] After February 24, new stocking densities were used (12 and 24 juveniles per cage[-1]).

* Indicates a significant result, P < 0.05.

TABLE 4.

Survival rates of juveniles grown at different stocking densities and depths in O Grove, Ría de Arousa.

Date	6 m		10 m	
	12 juveniles cage[-1]	24 juveniles cage[-1]	12 juveniles cage[-1]	24 juveniles cage[-1]
5/27/1999	99.8	100	100	100
7/20/1999	96.9[a]	100[b]	100[b]	100[b]
9/28/1999	92.7[b]	95.8[b]	100[a]	91.8[b]
2/17/2000	83.3	92.7	93.5	83.7
5/20/2000	78.1[a,b]	84.4[b,c]	87.0[c]	73.5[a]

Presented as %. Values with a common superscript letter do not differ significantly in each month.

at that time, as the temperature varied very little at the different depths. Similar results have been reported by Román et al. (1999) for *Aequipecten opercularis*. The results confirm those of Lodeiros and Himmelman (1994), who observed that in temperate and northern regions food availability is more important than temperature for the growth of pectinids. The results, however, contrast with those of Laing and Utting (2001), who suggest that temperature is the most important factor for the growth of scallop seed in the sea. However, they worked with a wider temperature range (5–23°C) than that recorded in the present study (14–21°C in Fuengirola; 13–18°C in O Grove).

From autumn until the following spring, the rate of growth of the scallops maintained at 6 m was slightly higher than that of scallops cultivated at 10 m, and there were no longer any significant differences. The higher growth rate in the scallops maintained at 6 m may have been partly due to the sharp decrease in levels of chlorophyll *a* at 10 m between September and October, and partly due to compensatory growth. In May 2000, the growth rate of the scallops maintained at 10 m was again higher than that of the scallops maintained at 6 m, although the reasons for this were not clear. The slight decreases in salinity that were registered may have been sufficient to produce differences in the growth rates. Fouling of the cages and of the scallops may be an important factor affecting the growth rate, although no specific study has been carried out to test this.

In O Grove, minimum temperatures (12–13°C) and levels of chlorophyll *a* (1–1.5 µL^{-1}) were registered during the period of spat culture (November 17, 1998, to April 6, 1999). However,

TABLE 5.

Survival rate of spat and juveniles grown at different stocking densities, depths, and dates in Fuengirola, Málaga.

Stocking Density (Spat/Cage)	26 m	23 m	18 m	13 m
Spat, February 1999				
25	100	100	100	92.0 ± 4.0
50	100	94.0 ± 2.0	100	100
100	96.0 ± 2.0	100	98.0 ± 2.0	96.0 ± 2.0
200	94.0 ± 2.0	100	92.0 ± 2.0	91.0 ± 4.0
Juveniles, May 1999				
24	93.8 ± 6.3	92.8 ± 1.0	89.6 ± 2.1	88.5 ± 5.2
12	93.8 ± 2.1	95.8 ± 0.0	83.3 ± 16.7	95.8 ± 4.2

Presented as mean (±SD) %.

growth rates of between 0.131 and 0.089 mm d^{-1} were recorded between November and March, when scallops were not handled, and of only between 0.047 and 0.024 mm d^{-1} between March and April. The decrease in the growth rate may have been caused by stress according to Laing et al. (1999), who observed an increasing level of stress in scallops disturbed monthly during a period when there was little food available for growth. In the previous winter months, undisturbed scallops grew faster, even with lower or similar food availability and temperature levels.

Effect of Stocking Density and Areal Coverage

When spat reached a height of approximately 35 mm, lower growth rates were recorded in both areas at stocking densities of 50/cage^{-1} and higher (corresponding to at least 38% areal coverage).

In O Grove, there were no significant differences in the growth of juveniles (those of initial size range of 39.9–41.4 mm on April 6, 1999), until 6 mo after the start of the experiment (September 28, 1999, height reached 57–65 mm), despite the different stocking densities used (12–24 juveniles/cage^{-1}). However, in Fuengirola, where growth was faster, significant differences in growth rates at different stocking densities were observed in the first month after the start of the cultivation period. Different growth rates were apparent when areal coverage reached 23% and 47%, respectively, for 12 and 24 scallops/cage^{-1} (height, approximately 55 mm).

In O Grove, juveniles scallops maintained at 12/cage^{-1} from September onward showed a significantly higher growth rate than those maintained at 24/cage^{-1}. These densities corresponded to mean pooled areal coverages of 29.7% and 54.6%, respectively. Growth almost ceased between February and May 2000 at both stocking densities, when areal coverage was 39.7% at 12 scallops/cage^{-1}.

For the range of sizes used in this study, *P. maximus* showed low growth rates at areal coverages of between 30% and 40%. This is in accordance with the guidelines for growing scallops in net culture (i.e., that the area of the floor space occupied by scallops should not exceed 33%; Paul et al. 1981). When growing aequipectenoid pectinids (*A. opercularis*, *Placopecten magellanicus*, and *Argopecten irradians*), higher coverage rates can be used, but the same is not true for pectinoid Pectinids. Perhaps because they are not byssus-attached, there is a higher incidence of biting, and they are more affected by sea swell.

Comparison Between Areas

In both regions, there was sustained growth of spat during the winter. From November 1998 until May 1999, growth in the cultures held under the most favorable conditions (i.e., those held at a stocking density of 25/cage^{-1} as spat, and at 12/cage^{-1} as juveniles) was higher in Fuengirola (final mean size 64.7 ± 4.5 mm) than in O Grove (final mean size 52.6 ± 4.1 mm; ($P < 0.001$ by t test). The faster growth rates in Fuengirola may have been due to the higher availability of food (measured as chlorophyll a). Furthermore, the temperature in Fuengirola was higher, as during

most of the experimental period it ranged between 14°C and 16°C, compared with between 12°C and 13°C in O Grove.

In Fuengirola, during the first 2 mo of cultivation (September 24 to November 23, 1999) the spat increased in height from 20.4 to 34.9 mm (mean values), at the same time as the maximum temperature was recorded (21°C). However, in natural populations in Galicia, we have observed the formation of false growth rings between September and October (Román, unpub. results). These rings are associated with an arrest in growth that coincides with the maximum temperatures that occur during the year (~18.5–20°C). It is therefore possible that the scallops in Málaga are genetically adapted to the higher temperatures.

In O Grove, the rate of growth between February and April was very slow; by this date scallops reach the size when juveniles maintained in suspension stop growing, as has been described in other areas. According to Slater (1995), the cultivation of *P. maximus* in baskets or cages is easily carried out until the spat reach a size of 45 mm, but thereafter growth is retarded. Although Cano et al. (2000) obtained scallops of commercial size (100 mm length) after 18 mo of cage culture in Málaga, only a small proportion of the spat culture in O Grove reached commercial size when maintained in cages. In O Grove, the scallops detached from collectors in November and cultivated in cages had grown sufficiently (>60 mm) by the following September to be ear-hung; taking into account that in the latter months of cage culture (until May 2000) growth was very slow, it may be advisable to begin ear-hanging culture in September.

The culture of *P. maximus* in suspension is complicated and is influenced by many factors, not all of which have been thoroughly studied or are well understood. In addition to the factors usually considered, such as food availability, temperature, stocking density, and depth, other factors such as handling frequency and fouling of both shells and cages, and the interactions among these also should be taken into account. This species lives recessed in the sediment and under natural conditions is not usually heavily fouled. However, when grown in suspension the animals are heavily fouled, apparently more than other epifaunal pectinids, such as *A. opercularis* and *Chlamys varia*. The effect of fouling should be studied because, as well as the negative effects (i.e., competition and reduction of water flow), there may be positive effects, such as the prevention or reduction of mobility within the cages, thereby reducing biting and malformations.

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INTRASPECIFIC GENETIC VARIATION IN MITOCHONDRIAL 16S RIBOSOMAL GENE OF ZHIKONG SCALLOP *CHLAMYS FARRERI*

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ABSTRACT A 592 base-pair fragment of the mitochondrial 16S ribosomal gene in 47 Zhikong scallop (*Chlamys farreri*) specimens was sequenced to examine its intraspecific genetic variation and geographic structure. These samples were collected from six populations [four from China, and one each from South Korea (SK) and Japan] across its range. Thirty-one nucleotide positions were found variable, and twenty-three haplotypes were detected in all samples, which showed that more 16S rDNA variation existed in *C. farreri* when compared with several other oyster species. Analysis at the intrapopulation level showed that the SK sample had the richest sequence diversity. However, an analysis of haplotype frequency distribution and analysis of molecular variance indicated that little geographic structure was present among all samples, and an absolute majority (99.65%) of the genetic variation was distributed within populations, suggesting that the populations in this study may belong to a single panmictic unit. A relatively smaller distribution range and various currents may account for sufficient gene flow among these populations for this benthic species.

KEY WORDS: *Chlamys farreri*, genetic variation, geographic structure 16S rRNA gene, scallop

INTRODUCTION

Distributed mainly along the coast of northern China, North Korea, South Korea (SK), and Japan (JP), the Zhikong scallop, *Chlamys farreri*, has been one of the major species of the shellfish aquaculture industry on the northern coast of China for several decades (Qi 1989, Wang et al. 1993). This species comprises about 75 to 80% of the total production of scallops in China (other species include bay scallop *Argopecten irradians*, Japanese scallop *Patinopecten yessoensis*, and *Chlamys nobilis*). In 1996, some 780,000 metric tons of the scallop *C. farreri* was produced in China (Guo et al. 1999). In recent years, however, scallop culture has been haunted by a high mortality problem. Mortality rates varied from 20% to as high as 80% at a variety of areas in late summer and early fall before harvest season. It is believed that the problem was caused by a combination of overcrowding, high summer temperature, and deteriorating water quality. Additionally, one more possible reason for the problem is that, to some extent, the scallop stock may be deteriorating genetically. This is possible because, although collected from the wild, most of the scallop seed was primarily collected from restricted waters (Changdao, Yantai district, Shandong province) where the wild population is believed to have originated from hatchery production from the late 1970s to the early 1980s (Guo et al. 1999). For this reason, refreshing the scallop stock by introducing new stocks from other populations outside the coast of north China was considered. Consequently, investigation and evaluation of its stock structure throughout its geographic range are required.

It has been proposed that benthic marine species with pelagic larvae have population genetic structures reflecting the dispersal capacity of larvae. Most of them are thought to have little genetic structure. Considerable work has been conducted to test the hypothesis on many species, including oysters (Buroker 1983, Reeb & Avise 1990, Karl & Avise 1992, Small & Chapman 1997), mussels (Karakousis & Skibinski 1992, Geller et al. 1993), scallop (Wilbur et al. 1997), gasptropods (Hoskin 1997, Kyle & Boulding 2000), abalone (Huang 2000), and many other invertebrates with planktonic larvae (Palumbi & Wilson 1990, Arndt & Smith 1998, Schizas et al.1999). Mitochondrial DNA sequences (including 16S rDNA) were used for many of these studies. In the scallop *C. farreri*, this hypothesis has almost never been checked. The intraspecific genetic variation of *C. farreri* was investigated using allozyme frequency data with five populations along the northern coast of China (Zhang & Zhang 1997), but the question of whether significant geographic structure exists was not directly answered. Moreover, as the results of other researchers have shown, when different genetic systems are used in the same species, the resulting population genetic structures may differ (Karl & Avise 1992). So, this also led to our interest in examining the genetic structure of the population using mitochondrial gene sequence data with samples from the geographic range of the species.

MATERIALS AND METHODS

Sampling and Polymerase Chain Reaction Amplifications

Scallop *C. farreri* samples were collected by scuba divers or were dredged from Daliang (DL), Changdao, Yantai (YT), Rongcheng (RC), and Qingdao (QD) along the northern coast of China. Samples from SK and JP were obtained through commer-

Figure 1. A map of the sampling area for the Zhikong scallop *C. farreri*. The numbers 1, 2, 3, 4, 5, and 6 represent, respectively, DL, YT, RC, QD, SK, and JP.

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TABLE 1.

The polymorphic sites and haplotype frequencies of 16S rRNA gene in Zhikong scallop *C. farreri*.

Haplotype	8	114	23	33	63	82	160	165	166	176	247	260	286	289	312	319	325	327	329	342	350	351	360	365	403	405	419	446	517	553	DL	YT	QD	SK	JP	RC	Total
A	T	A	A	C	G	G	A	A	A	A	C	G	T	C	T	C	A	G	A	A	T	G	T	T	G	C	C	C	G	G	4	1	3	1	2	3	14
B	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	A	.	.	.	.	.	.	.	.	.	.	.	.	.	.	2	1	1	1	2	1	8
C	.	.	.	.	.	.	.	.	.	.	T	.	.	.	.	A	.	.	.	.	.	.	.	.	.	.	.	.	.	.	1	1	—	—	—	1	3
D	.	.	.	.	.	.	.	.	.	.	T	.	.	.	.	A	.	.	.	.	.	.	.	.	.	.	.	.	.	.	1	—	—	—	—	—	1
E	.	.	.	.	.	.	.	.	.	.	.	.	.	C	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	—	1	—	—	—	—	1
F	.	.	.	.	.	.	.	G	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	—	1	—	—	—	—	1
G	.	.	.	.	.	.	.	.	.	G	.	.	.	.	G	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	—	1	—	—	—	—	1
H	.	.	.	.	.	.	.	.	.	.	.	.	.	T	.	.	.	.	.	.	.	.	.	.	.	.	.	G	.	.	—	—	1	—	—	—	1
I	.	.	.	.	.	.	.	.	T	.	.	.	.	.	.	.	.	.	.	.	T	T	.	.	.	.	.	.	.	.	—	1	1	—	—	—	1
J	.	.	.	.	.	.	.	.	.	.	.	C	.	.	.	.	.	.	.	.	.	T	.	.	.	.	.	.	.	.	—	—	1	—	—	—	1
K	.	A	A	.	.	A	.	.	.	.	.	.	.	.	.	.	.	.	.	.	C	.	.	.	.	.	.	.	.	.	—	—	1	—	—	—	1
L	C	.	.	.	.	.	G	.	G	.	.	.	.	.	.	.	.	.	.	T	.	.	.	.	.	.	.	.	.	.	—	—	1	—	—	—	1
M	.	.	.	T	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	—	—	—	—	1	1	2
N	.	G	.	.	A	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	C	.	.	.	C	.	.	.	.	.	—	—	—	1	—	1	2
O	.	.	A	A	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	C	.	.	A	.	.	.	.	—	—	—	1	—	—	1
P	.	.	.	.	A	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	T	.	.	.	.	T	.	.	.	.	—	—	—	1	—	—	1
Q	.	.	.	.	.	.	.	.	.	.	.	.	G	T	.	.	.	.	.	.	.	.	C	C	.	.	.	.	.	.	—	—	—	1	—	—	1
R	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	C	.	C	.	.	.	.	.	—	—	—	1	—	—	1
S	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	C	.	.	A	.	.	T	—	—	—	—	1	—	1
T	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	T	T	—	—	—	—	1	—	1
U	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	A	.	.	.	.	.	.	.	.	.	.	.	.	.	—	—	—	—	1	—	1
V	G	.	G	.	.	.	G	.	.	.	.	.	.	.	.	.	.	.	G	.	.	.	.	.	.	.	.	.	.	.	—	—	—	—	—	1	1
W	G	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	—	—	—	—	—	1	1

Variable Nucleotide Sites   Haplotype Frequency

TABLE 2.

The sequence indices of intrapopulation level of 16S rRNA gene in Zhikong scallop *C. farreri*.

	DL	YT	RC	QD	SK	JP
No. polymorphic sites	3	8	7	7	14	6
No. haplotypes	4	7	6	6	8	6
Haplotype diversity	0.750	1.000	0.893	0.893	1.000	0.929
Nucleotide diversity	0.00175	0.00418	0.00296	0.00326	0.00591	0.00314
Average No. nucleotide differences	1.0357	2.4762	1.7500	1.9286	3.5000	1.8571

cial catch practice in Gunsan, SK, and in Kanazawa, JP, respectively (Fig. 1).

Total DNA was extracted from adductor muscle tissue using a standard phenol/chloroform method (Sambrook et al. 1989). 16S fragments of the 16S rDNA were amplified using a pair of universal primers: 16sar-L/16sbr-H: 5'-CGCCTGTTTATCAAAAA-CAT-3'/5'-CCGGTCTGAACTCAGATCACGT-3' (Palumbi 1991).

Amplification of the products was performed using a PTC-100 thermal cycler (MJ Research, USA). The 100-µL amplification reaction contained the following: 2.0 mM MgCl₂; 200 µM each dNTP; 0.2 µM each primer; 2.5 µL of template DNA; 2.5 units of *Taq* polymerase (Sangon, China) with supplied buffer. For all amplifications, a hot-start polymerase chain reaction (PCR) was initiated by the addition of polymerase and primers following an initial 2-min denaturization at 80°C. The PCR cycling profile was as follows: 35 cycles at 94°C for 45 sec, 50°C for 1 min, and at 72°C for 1 min; with a final extension at 72°C for 7 min.

Figure 2. A median network diagram elucidating the relationship of the 23 haplotypes of the 16S rRNA gene in the Zhikong scallop *C. farreri*. Haplotype codes are defined in Table 1.

Sequencing

PCR products were purified using UNIQ-5 Column PCR Product Purification Kit (Sangon, China), were ligated into pMD18-T vector by following the instruction of the Takara DNA Ligation Kit, version 2 (Takara, Japan), and were used to transform competent JM109 *Escherichia coli* cells using standard protocols. Recombinant colonies were identified by blue-white screening. Inserts of the correct size were detected via restriction enzyme digestion by *EcoR*I and *Hind*III. Vector DNA containing the desired insert was further purified using the Pharmacia EasyPrep Kit (Sweden). Sequencing was performed for both strands of every sample on an ABI PRISM 377XL DNA Sequencer using ABI PRISM BigDye Terminator Cycle Sequencing Ready Reaction Kit with AmpliTaq DNA Polymerase, FS (Perkin Elmer).

Data Analysis

Initially, 16S sequences from individual specimens were aligned with CLUSTAL X (Thompson et al. 1997) and then were assigned a haplotype on the basis of discrete combinations of nucleotide sites. Population-specific haplotypic diversity (Nei & Tajima 1981) and nucleotide diversity (Nei 1987) were quantified, respectively. A haplotype median network diagram describing the relationships of observed haplotypes was built using Network 3.1.1.1 (Röhl 1999, Bandelt et al. 1999). All populations were nested into three groups (China, SK, and JP), respectively, and then analysis of molecular variance was conducted to determine the genetic differentiation of the populations with ARLEQUIN (Schneider et al. 1997). Haplotype frequency distributions also were analyzed by exact test (Raymond & Rousset 1995) with the same software. Genetic differentiation at different hierarchical levels was assessed by Φ statistics (Weir & Cockerham 1984). A pairwise matrix of interpopulation nucleotide divergences (Nei 1987) among all populations was calculated, and it was used to construct an unweighted pair group method with arithmetic mean (UPGMA) phenogram employing the NEIGHBOR program, and the tree was drawn using the DRAWGRAM program in the PHYLIP package (version 3.56C; Felsenstein 1989).

RESULTS

Sequences of the 592-base pair 16S rRNA gene of all 47 specimens were obtained, and 31 nucleotide positions were found variable. Twenty-three haplotypes were detected among all samples, and their frequencies are shown in Table 1. Haplotype A and B were the most common ones and were observed in all populations. Their frequencies were 29.8% and 17.0%, respectively. Haplotype C was shared by three populations (DL, YT, and RC), haplotype M was present only in the SK and JP populations, and haplotype N was observed in both the SK and RC populations. All others were
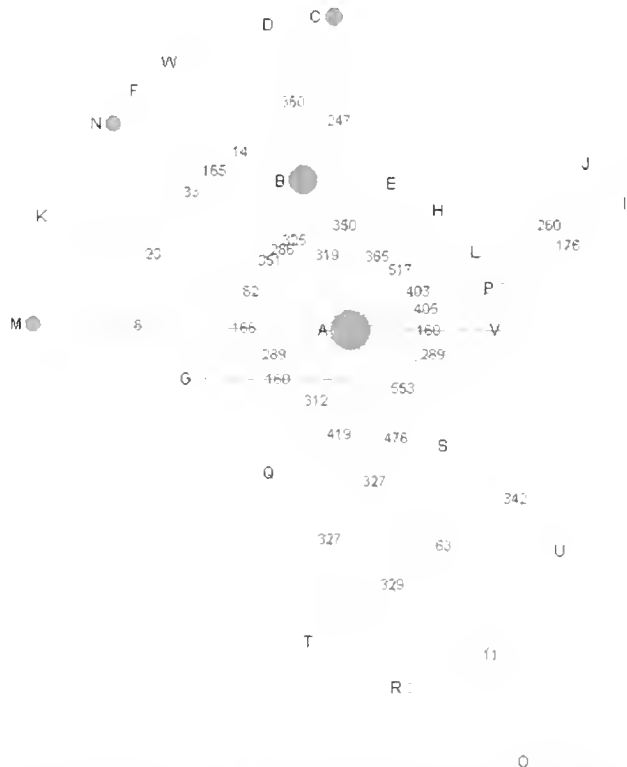
TABLE 3.

Analysis of molecular variance of 16S rRNA gene haplotypes in Zhikong scallop *C. farreri.*

Source of Variation	Df	% Total	Φ Statistics	*P* Value
Among groups	2	2.00	Φ_{CT}: 0.0202	0.195
Among samples within groups	3	0.00 (−1.67)	Φ_{SC}: −0.0171	0.337
Within samples	41	99.67	Φ_{ST}: 0.0035	0.532

Df, degree of freedom.

population-specific haplotypes. Haplotypic diversity, nucleotide diversity, and other population-specific diversity indices are presented in Table 2. For all numbers, SK had the greatest value, with YT the next greatest (also the greatest among the four populations from China), and the DL population has the lowest frequency. Construction of a median network based on nucleotide divergences among the haplotypes detected in this study indicated that most haplotypes were closely related, with the dominant haplotype (A) as the center of radiation (Fig. 2). Many adjacent haplotypes differed from each other by one nucleotide, and some haplotypes were two mutational steps removed from A.

The analysis of haplotype frequency distribution showed that there were no significant differences among all samples ($P = 0.925$), and that there were none between any pairs of samples. The analysis of the partitioning of the haplotype diversity indicated that an absolute majority (99.65%) of the genetic variation was distributed within populations (Table 3). No variance was attributable to differentiation among populations within groups, and <2% could be attributed to variation among different geographic regions, which was not significant ($P = 0.124$).

Interpopulation nucleotide divergences are presented in Table 4. While the greatest value of pairwise divergence among populations was observed between the SK and YT populations, the smallest value was present between the DL and JP populations, which are the most distant population pair in this study. The UPMGA tree generated from these divergence data is shown in Fig. 3. The tree clustered six populations included in this analysis into one major branch that separated DL population from the other five, and connected YT and SK first with other populations joining sequentially in a semi-random pattern.

DISCUSSION

As Table 1 and 2 show, 16S rRNA gene sequences of *C. farreri* presented a reasonable degree of variation, although it is usually a low variation region in mitochondrial genome (Hixson & Brown 1986). The richest variation in YT population among the four populations in China supported the fact that YT has been the center of wild resources of this species in China. Considering this, it is difficult to confirm that the YT stock is deteriorating genetically based upon our results. With 14 polymorphic sites and 8 haplotypes, the SK population showed the richest variation in all six populations, which may support the idea of stock introduction from SK. Zhang et al. (1997) studied genetic variation with five populations from China in this species using allozyme starch gel electrophoresis. Four of these populations were from the same locations as ours (DL, YT, RC, and QD). Their results indicated that YT samples showed highest heterozygosity (observed and expected) among these populations, which matched our result from the 16S sequence data.

When compared with oysters, the sequences of the scallop *C. farreri* 16S rRNA gene seems more variable. In a 400-nucleotide (nt) 16S rDNA sequence of *Crassostrea gigas* and *Crassostrea sikamea*, Banks et al. (1993) did not detect any polymorphism from nine individuals. O'Foighil et al. (1995) found that both five *C. gigas* and five *Crassostrea ariakensis* exhibited no variation in a 443-nt 16S rDNA sequence, and only two nucleotide sites showed polymorphisms among 20 specimens in *Crassostrea virginica* (five haplotypes). Similarly, just one, one, two, and two haplotypes, respectively, were observed in the same 443-nt 16S rDNA sequence for 8 *C. gigas*, 10 *Crassostrea plicatula*, 7 *C. ariakensis*, and 10 *Crassostrea talienwhanensis* individuals in a recent study (Yu et al. 2003). While a longer sequence (592 nt) of scallop *C. farreri* was examined in this study than that of oysters (400 or 443 nt) may account for part of the reason, species difference at the degree of sequence variation should be the greater part of the explanation.

Although some degree of variation was observed in populations of *C. farreri*, the results of a statistical analysis of 16S rDNA haplotypes indicated that little geographic structure was present among populations and regions. This lack of significant divergence implied that there has been sufficient gene flow among these populations. It was supported by estimated rates of migration ($N_e m$) ranging from 15 to infinity per generation among populations. Further evidence of the lack of population divergence is also indicated by Table 1 and Fig. 2, which show that the most common haplotypes, A and B, were shared by all populations, and that

TABLE 4.

Sequence divergences at interpopulation level of six populations of Zhikong scallop *C. farreri.*

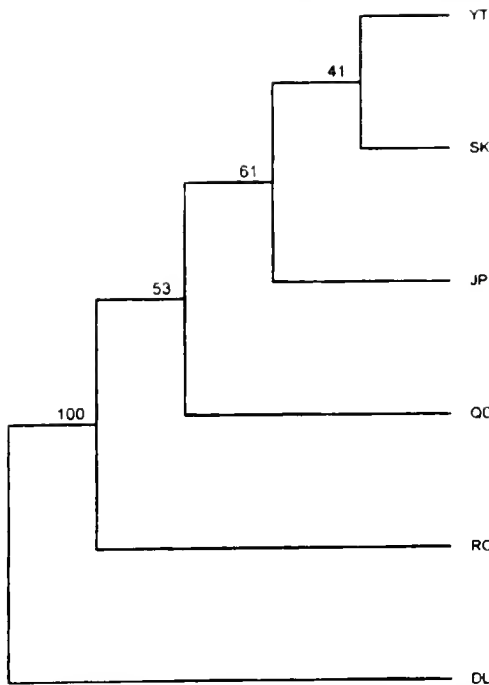|	DL	YT	RC	QD	SK	JP
DL	0					
YT	0.00274	0				
RC	0.00250	0.00348	0			
QD	0.00259	0.00356	0.00332	0		
SK	0.00385	0.00477	0.00440	0.00449	0	
JP	0.00243	0.00350	0.00326	0.00327	0.00443	0

Figure 3. An UPGMA tree constructed with the interpopulation sequence divergences of 16S rRNA gene from six populations in the Zhikong scallop *C. farreri*. Numbers on the branches indicate the percentage of 200 bootstrap replications.

almost all other haplotypes were one or two steps removed from haplotype A. This star phylogeny is generally viewed as a possible sign of an expanding population.

Usually, much less or no genetic structure was found in many marine species with longer periods of planktonic larvae than those with short or no period of planktonic larvae (Hellberg 1996, Hoskin 1997, Arndt & Smith 1998, Kyle & Boulding 2000), with some exceptions. In molluscs, Wilbur et al. (1997) compared a Siberian population with Japanese populations in the Japanese scallop *Patinopecten yessoensis* using PCR-restriction fragment length polymorphism (RFLP) analysis of three mitochondrial coding regions. They found that there was not a significant variation of restriction sites between these two regions, but haplotype frequency distributions were found to be significantly different between regions. The Sea of Japan and the prevailing current patterns between these populations were considered to be obstacles to gene flow.

However, using the RFLP analysis of mitochondrial (mt) 16S rDNA, Small and Chapman (1997) did not detect any significant population structure for *C. virginica* sampling along the Atlantic coast to the Gulf of Mexico, where a distinct genetic break along the Florida coast was found by Reeb and Avise (1990) with RFLP analysis of the whole mtDNA, and by Karl and Avise (1992) with RFLPs of a few anonymous single-copy nuclear DNA sequences. So, it was concluded that the restriction sites within 16S rRNA

gene were more conserved than other sites and other regions in the mt genome of the species. We conducted restriction analyses of all 23 haplotypes with the set of 11 restriction endonucleases used in the study of Wilbur et al. (1997) through WEBCUTTER 2.0 (http://www.firstmarket.com/cutter/cut2.html) and found that 4 of 16 restriction sites were among the 31 variable sites. The proportion of polymorphic sites among all sites detected by these enzymes was 5.88% (4 of 68 sites), and that of sites detected by sequencing was 5.24% (31 of 592 sites). It seems that restriction sites are not necessarily more conserved than other sites within this gene in Zhikong scallop *C. farreri*.

Compared with the American oyster, *C. farreri* has a smaller geographic range of distribution. Although collected from China, SK, and JP, the sampling region actually is not very large. The distances among the four samples next to each other in China are <250 km, the RC and SK populations are around 400 km away from each other, and only the JP is about 800 km from the SK population. With the species having pelagic larvae for a few weeks and with the various currents flowing among these regions, gene flow among these populations seems not to be significantly blocked in this relatively smaller range. It is difficult to deny that these populations in this study belong to a single panmictic unit.

Some stocks from SK have been introduced into a few commercial hatcheries in China. Initial efforts to produce seeds and grow-out have been made. Faster growth rates and slightly lowers mortality rates than those from China were observed (Dr. L. Song, pers. Comm.). However, caution should be taken when comparisons are made between these data and those from other stocks in China, because it is very possible that the SK stock and their resultant seeds received better care or grew in better culture conditions (e.g., better nursery of seeds, lower density for the culture, and better culture area provided) during the culture period. This was normally the case for introduced stocks. Therefore, we may not be able to say that the SK stock is not included in the same panmictic unit.

Because of the nature of maternal inheritance and the ability to reveal sequence variation to the highest degree, mitochondrial DNA sequencing usually may not require as large a sample size as other mitochondrial/nuclear techniques [e.g., PCR-RFLP, PCR-SSCP, allozyme, microsatellites, and amplified fragment length polymorphism (AFLP)] of sequence variation do (a sample size as small as 10 can also clearly detect genetic structure with nuclear markers, as shown in Huang et al. 2000). However, since more haplotypes were detected in this study when compared with oysters, it may give a clearer picture of the stock structure in *C. farreri* if the sampling size is somewhat larger.

ACKNOWLEDGMENTS

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PERKINSUS SP. INFECTION RISK FOR MANILA CLAMS, VENERUPIS PHILIPPINARUM (A. ADAMS AND REEVE, 1850) ON THE PACIFIC COAST OF NORTH AND CENTRAL AMERICA

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ABSTRACT Manila clams (*Venerupis philippinarum*, A. Adams and Reeve 1850) are an important aquaculture species on the west coast of North America and are also cultured in Europe, Asia, and other locations. Clams cultured on the west coast of North America are free of *Perkinsus* sp. infections, while clams from certain Asian and European sources are infected. Infection in Korean Manila clams is reportedly associated with high morbidity and mortality. We evaluated the health status of readily accessible Manila clam juveniles from Korea that were proposed for importation into Mexican waters where they would increase in size, and then be shipped into the United States, either to market destinations or to receiving waters. The examination of the clams was performed as a preliminary assessment for a producer considering the importation of Korean Manila clams. We report finding a high prevalence of a *Perkinsus* sp. causing significant tissue damage in juvenile Korean Manila clams. Parasite taxonomic verification was made using a genus-*Perkinsus* SSUrRNA gene-specific DNA probe for *in situ* hybridization. The use of this probe is validated and reported for the first time. As a result of this finding, no importation of this clam stock took place. It is urgently important to make widely known the risk of the spread of this disease into the clam stocks of the west coast of North and Central America to prevent such an introduction. In addition, we report new information regarding the prevalence and intensity of this disease in juvenile clams available for export, as well as pathologic features of the disease.

KEY WORDS: Venerupis (Tapes) philippinarum, juvenile clam infection, *Perkinsus* sp., DNA probe, *in situ* hybridization

INTRODUCTION

Manila clams (*Venerupis philippinarum*, A. Adams and Reeve 1850) are an important aquaculture species on the west coast of North America. More than 7 million pounds of littleneck clams, predominantly *V. philippinarum*, were produced in Washington, California, and Oregon in 2000 (Pacific Coast Shellfish Growers Association 2003), and additional production occurs in British Columbia, Canada. Although Alaska produces native littleneck clams, *Protothaca staminea* (Conrad 1837), Manila clams are exotic, and importation for aquaculture purposes is prohibited. *Venerupis philippinarum* is also an important aquaculture species in Europe and Asia, and is infected with *Perkinsus* sp. on both continents. Specifically, *Perkinsus atlanticus* occurs in Europe (Navas et al. 1992), a *P. atlanticus*-like parasite occurs in Japan (Hamaguchi et al. 1998), and *Perkinsus* sp. occurs in Korea (Choi & Park 1997) and China (Liang et al. 2001). Consistent with the close homology noted between DNA sequences at several *P. atlanticus* and *Perkinsus olseni* loci by diverse investigators, Murrell et al. (2002) assert these parasitic species to be synonymous, with taxonomic priority to the *P. olseni* name.

In contrast, clams from the west coast of North America are free of *Perkinsus* sp. infections. A survey of Manila clam health and conditions on the west coast of North America (Pacific Shellfish Institute 2001), and the required examination of over 3000 clams for health certifications from 1991 to 2002, showed no evidence of *Perkinsus* sp. infection. Moreover, such infections have not been reported elsewhere on the west coast during routine annual examinations and frequent health examinations of brood stocks and seed clams since 1985. In addition, *Perkinsus* sp. infection has not been reported in the native littleneck clam *P. staminea* or any other bivalve species from the west coasts of North or Central America.

Manila clams may be imported as a live market product from Korea, Japan, or other Asian countries into North America. In 1998, we evaluated the health status of juvenile Manila clams from Korea that had been proposed for importation into Mexican waters, where they would gain size before shipment to the United States, either to market destinations or to receiving waters for further grow out. The examination of clams was performed as a preliminary assessment for a producer considering the importation of Korean Manila clams. We report the finding of a high prevalence of a *Perkinsus* sp. causing significant tissue damage in juvenile Korean Manila clams.

As a result of this finding, no importation of this clam stock took place. It is urgently important to make widely known the risk of the spread of this disease to west coast North American clam stocks to prevent the introduction of this debilitating and lethal clam parasite. In addition, we report here new information regarding the prevalence and intensity of this disease in juvenile clams that are available for export, as well as pathologic features of the disease. Finally, a novel genus-*Perkinsus* DNA probe for *in situ* hybridization (ISH) assays on histologic samples is described.

Taxonomic references to the Manila clam (also commonly referred to as the Japanese littleneck clam) in the scientific literature are particularly confusing. We have designated the species as *V. philippinarum* in accordance with the Committee on Scientific and Vernacular Names of Molluscs within the Council of Systematic Malacologists, American Malacological Union (American Fisheries Society 1998). The common name Manila clam is also found in the literature, apparently in reference to the same species, associated with scientific designations of *Tapes philippinarum*, *Ruditapes philippinarum*, *Tapes semidecussatus*, and *Tapes japonica*.

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TABLE 1.

ISH assay results with genus-*Perkinsus* SSUrRNA probe, Perksp700DIG.

Parasite	Host	Sample	± Probe Hybridization	Sample Source	Reference
Perkinsus sp.	*V. philippinarum*	98-SH14-5		R. A. Elston	this article
Perkinsus sp.	*V. philippinarum*	98051504-2		Y. Maeno	Maeno et al. 1999
P. atlanticus	*R. decussatus*	685a		C. Azevedo	Azevedo 1989
P. olseni	*H. laevigata*	ST389-35		C. L. Goggin	Goggin et al. 1989
P. chesapeaki	*M. arenaria*	CHBRMa-14		C. Dungan	Dungan et al. 2002
P. andrewsi	*M. balthica*	MB3a2		F. G. Kern	Coss et al. 2001
P. marinus	*C. virginica*	221, 556-15		K. S. Reece	Mackin et al. 1950
P. mediterraneus	*O. edulis*	08 and 016		A. Villalba	Casas et al. in press
Perkinsus sp.	*C. pacificus*	CH02882		C. L. Goggin	Goggin et al. 1989
P. qugwadi	*P. yessoensis*	6492-A5	−	S. M. Bower	Blackbourne et al. 1998
Haplosporidium nelsoni	*C. virginica*	201, 239	−	E. Burreson	Haskin et al. 1966
H. costale	*C. virginica*	196, 774	−	E. Burreson	Couch 1967
haplosporidian-like sp.	*P. platyceros*	90-568J	−	S. M. Bower	Bower & Meyer 2002
Hematodinium sp.	*C. sapidus*	98-513	−	J. D. Shields	Shields 1994
Hematodinium sp.	*N. norvegicus*	990427Nnor-1	−	G. Stentiford	Field & Appleton 1995

MATERIALS AND METHODS

A total of 64 Manila clams [16–32 mm shell length (SL)] from Inchon Bay, South Korea, were clinically examined in February 1998 and were fixed whole in Davidson's shellfish fixative (Shaw & Battle 1957). These tissues were processed for routine histologic examination.

A representative tissue section containing parasites was evaluated by ISH. The genus-*Perkinsus* DNA probe was designed to specifically target SSU rRNA sequences of *Perkinsus* species by aligning the available SSU rRNA gene sequences, while not hybridizing to the sequences of closely related parasite taxa including dinoflagellates and apicomplexans. An SSU rRNA gene sequence is not available for *Perkinsus qugwadi*. The resulting probe Perksp700DIG (5′-CGCACAGTTAAGTRCGTGRGCACG-3′) was 5′ end-labeled with digoxigenin (Sigma-Genosys, The Woodlands, TX). ISH assays were performed as previously described (Stokes & Burreson 1995, Stokes & Burreson 2001), except that 125 μg/mL pronase was used for permeablization, instead of proteinase K, for a 30-min digestion, and a probe concentration of 7 ng/μl was used for hybridization. The probe was tested on an array of *Perkinsus* sp.-infected, paraffin-embedded tissues (Table 1), including *Perkinsus marinus* in *Crassostrea virginica*, *P. atlanti-*

cus in *Ruditapes decussatus*, *P. olseni* in *Haliotis laevigata*, *Perkinsus andrewsi* in *Macoma balthica*, *Perkinsus* sp. in *Vereurupis philippinarum* from Japan, *Perkinsus chesapeaki* in *Mya arenaria*, *Perkinsus mediterraneus* n. sp. in *Ostrea edulis* (Casas et al. in press), *Perkinsus* sp. in *Chama pacificus*, and *P. qugwadi* in *Patinopecten yessoensis*. Probe specificity was validated by testing tissue sections of the blue crab *Callinectes sapidus*, which was infected with the parasitic dinoflagellate *Hematodinium* sp. (Shields 1994), *Hematodinium* sp.-infected Norway lobster *Nephrops norvegicus* (Field & Appleton 1995), *Haplosporidum nelsoni*-infected and *Haplosporidum costale*-infected *C. virginica* oysters, and spot prawn *Pandalus platyceros*, infected by an undescribed haplosporidian-like protozoan parasite (Bower & Meyer 2002). Replicate sections of nonspecific ISH assay signal controls of each sample were tested identically, except that they received hybridization buffer without probe during the overnight hybridization step.

RESULTS

Histologic Evaluation of Infected Clams

The prevalence of juvenile clams infected with the presumptive *Perkinsus* sp., was 59 of 64 (92%), based on histologic examination. The protozoa were systemically distributed in a variety of organs, most typically in subepithelial areas of the gills, and fre-
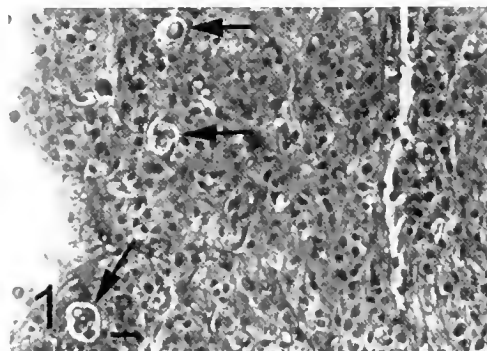
Figure 1. Gill tissue of a juvenile Korean Manila clam infected with *Perkinsus* sp. (arrows). Note the dense cellularity (hemocytosis) in the vicinity of the parasites. Bar, 10 μm, H&E.

Figure 2. Higher magnification of a cyst of *Perkinsus* sp. trophozoites in the gill tissue of the Manila clam (arrow). Bar, 10 μm, H&E.
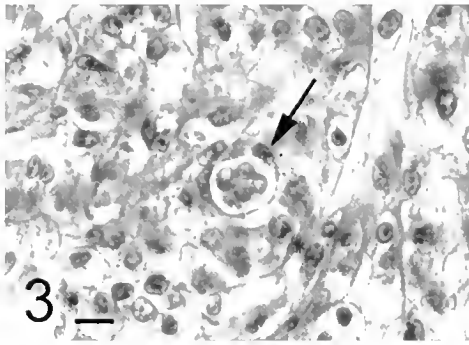
Figure 3. Cyst of *Perkinsus* sp. trophozoites encapsulated by a hemocyte within the gill of a Manila clam. Bar, 10 μm, H&E.

quently in the mantle and labial palps. Parasites were often associated with tissue hemocytosis (Fig. 1) and occurred as single or multiple trophozoites (Fig. 2). In severe infections, the parasites were more abundantly distributed in the tissues, including the vascular sinuses around the digestive diverticula. Broad areas of the subepithelial connective tissues were composed of solid masses of parasite cysts in the most severe infections. In many cases, the parasites were contained within a thin-walled cyst formed by one to several host cells (Fig. 3). Such encapsulations contained up to

10 protozoan cells and associated hemocytosis. The parasites were often characterized by the presence of an eccentric vacuole (Fig. 1 and 3), characteristic of *Perkinsus* sp. trophozoites.

Confirmation of Perkinsus sp. by ISH

The genus-*Perkinsus* SSUrRNA gene probe Perksp700DIG demonstrated strong hybridization to *Perkinsus* sp. cells in all of the tissue sections, except those of *P. qugwadi* infecting *P. yessoensis* (Table 1 and Fig. 4A–I). No hybridization to parasite cells of other genera was observed. ISH of parasite cells in tissue sections of infected Korean Manila clams with this genus-*Perkinsus* probe confirmed the genus level affiliation of the parasites in our sample of juvenile Korean Manila clams (Fig. 5).

DISCUSSION

We report the confirmation by ISH assays and histology of *Perkinsus* sp. infections in Manila clam seed proposed for the introduction into Mexican waters and the subsequent transport to growout sites on the Pacific coast of the United States. This is the first confirmation by a molecular diagnostic probe of *Perkinsus* sp. infection of Korean Manila clams As a result of these findings, the plan for importation of these clams was rejected by the shellfish producer, and no Korean seed clams were imported to the west coasts of Mexico or the United States. However, the ready avail-
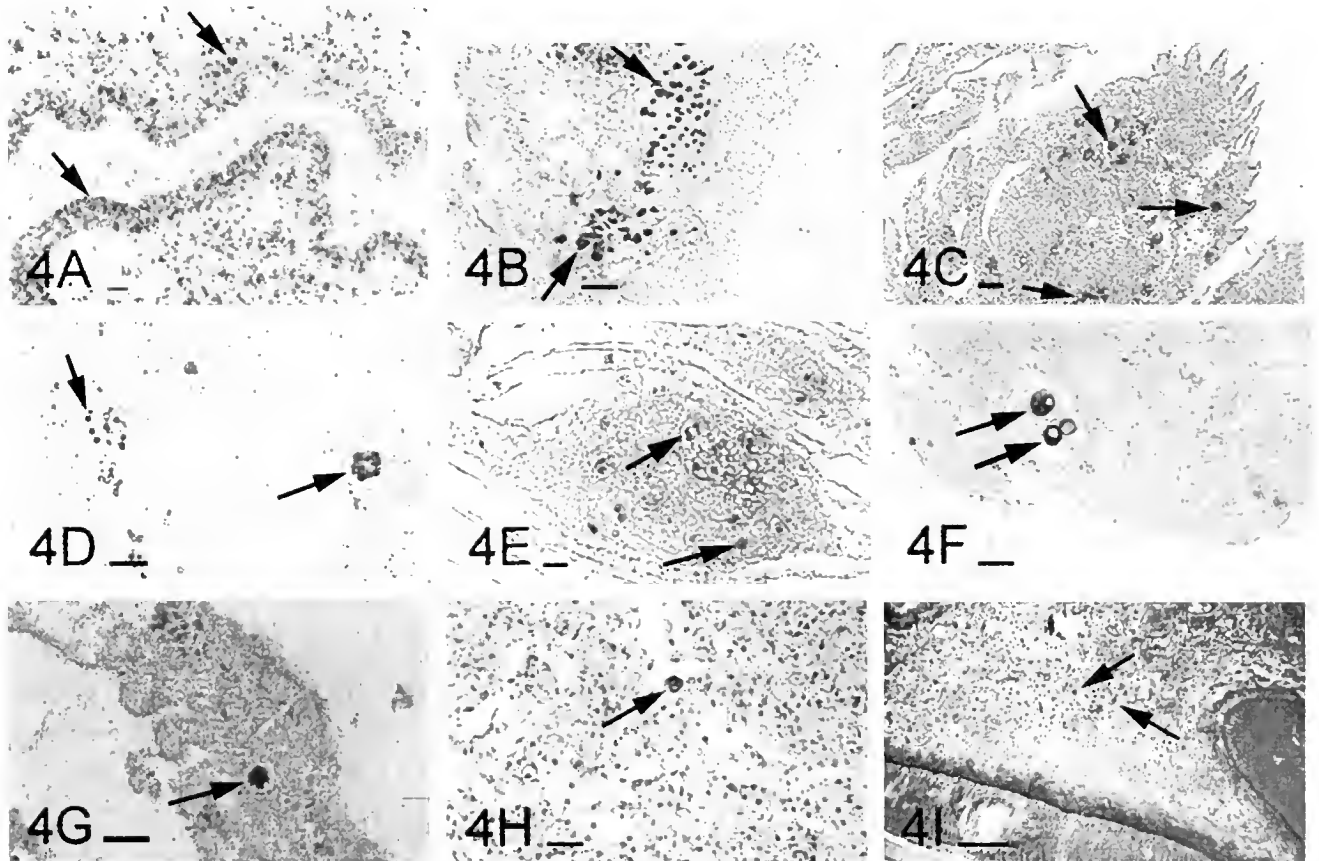
Figure 4. Tissues sections of host tissues reacted with the genus-*Perkinsus* probe Perksp700 by ISH. Positively stained *Perkinsus* sp. parasites are shown by arrows. (A) *P. marinus* in *C. virginica* intestine (bar, 10 μm). (B) *Perkinsus* sp. in *C. pacificus* (bar, 10 μm). (C) *P. atlanticus* in *R. decussatus* (bar, 10 μm). (D) *P. olseni* in *H. laevigata* gill and mantle (bar, 25 μm). (E) *Perkinsus* sp. in *M. balthica* (bar 25 μm). (F) *Perkinsus* sp. in Japanese *V. philippinarum* (bar, 10 μm). (G) *P. chesapeaki* in *M. arenaria* (bar 10 μm). (H) *P. mediterraneus* n. sp. in *O. edulis* (bar, 10 μm). (I) *P. qugwadi* in *P. yessoensis* (no hybridization observed) (bar, 25 μm).
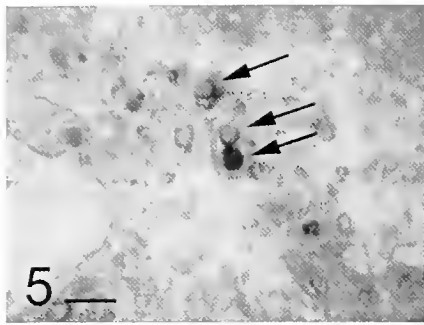
Figure 5. Tissue section of Korean Manila clam reacted with genus-*Perkinsus* probe Perksp700DIG by *ISH*. Positively stained *Perkinsus* sp. parasites are shown by arrows. Bar, 10 μm.

ability of such infected seed clams from Korean or Japanese producers requires vigilance to ensure that no such importations take place into areas that are free of the pathogen, such as the west coasts of North and Central America. Reports of lethal *Perkinsus* sp. infections in European and eastern Asian Manila clams from latitudes as far north as that of northern Oregon, confirm the high likelihood that such infections, if introduced, could persist and be transmitted, with damaging results to both wild and cultured clam stocks along the Pacific coasts of North and Central America.

This study demonstrated that infection prevalence in seed clams ranging from 16 to 32 mm SL can be nearly 100% and that high parasite intensities cause significant histologic damage to the organs of infected clams, particularly the gills.

Choi and Park (1997) studied five species of Korean clams for infections by *Perkinsus* sp. using Ray's fluid thioglycollate medium (Ray 1966) and found infected Manila clams along the south coast of Korea. While no infection occurred in clams of <15 mm SL, nearly 100% infection prevalence occurred in clams of >20 mm SL. Park et al. (1999) reported mass mortality of Manila clams along the west and south coasts of Korea over a period of several years, which was associated with *Perkinsus* sp. infections. They reported 100% infection prevalence in 142 clams from Komsoe Bay on the west coast of Korea with moderately severe mean parasite intensities of 2.87 based on the infection intensity scale of Choi et al. (1989). A negative correlation was found between the intensity of *Perkinsus* sp. infections and the clam condition index, while clam size was positively correlated with infection intensity.

Maeno et al. (1999) reported *Perkinsus* sp. parasites in Manila clams from an inner bay of the western part of Japan in April 1998, using genus-*Perkinsus*-specific antibodies. These authors concluded that the parasites were *Perkinsus* sp. based on a positive reaction with both single and clustered trophozoites. Hamaguchi et al. (1998) have reported the first detection of *Perkinsus* sp. in Japanese Manila clams. Anecdotal information that we received from the Korean supplier of the seed clams and their Japanese

customers indicated that the Manila clam seed had been transported from the Korean source to Japan for at least 20 y with no unusual mortalities or loss of growth reported. This anecdotal report and the multiple reports of the *Perkinsus* sp. parasite occurring about 1997 or 1998 in Japan and Korea suggest that it could have been a new introduction to the Korean clams, as well as the Japanese clams, at about this time.

Manila clams and other bivalve species from Europe reportedly have been infected with *Perkinsus* sp., as follows: *P. atlanticus* from the Mediterranean coast of Spain (region of the Ebro Delta, Tarragona, Spain) infected *R. philippinarum* (Sagrista et al. 1996); Manila clams from the Lagoon of Venice in northeast Italy infected with a *Perkinsus* sp. (DaRos et al. 1998); and *P. atlanticus* infected the carpet shell clam (*R. decussatus*) from European locations (Ordas et al. 2000). Villalba et al. (2000) reported a significant correlation between the SL of *R. decussatus* and *P. atlanticus* infection intensity. No clams of <20 mm SL were infected, and the highest seasonal parasite intensities occurred in spring and late summer to early autumn.

The relationship of *Perkinsus* sp. in European waters to the *Perkinsus* sp. found in Korea and Japan is unknown at this time. Nonetheless, this and other studies cited in this report indicate the presence of this damaging parasite in Korean and Japanese Manila clams, confirmed first in this study by histology and then definitively by the *Perkinsus* sp.-specific probe presented for the first time in this article. This knowledge can be used to prevent the unintentional introduction of this parasite to west coast of North and Central America. We urge that the science presented in this article be applied by shellfish growers, and by natural resource and conservation managers to prevent such a damaging introduction.

ACKNOWLEDGMENTS

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TOLERANCE AND RESPONSE OF MANILA CLAMS, *VENERUPIS PHILIPPINARUM* (A. ADAMS and REEVE, 1850) TO LOW SALINITY

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ABSTRACT To determine under what conditions winter mortalities of the Manila clam (*Venerupis philippinarum*, A. Adams and Reeve, 1850) might be the result of excessive exposure to low salinities, a series of experiments was conducted. Clams were exposed to various concentrations of salinity to determine their physiologic lower limit of tolerance to salinity concentration, the duration they could withstand lethal or marginal low salinities through the mechanism of shell closure, and diagnostic structural changes in tissues indicative of low salinity exposure. Salinities of ≤ 10 parts per thousand (ppt) were not tolerated in long-term exposures of 13 groups of clams. This lethal low salinity was also confirmed by the exposure of clams with a resection of a portion of shell. A salinity of 12.5 ppt was considered marginal, and various proportions of the different populations were able to tolerate this salinity, while no significant mortality occurred at ≥ 15 ppt. Clams could withstand lethal low salinities of 5 ppt and 10 ppt for between 6 and 8 days, but all populations exposed to lethal low salinities for 14 days and then placed at high ambient salinity (~31 ppt) showed a high cumulative mortality. Clams may not die until several days after exposure to lethal low salinity followed by placement in a recovery tank at their normally tolerated high salinity. We found no significant difference in the responses of several groups of clams to the marginal salinity of 12.5 ppt when exposed at temperatures of 6 C, 12°C, and 18 C. Histologic examination showed that the following sequential changes occurred in the digestive gland in clams exposed to 10 and 12.5 ppt for between 2 and 14 days: loss of granulation of the digestive tubular absorptive cells; swelling of these cells and occlusion of the tubular lumina; and finally the shedding of necrotic tubular epithelium into the digestive gland tubular lumina.

KEY WORDS: *Venerupis (Tapes) philippinarum*, low salinity tolerance, Manila clam

INTRODUCTION

Over 3000 tons of Manila clams (*Venerupis philippinarum*, A. Adams and Reeve, 1850), valued at over $22 million (US dollars), were produced on the west coast of the United States in 2000 (Pacific Coast Shellfish Growers Association 2003). Most production occurs in Washington, but clams are also produced in California, Oregon, and British Columbia, Canada. An unfilled domestic and overseas demand is driving attempts to increase the production of this clam. In addition, a significant Manila clam seed production industry has developed, with production facilities in Washington, Oregon, California, and Hawaii. Native littleneck clams (*Protothaca staminea*, Conrad 1837) are also produced in Washington and Alaska, but production is limited due to a short shelf life, a lower price for the producer, and the preference of consumers for the Manila clam.

One constraint to the growth of the Manila clam industry on the west coast of the United States is the occurrence of sporadic mortality and poor growth due to unknown causes. With some exceptions, mortalities are usually reported between November and March. Freezing damage may be a factor in Manila clam mortalities during the winter (Bower 1992). No highly pathogenic infectious diseases of Manila clams are known to occur on the west coast of North America (Elston et al. 2003).

Clams may be reared in locations near freshwater streams or rivers with occasional high outflows in winter. We therefore suspected that at least some of the reported winter mortality events could be the result of exposure to salinities below the physiologic tolerance of the clam or from exposures to low salinity of duration longer than that for which clams can maintain shell closure. The clams burrow into the substrate, and clam deaths may only be observed at some time after the mortality event. A survey of the

literature revealed limited information on the low-salinity tolerance of juvenile and adult Manila clams (Kim et al. 2001, Kurata 2000, Numaguchi 1998). Therefore, we conducted the studies reported here (1) to determine the lowest salinity at which Manila clams from several populations could survive over an extended time period, (2) to determine the duration of exposure that adult and juvenile clams can survive when exposed to lethal and marginal low salinities, (3) to determine the relationship of water temperature to clam survival at a marginal low salinity, and (4) to determine histologic changes that could be used to diagnose the exposure of clams to low salinity.

Taxonomic references to the Manila clam (also commonly referred to as the Japanese littleneck clam) in the scientific literature are particularly confusing. We have designated it *Venerupis philippinarum* in accordance with the Committee on Scientific and Vernacular Names of Molluscs of the Council of Systematic Malacologists, American Malacological Union (American Fisheries Society 1998). The common name of Manila clam is also found in the literature, and, apparently in reference to the same species, the clam is associated with scientific designations of *Tapes philippinarum*, *Ruditapes philippinarm*, *Tapes semidecussatus*, and, less recently, as *Tapes japonica*.

MATERIALS AND METHODS

Apparatus for Low Salinity Exposure Assessment

We conducted initial salinity exposure experiments in static aerated aquaria over a 3-day period. In these experiments, the clams were not fed. However, the majority of experimental evaluations of low-salinity effects were made in two flowing seawater systems that we designed and built for this purpose, and that were operated at a commercial shellfish hatchery facility in Quilcene, Washington, where the ambient salinity ranged from 29 parts per thousand (ppt) to 32 ppt. These systems provided several flow-

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through tanks capable of holding large numbers of test animals at constant levels of reduced salinities (up to four treatments simultaneously) for extended periods of time. This system was later modified to allow for multiple temperature treatments across a single salinity.

In the initial configuration of this system, sand-filtered seawater and unchlorinated fresh water were pumped into separate headtanks (~200 L) the levels of which were kept constant by standpipes and float valves. A coiled length of vinyl tubing was used as a heat exchanger for the fresh water line to help equalize the temperature of the two water sources. Each of these two tanks fed a manifold fitted with four outlets restricted by variously sized orifices that flowed into mixing tanks. Each mixing tank (~20L) flowed in turn into a treatment tank (~40L) where the test animals were held. Altering the sizes of each orifice feeding into the mixing tanks thereby controlled the salinity of the water within each treatment tank. A continuous flow of mixed algal food species provided by the commercial hatchery production system was introduced into the saline headtank at a rate sufficient to allow excess food in all treatments. Airstones were used in each mixing tank to ensure the adequate mixing of the two water sources and to maintain dissolved oxygen saturation prior to the water being allowed to enter the treatment tanks. Salinity loggers and periodic manual checks were used to track treatment salinity and temperature levels. Overall, the actual salinities varied no more that ±1.0 ppt from target salinities based on logger checks and spot manual checks, with the exception of two instances where actual salinity was 2.2 ppt higher than the target or 1.2 ppt lower than the target. Flow apertures were checked, and any salinity deviations approaching or greater than 1 ppt from target were corrected at least twice per week during experiments.

Method of Testing Clams

Initially, we tried to maintain shell opening by inserting wooden wedges between the valves, but we abandoned this method because the clams usually rejected the wedges, although the method has been used successfully in other species such as *Mytilus edulis* (Shumway 1977). Alternatively in the initial experiment, we cut a wedge-shaped opening in the shell of clams (Fig. 1) to force exposure of tissues to the exposure salinities. While this method appeared to have some utility for determining physiologic tolerance to low salinity levels, it was time-consuming and success
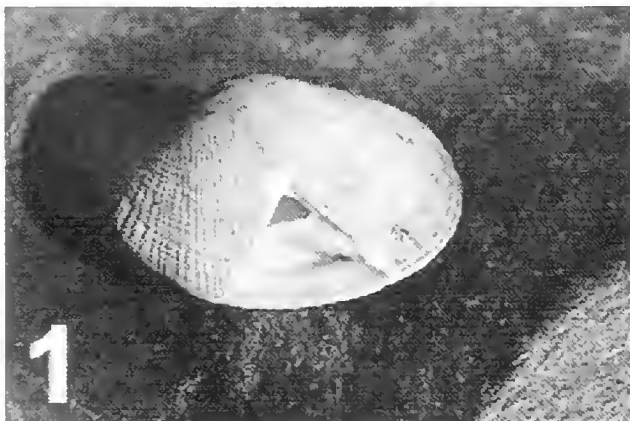
Figure 1. Manila clam with portion of shell resected to isolate the physiologic response to low salinities.

required extensive operator practice to avoid damage to soft tissues. Therefore, we abandoned this method in favor of long-term exposures (4 wk) to evaluate physiologic adaptation or lack thereof to various salinity concentrations.

Clams were obtained from locations in Washington, California, and Hawaii, and were placed in trays in the flowing seawater tanks without sand. Water temperatures were maintained at a constant level within experiments but varied between experiments from 10.0 to 14.0 C, except for the trial in which we tested the effect of temperature on tolerance to a marginal low salinity concentration.

Clams were removed from the experiments and considered dead when their shells gaped and they were unresponsive to probing. Alternatively, clams that were counted as alive when returned to recovery tanks at ambient salinity had active shell adduction and extension of the siphons.

Two experiments were conducted to observe the histologic effects of low-salinity exposures on the gills and digestive gland of clams, two organs that in preliminary experiments appeared sensitive to low-salinity exposure. Adult clams [40–50 mm shell length (SL)] were used in both experiments, which lasted 9 days and 14 days, respectively, with samples collected at the initiation of the study and at 2, 4, 7, 9, and 14 days of exposure to 10 ppt and 12.5 ppt, along with control clams at ambient (~30 ppt) salinity.

We compared the 4-wk mortality rate for the groups containing two replicates (Table 1) using the probability density function for a binomial distribution (Samuels & Witmer 1999). Analysis of variance was not used as it did not meet the requirements for normality and sample size. The probability density function for the binomial distribution is

$$f(x) = \binom{n}{x} p^x (1-p)^x, \; x = 0,1,2,\cdots,n.$$

where n is the number of trials and p is the probability of "success." Applied to the data in Table 1, there are only two outcomes for each clam, dead or alive, with dead clams corresponding to the success of a trial. For example, to test whether there is any significant mortality difference between two locations (e.g., Stoney Point-Willapa and Little Skookum Creek in the 10 ppt treatment), the probability of success for the Stoney Point-Willapa site is estimated as $p_0 = 13/30 = 0.4333$. Our null hypothesis (H_0) is $p = p_0$. And the alternative hypothesis (H_1) is $p > p_0$. The P value for observing $x \geq 21$ is $P(X \geq 21) = 0.0002$, where X is a random variable following a binomial distribution with 30 trials and the probability of success for each trial is 0.4333. Since the P value for testing H_0 vs H_1 is so small, we reject the null hypothesis (at least at a 5% level of significance). This means that there exists a significant difference in the mortality rate at the two different locations. Where applicable, results also were compared using t-tests and determination of 95% confidence intervals for sequential time points in serially sampled experiments.

RESULTS

Measurement of Physiological Tolerance to Low Salinity by Partial Shell Removal

Figure 2 shows the results when clams with a shell wedge removed were exposed to six salinity levels for 3 days in a static aquarium at 10.5°C, followed by a 19-day recovery period in flowing seawater. There was no mortality in either of the control groups (shell cut or intact clams). Although the salinity treatment at 20 ppt

TABLE 1.

Cumulative mortality of Manila clams held in designated salinity concentrations for 4 wk*

Clam Source‡	Replicates	n	Salinity Concentrations Tested† (%)					
			25 ppt	20 ppt	17.5 ppt	15 ppt	12.5 ppt	10 ppt
Oakland Bay	1	30	3	0		0		97
Chelsea ground	1	30	0	0		7		67
Chelsea yearling	1	30	0	0		0		67
California nursery seed clams	1	30			20.0	13.3	66.7	100
Survivors of low-salinity event	1	30			6.7	10.0	16.7	90.0
Hawaii nursery seed clams	1	50			0	6	82	100
Little Skookum Creek	2	15			0	3 ± 4.7	27 ± 9.4	73 ± 4.7
Little Skookum Slough	2	15			0	0	7 ± 0	80 ± 6.7
Chelsea Seafarms Creek	2	15			0	0	27 ± 9.4	80 ± 12.7
Chelsea Seafarms Beach-N	2	15			3% ± 4.7	0	30 ± 4.7	93 ± 4.1
Stoney Point-Willapa	2	15			7% ± 0	7 ± 0	13 ± 5.0	43 ± 2.0
Oakland Bay	2	15			0	0	80 ± 18.9	97 ± 4.7
Thorndyke Bay	2	15			0	17 ± 4.7	7 ± 9.4	97 ± 4.7

* The results are the cumulative mortality rate after 4 wk exposure at the indicated salinity. Further mortality was observed in many groups within 7 days after the 4-wk exposure, when the clams were placed in an ambient (30 ppt) salinity tank. Replicated treatment results are expressed as the average ± SD
† Target salinity concentrations are shown.
‡ All clam sources are from Washington except as noted and, except as noted, are adult clams with 40 to 50 mm SL.

resulted in a cumulative mortality rate of 20% and the salinity treatment at 15 ppt resulted in a 10% loss, these losses were attributed to nontreatment effects. It was clear from this experiment that 5 ppt and 10 ppt were lethal low salinities from which a 3-day exposure resulted in 100% mortality within 17 days postexposure. Most of the clams in these two groups died between 3 and 6 days after removal from the static salinity treatment tanks.

Physiological Tolerance to Low Salinity Measure by Exposures of 4 Week Duration

Table 1 shows that there was relatively little mortality in any group tested at 15 ppt or higher, in comparison with control group mortalities, and no significant differences were found between the tested groups at these higher salinities. At 12.5 ppt salinity, the binomial distribution test showed a significant difference at the 5% level between Stoney Point-Willapa and Little Skookum Creek, and between Chelsea SeaFarms Beach-N and Oakland Bay. In terms of significant difference at the 5% level, the mortality of clams at 12.5 ppt can be split into three groups that are different

Figure 2. Experiment 1 results showing the response of Thorndyke Bay adult clams with shell wedge removed to six salinity concentrations in a 3-day static tank exposure (n = 10 clams per group; 41 ± 1.9 mm mean SL; test temperature 10 to 11°C).

from each other: (1) Thorndyke Bay, Little Skookum Slough, and Stoney Point Willapa; (2) Little Skookum Creek, Chelsea Sea-Farms Creek, and Chelsea SeaFarmsBeach-N; and (3) Oakland Bay. At 10 ppt salinity, there is a significant difference (5% level) in salinity between Stoney Point-Willapa and Little Skookum Creek. Stoney Point-Willapa is significantly different from the rest of the group. At the 5% level of significance for the 10 ppt exposures, the seven locations can be split into three groups, which are significantly different from one another: (1) Stoney Point-Willapa; (2) Little Skookum Creek, Little Skookum Slough, and Chelsea SeaFarms Creek; and (3) Chelsea SeaFarms Beach-N, Thorndyke Bay, and Oakland Bay.

Overall, Table 1 shows that, of the 13 groups of clams, in all but one group very few clams could survive a 4-wk exposure to 10 ppt salinity. However, the ability to survive at a salinity of 12.5 ppt varied greatly between groups of clams. Unlike Oakland Bay clams, adult clams from Thorndyke Bay (Fig. 3) were tolerant to salinity of 12.5 ppt. The highest mortality rate at 15 ppt was 17% and apparently was due either to factors other than salinity or to within-group variation since the mortality rate of the same group of clams (Thorndyke Bay) at 12.5 ppt was only 7%. The clams were removed from the salinity exposures after 4 wk to ambient salinity (~28 ppt) for 1 wk, during which additional mortality occurred at 10 ppt and 12.5 ppt, bringing the mortality rate in all 10 ppt groups to nearly 100%, possibly as a result of partial acclimation to the lower salinity and the inability to readapt quickly to the higher salinity. Only the 4-wk mortality rates are shown in Table 1. The 4-wk exposure of the Thorndyke Bay clams demonstrated that mortality reached nearly 100% after 3 wk of exposure at a salinity of 10 ppt. Figure 3 also shows that differences in mortality rate in clams from the Thorndyke Bay population at 12.5, 15.0, and 17.5 ppt were not statistically significant (P < 0.05).

We sampled two paired groups of clams (Chelsea Seafarms and Little Skookum) from locations near intermittent high-flow streams and paired locations distant from the freshwater sources. Location near the creek outflows did not correspond to lower mortality at 4 wk, and, in fact, the Little Skookum Creek clams ha[
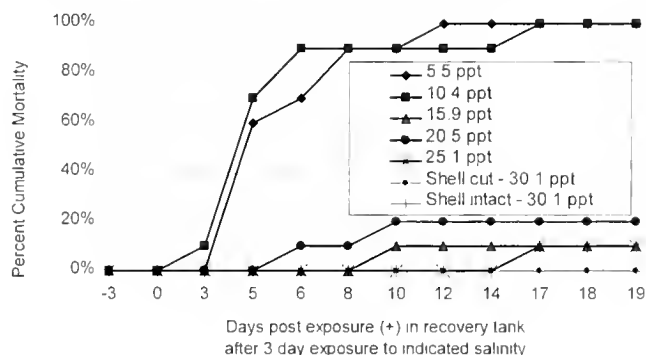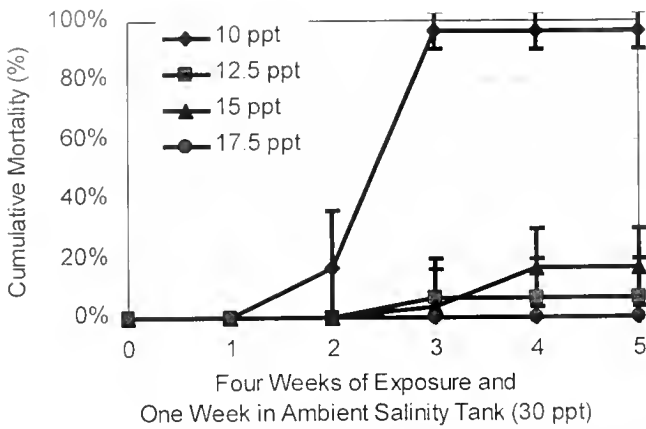
Figure 3. Cumulative mortality of Thorndyke Bay adult clams held at four salinity levels for 4 wk and a fifth week at ambient salinity (~30 ppt). Each treatment was replicated twice with 15 clams in each replicate group (41 ± 1.9 mm SL; vertical bars are 95% confidence intervals; test temperature 11–13 C).

Figure 5. Thorndyke Bay adult clams exposed to the lethal low salinity of 10 ppt for various durations show a graded mortality response that is directly correlated with the duration of exposure and exhibit the ability to survive longer than clams similarly exposed to the lethal low salinity of 5 ppt (Fig. 5) (n = 15 clams per treatment and control at ambient salinity; 41.7 ± 2.9 mm SL; test temperature 10–11 C).

a significantly higher mortality rate than did the Little Skookum Slough clams.

Overall, the results establish that 12.5 ppt is a marginal salinity for most populations of clams, with variable numbers of individuals able to survive this salinity concentration, and some populations, such as the Oakland Bay clams, contained very few clams able to survive at 12.5 ppt.

Duration of Lethal and Marginal Salinities That Can Be Survived

Groups of 15 clams each [41.7 ± 2.9 mm average (±SD) SL] from Thorndyke Bay were exposed to lethal low salinities in flowing seawater of 5 ppt and 10 ppt for intervals ranging from 2 to 14 days and returns to ambient salinity (~28 ppt). The postexposure mortality results (Figs. 4 and 5) showed that at 5 ppt a mortality response occurred in all groups exposed for ≥8 days. The response was graded, but there was nearly a 50% greater mortality rate at 12 days of exposure in comparison to 10 days of exposure. No mortality was seen in clams exposed for 2, 4, or 6 days to 5 ppt. At 10 ppt a mortality response occurred in all groups exposed for ≥10 days, and the response was graded from 10 to 14 days of exposure. No mortality was seen in clams exposed for 2, 4, 6, or 8 days at 10

ppt. The results demonstrate that a population containing a high proportion of marginal (12.5 ppt) salinity-tolerant clams (Table 1) could withstand 5 ppt exposure for 6 days without losses, and exposure to 10 ppt for 8 days without losses.

We conducted two additional experiments to evaluate the duration of tolerance to lethal and marginal salinities. We compared the response of two groups of adult clams to the lethal low salinity concentration of 10 ppt followed by placement in an ambient salinity (~28 ppt) tank. This included one population that showed a low proportion of individuals with tolerance to marginal low salinity (Oakland Bay clams) and another group of clams believed to contain individuals with a high degree of tolerance to low salinity (clams from Totten Inlet, Washington). Clams were exposed for durations of between 1 and 14 days, and were observed over a total of 4 wk, including the time in the low-salinity exposure tank and the ambient salinity (~28 ppt) recovery tank (Figs. 6 and 7). These results indicated that no mortality occurred in Totten Inlet clams held for 7 days at 10 ppt but that an intermediate mortality rate of 40% occurred in Oakland Bay clams held for 7 days at 10 ppt. Nearly 100% mortality occurred in both groups of clams held at 10 ppt for 14 days.

An experiment was conducted using juvenile clams to examine their duration of tolerance to lethal (10 ppt) and marginal (12.5 ppt) low salinities, followed by a return to the ambient salinity. The results showed that when exposed to salinity of 10 ppt (Fig. 8)

Figure 4. Thorndyke Bay adult clams exposed to the lethal low salinity of 5 ppt for various durations show a graded mortality response that is directly correlated with the duration of exposure (n = 15 clams per treatment and control at ambient salinity; 41.7 ± 2.9 mm SL; test temperature 10–11°C).
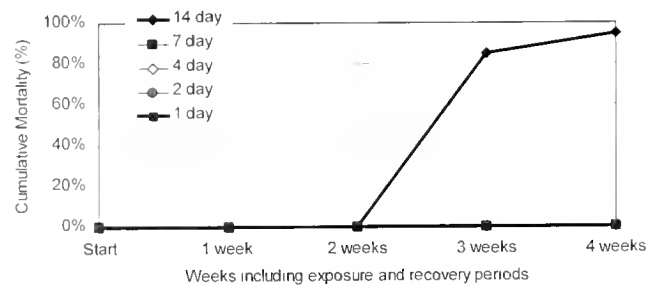
Figure 6. Totten Inlet adult clams exposed to salinity of 10 ppt for intervals ranging from 1 to 14 days followed by a recovery period in ambient (~30 ppt) salinity (n = 20 clams per group; 46.1 ± 5.3 mm SL; test temperature 10–11°C).
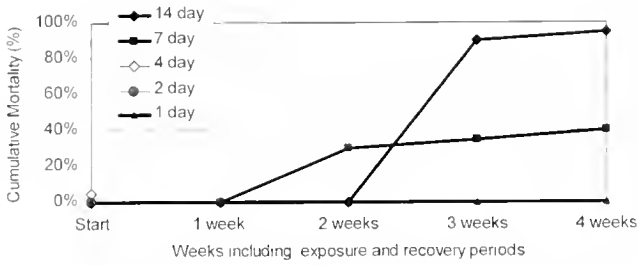
Figure 7. Oakland Bay adult clams exposed to salinity of 10 ppt for intervals ranging from 1 to 14 days followed by a recovery period in ambient (~30 ppt) salinity (n = 20 clams per group; 43.6 ± 3.9 mm SL; test temperature 10–11 C).

there was no significant difference in cumulative mortality between exposures for 1, 2, 4, and 7 days, which all had cumulative mortality rates below 20%, but that a 14-day exposure resulted in about a 90% cumulative mortality rate within 2 wk after placement in the recovery tank. At 12.5 ppt, the mortality responses were similar, but there was higher variability among replicate groups, and the mortality rate was not statistically significantly different between any of the treatment groups. The final mortality rates for all treatments at 12.5 ppt were very similar to those resulting from similar exposures of clams to salinities of 10 ppt. The high variability was not due to the smaller size clam replicate (5 mm SL compared with 13 mm and 14 mm SL) being more sensitive to 12.5 ppt salinity, because one of the larger size groups showed very high mortality compared with the other two groups.

Effect of Temperature on Tolerance to Marginal Salinity

We examined the effect of temperatures from 6 to 18°C on the survival of seed clams exposed to the marginal salinity of 12.5 ppt (Fig. 9) There were no statistically significant differences in cumulative mortality rate over the 4-wk exposure period. Mortality was highest at 4 wk in the 6°C treatment, but this was due to a high cumulative mortality rate in one of three replicate groups. The maximum cumulative mortality rate was 20% over the 4-wk exposure period.

Evaluation of Histological Changes at Lethal and Marginal Salinities

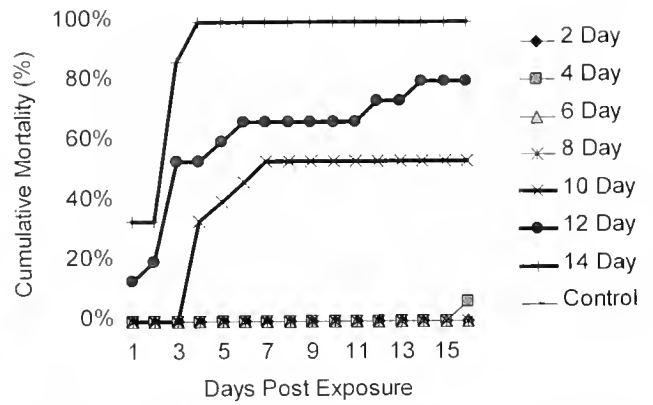Although the histological observations were variable between individuals, clear trends emerged that can be useful in the presumptive diagnosis of low salinity exposure. The following sequential changes occurred in the digestive glands of clams exposed
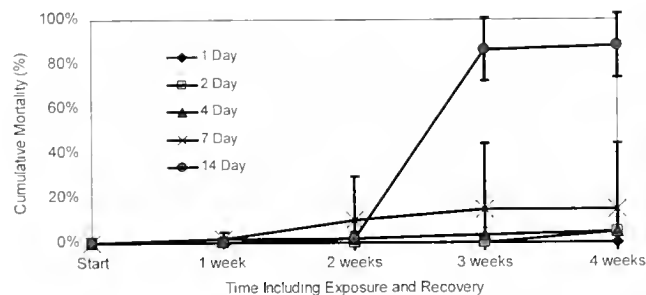
Figure 8. Replicate groups of 20 juvenile clams, each exposed for various durations to lethal salinity concentration of 10 ppt and a recovery period at ambient salinity (~30 ppt). Three seed replicate groups measured 5 ± 1.5 mm, 13 ± 1.3 mm, and 14 ± 1.4 mm SL each (test temperature 10–11 C).
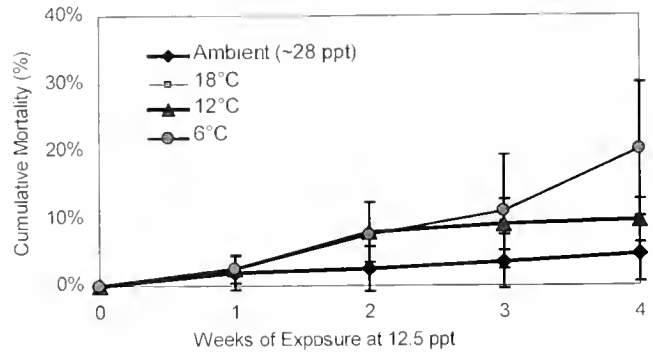
Figure 9. Evaluation of the effect of three temperatures on juvenile clam survival at the marginal salinity of 12.5 ppt. Three clam groups: group 1, two replicates of 22 clams each (5 ± 1.5 mm SL); group 2, three replicates of 25 clams each (13 ± 1.3 mm SL); and group 3, three replicates of 25 clams each (14 ± 1.4 mm SL).

to 10 ppt and 12.5 ppt: loss of granules in the absorptive cells (presumably from lack of feeding) (Figs. 10 and 11); swelling of the absorptive cells of the digestive gland so that the luminal spaces of the terminal digestive tubules became occluded (Fig. 12); and sloughing of absorptive cells of the digestive tubules into the cell lumina where they appeared as necrotic cells and cellular debris (Fig. 13). Loss of granules in the digestive tubular absorptive cells occurred within 2 days of exposure to both 10 ppt and 12.5 ppt salinity. In the shorter experiment, the digestive tubule absorptive cell lumina remained patent and normal-appearing through 7 days of exposure to both 10 ppt and 12.5 ppt, but by 9 days of exposure the luminal spaces were occluded in all clams exposed to 10 ppt salinity, and in about one half of the clams exposed to 12.5 ppt salinity. At 9 days, mild evidence of digestive absorptive cell sloughing was noted in a few clams exposed to 10 ppt only. In the 14-day experiment, using two different groups of
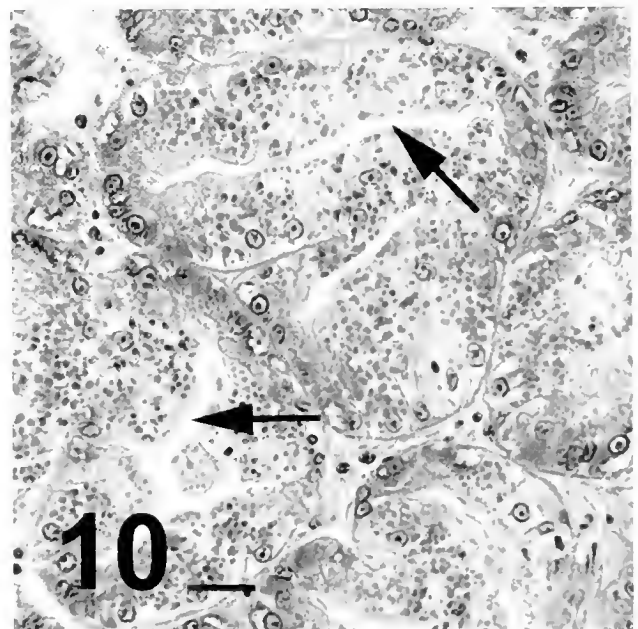
Figure 10. Histological section of normal Manila clam digestive gland showing granules in absorptive cells and open digestive tubular lumina (arrows). Bar, 20 μm, H&E.
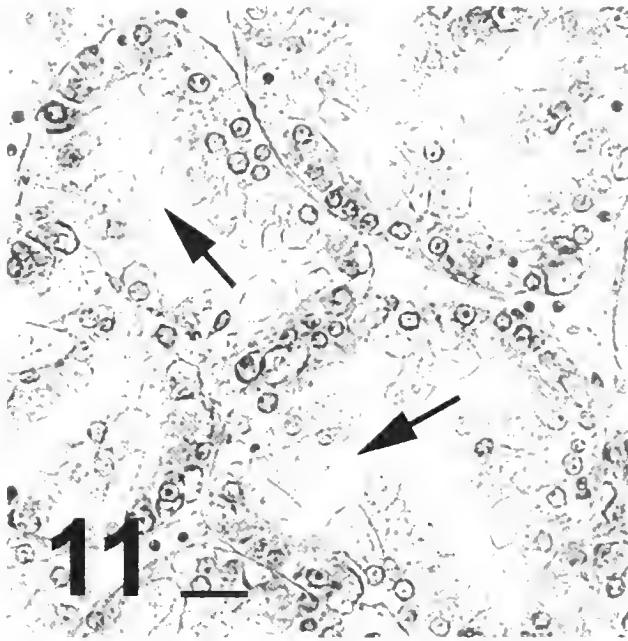
Figure 11. Histological section of normal Manila clam digestive gland without granules in absorptive cells but with patent digestive tubular lumina (arrows). Bar, 20 μm, H&E.

clams, about half of the clams exhibited swelling and luminal occlusion of the digestive tubule absorptive cells after 4 days of exposure to both 10 and 12.5 ppt salinity. A similar proportion showed these changes at 7 days as well as mild cell sloughing at the lower salinity concentration. By 14 days of exposure, clams from one group had uniformly sloughed and necrotic cells in the tubular lumina, while in the other group about one half of individuals had occluded swollen lumina and one half had shed necrotic cells into tubular lumina at 10 ppt salinity. At salinity of 12.5 ppt and 14 days of exposure in both groups, about one half of the clams had swollen occluded tubular lumina, and about one half had sloughed necrotic cells in the tubular lumina.

The gills showed sloughing of epithelium in exposed clams, but, due to the random planes of section typical in histological preparations of the gill and the fact that at least mild epithelial loss was observed in apparently normal clams, the gills were a less reliable measure of low salinity exposure and were therefore not systematically evaluated.

DISCUSSION

Other limited studies of juvenile or adult Manila clams have shown similar low salinity tolerance. Kurata (2000) found that when tested at a temperature of 1°C, salinities below 15 ppt limited the survival of Manila clams. Manila clam larvae have been found to have an optimal salinity range of 20 to 30 ppt in hatchery studies (Robinson & Breese 1984). Numaguchi (1998) reported that D-hinge Manila clam larvae could survive for 72 h at 12 ppt but that swimming was abnormal. Larvae did not survive at 8 ppt, but at salinities of ≥15.5 ppt survival and swimming behavior did not differ from those of control larvae held at higher salinities. On the other extreme of salinity, Shpigel and Fridman (1990) found that Manila clams (referred to in the article as *Tapes semidecussatus*) grew well in a salinity of 41 ppt.

Lethal and Marginal Salinity Concentrations

These experiments clearly showed that salinity of ≤10 ppt is a lethal concentration for Manila clams, at least for all of the populations of clams used in this study. Although we placed clams in a recovery tank after the fourth week, we have reported only the mortality that occurred after 4 wk at constant salinity. The fact that additional mortality occurred in the fifth week may indicate at least a partial adaptation to the lower salinity followed by an inability to adapt quickly to the higher salinity experience in the fifth week.

Figure 13. Histological section of digestive gland from Manila clam exposed to 12.5 ppt salinity for 14 days showing shed necrotic absorptive cells in the digestive tubular lumina (arrows). Bar, 20 μm, H&E.

Figure 12. Histological section of digestive gland from Manila clam exposed to 10 ppt salinity for 4 days showing swelling of the digestive tubular cells and occluded lumina (arrows). Bar, 20 μm, H&E.

This, in fact, represents likely environmental conditions that may compound the mortality effect of long-term low-salinity exposure. In fact, our other experiments showed that clams exposed to 10 ppt for only 2 wk (Figs. 6 and 7) or less (Figs. 4 and 5) and then removed to ambient high salinity (~28 ppt) succumbed at a high rate. Therefore, the survival of clams held in low salinities (10 to 12.5 ppt for extended periods (e.g., 4 wk) may depend on the rate at which they are reacclimated to higher salinities.

A salinity concentration of 12.5 ppt was shown to be a marginal concentration in which the survival of clams over a 4-wk period followed by 1 wk in a recovery tank was highly variable between populations and even within replicated groups. The average percentage mortality rate at 12.5 ppt ranged from 7 to 82%. Standard deviations were typically very high in replicated groups, indicating the high variance within given populations for survival at 12.5 ppt. The striking difference between two populations is demonstrated by the Thorndyke Bay clams (tolerant to 12.5 ppt) and the Oakland Bay clams (intolerant to 12.5 ppt). We were not able to statistically link high survival at 12.5 ppt to specific locations where the clams seemed likely to have adapted to low salinity due to freshwater inflows near the clam beds. However, the Thorndyke Bay clams, which had the greatest survival at prolonged exposure to 12.5 ppt, are located near streams that may occasionally subject them to low-salinity conditions. The results seem to indicate that most clam populations contain some individuals with the ability to withstand 12.5 ppt for extended time periods. Clams from many of the locations tested are the result of planting hatchery-produced juvenile clams and represent possibly mixed as well as undocumented heritage, which may, in part, explain the variation in the proportions of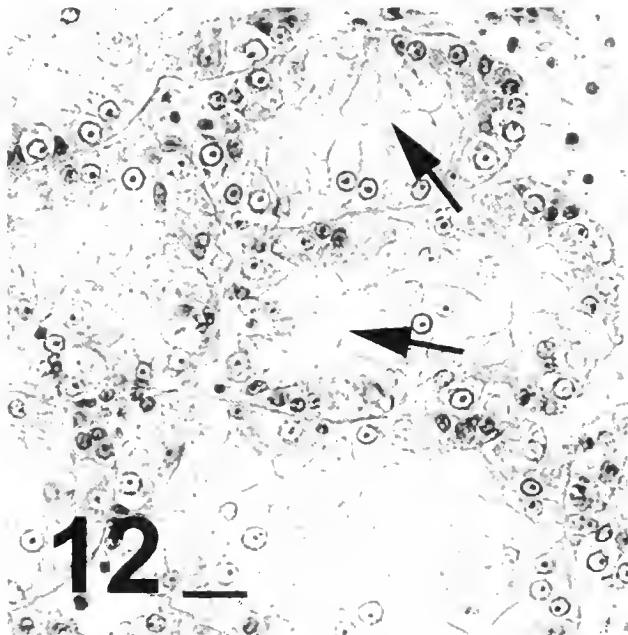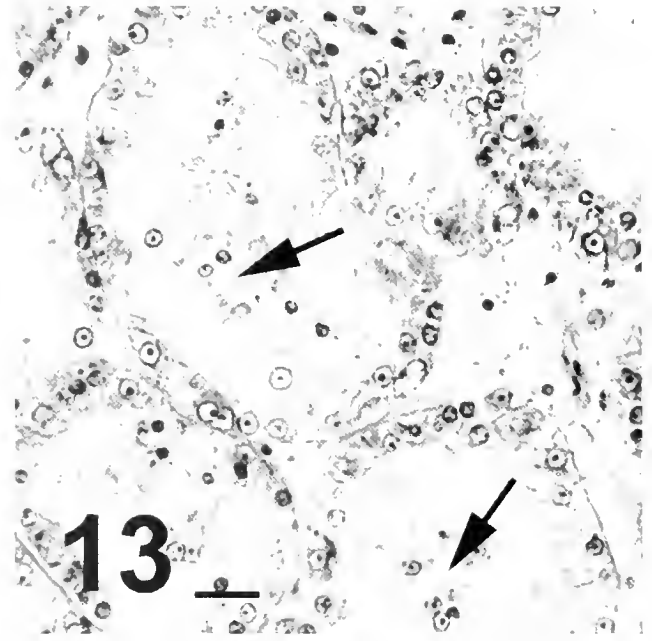 individuals that can survive at 12.5 ppt in various populations of Manila clams. However, if one had the objective of selecting clams with resistance to low-salinity concentrations, it would seem advisable to use a population such as the Thorndyke Bay clams as a founder population, since it appears to be enriched with individuals capable of withstanding a marginal salinity of 12.5 ppt.

Kim et al. (2001) reported that Manila clams recovered a typical endogenous circatidal rhythm of oxygen consumption when placed in reduced salinity as low as 15 ppt but not at salinities below 10 ppt. These authors concluded that Manila clams cannot maintain normal metabolic activity below 15 ppt. They also reported that all clams exposed to 5 ppt were dead within 7 days. The authors apparently did not evaluate the metabolic activity of clams at 12.5 ppt. The results of our study suggest that some clams may be able to respire normally at 12.5 ppt, based on their long-term survival at this salinity concentration.

Mechanism of Response to Low-Salinity Concentration in Manila Clams

In regard to low-salinity effects on Manila clams, our working hypothesis was that resistance to low salinity consists of two features: a physiological capacity of the tissues to tolerate a particular low salinity; and a survival response, consisting of the time for which the clam can maintain a closed shell condition, thus excluding lethal low salinities, as has been shown to occur in other bivalve species. For example, Shumway and Youngson (1979) showed that shell closure of *Modiolus modiolus* (Linnaeus 1758) occurred at 60% seawater. Burrell (1977) hypothesized that the greater resistance to low salinity in *Mercenaria mercenaria* (Linnaeus 1758) in comparison to Eastern oysters (*Crassostrea vir-*

ginica, Gemlin 1791) was due to the ability of clams to maintain shell closure for a longer period of time. Clearly, the survival response is complex, and depends both on aspects of the clam's metabolism (e.g., capacity for anaerobic metabolism) and possibly on environmental factors that remain undefined, although, surprisingly, temperature did not appear to affect the response, at least within the parameters of the experiment conducted in this study.

Our results suggest that while Manila clams can successfully resist lethal low salinities for a period of time, they are probably constantly testing salinity either by active subtle valve opening or seepage of low-salinity seawater into the mantle cavity. The fact that we observed swelling of digestive gland tubular absorptive cells at 4 days of exposure to both 10 and 12.5 ppt salinity, combinations of exposure time and salinity that we also showed to be clearly survivable, indicates that the clams do not totally exclude lethal and marginal low salinities during exposure by shell closure, although it is clear that they limit the exposure of their tissues to the low salinities by shell closure.

Effect of Temperature on Tolerance to the Marginal Salinity of 12.5 ppt

We were not able to demonstrate any significant effect of temperature on the tolerance of three groups of clams to the marginal salinity of 12.5 ppt, even though the populations tested included those that showed moderate to high mortality rates when exposed to 12.5 ppt in earlier experiments. However, Cain (1973) reported that survival was reduced in larval *Rangia cuneata* at high temperature-low salinity combinations. Laing and Child (1996) showed that 6°C, the lowest temperature that we tested, was compatible with the growth of Manila clams, while Mann (1979) showed that growth and spawning occurred at 18°C, the highest temperature that we tested.

Structural Response of Tissues to Exposure to Lethal and Marginal Low Salinities

These experiments provided data that can be used for the diagnosis or forensic evaluation of clams that are suspected of exposure to lethal or marginal low salinities. Individual variation in response is probably a result of the extent to which individuals open and test the ambient salinity or, conversely, their ability to remain tightly closed when they sense lethal or marginal salinities. In either case, the results showed that relatively short-term exposure (i.e., between 4 and 14 days) to salinity of 10 or 12.5 ppt resulted in the swelling of the absorptive cells of the digestive tubules, presumably from the absorption of hypoosmotic seawater, followed by the sloughing or loss of these cells into the digestive tubular lumina.

Mortality Due to Lethal Low Salinity Exposure May Occur Over Several Weeks

While our experiments on the duration of tolerance to 10 ppt, a lethal low salinity, showed that only about 7 days of exposure was required for a significant (approaching 100% in many cases) mortality response, all of the experiments in which clams were exposed and then placed in a recovery tank at ambient salinity tended to show a sharp increase in obvious shell gaping (our criteria of mortality) after placement in the recovery tank. The long-term (4-wk) exposures, for example, showed that while some of the clams were in the exposure tanks at 10 ppt they maintained shell closure and appeared normal for up to 4 wk followed by

sharp rise in the mortality response during the fifth week when the clams were placed in an ambient salinity recovery tank. The reason for this is not known, but it may be due to the fact that the low salinity stimulates a strong shell closure response that disappears when the stimulus is removed (i.e., the clams are placed in the recovery tank). However, structural damage to tissues, as demonstrated in this study, as well as stressful metabolic alterations (e.g., depletion of free amino acids) are significant and, in fact, are irreversible much earlier, although they are not manifested in the obvious death of the clam at gaping, until it is returned to an environment where the stimulus for protective shell closure is removed. Whatever the basic underlying mechanism, the results from this study show that the obvious mortality response to lethal low-salinity exposure may be delayed, depending on the salinity regimen and perhaps other factors. Therefore, as a practical application of our results, it would be incorrect to assess a clam population immediately after, for example, a 7-day exposure to salinity of ≤10 ppt and assume that clams showing tight shell closure were unaffected. It would be more accurate to assess the clam population several weeks later and, best of all, to additionally obtain tissue samples for histological analysis during and at intervals after the exposure to the low-salinity regimen.

Reasons for Control Mortality Losses

Control mortalities were generally <20% and often near zero. However, a control mortality rate even approaching 20% is a vex-ing issue and is one that will require further investigation to elucidate the causes. Clearly, the clams were held in a somewhat artificial environment in that a sedimentary substrate was not provided due to the necessity to evaluate their condition frequently and could have contributed to the losses. We determined from an extensive histological examination of the clam populations used in this experiment and from other studies that there were no significant known infectious diseases of Manila clams present.

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ON TWO NEW MACROSCOPIC INDEXES TO EVALUATE THE REPRODUCTIVE CYCLE OF *ENSIS MACHA* (MOLINA, 1782)

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ABSTRACT We describe the reproductive cycle of razor clam *Ensis macha*, during 1996 and 1997, in the Golfo de Arauco, Chile (37°14'S–73 29'W) based in the variation of the monthly averages of common and new macroscopic and microscopic indexes and scales. The common macroscopic indexes are weight ratio of soft tissues to valve weight or Somatic Valve Index and, weight ratio of the soft tissues to total weight or Somatic Tissue Index. The new Macroscopic Index and scale are the quantification of the width of the posterior foot or Morphometric Index and the quantification of digestive gland cover with ovary tissue plus the degree of ovary development or mature morphometric scale. The microscopic indexes consist of the quantification of ripe gamete over the bulk of the gonadic tissues, previously treated in formalin and without stain or gametic index and the same quantification over histologic preparations or Gametic Histologic Index. The somatic valve index and somatic tissue index results are not adequate to describe the reproductive cycle of this specie; however, the Morphometric Index and Mature Morphometric Scale are very useful. These last two methods, in addition to the Gametic Index and Gametic Histologic Index, show that the razor clam reproductive cycle, over these 2 years, is characterized by a resting period from March through July, and a progressive development of the gametes between August and October. The spawn starts in November and is widespread until February. The index dropped abruptly during November1997, showing a spawn rate more intensive than the previous year, which may be related to anomalous temperatures for the region. The reproductive *E. macha* cycle described here, is similar to the *Ensis minor* cycle in the Manfredonia Gulf, in Italy and the *Ensis siliqua* of Vilamoura on the southern coast of Portugal, but it is different to that observed for others authors in the Region X during 1994, and in the Golfo de Arauco and other locations of southern Chile during 1997.

KEY WORDS: gametic and gonadic indexes, reproductive cycle, razor clam, *Ensis*

INTRODUCTION

The razor clam *Ensis macha* (Molina, 1782) is a bivalve shellfish distributed from Caldera to Magallanes in the Chilean coast and up to San Matías Gulf in the Argentine coast. It is found in the shallow sandy bottom living buried deeply in the sand favored by its shape and a large foot.

Razor clam fishery became an important resource, exploited mainly for exportation and includes activities of artisanal fisherman, mediators and canning enterprises that commercialize the product mainly to Spain and Japan. The fishery started in south Chile in 1988, with a catch reaching 1.741 tones from Region X only. After that it reached a maximum landing of 8.617 tones in 1991 with contributions from the X and VIII Regions, but this year the landings in Region X diminished while the landings from Region VIII were increasing until 1995. Later on, the total landings diminished to 6.115 tones in 1999 when 88.3% came from the Region VIII, 10.1% from the Region X and the rest from the VII, XII, and IV Regions which has been slowly incorporated but at very small rate (Sernapesca 2000). This reduction in the landings led to additional fishery management and the support of the research projects to cover the basic biology and fishery aspects of this resource looking towards future aquaculture.

In this article, the background of the reproduction of this species in the Golfo de Arauco, Region VIII, Chile, during 1996 and 1997 is given. It has been characterized through macroscopic and microscopic methods in common use, plus two new methods not described previously, here proposed as and easy application, validated with the histology of the female ovary. (Lépez et al. 1997a, 1997b, Aracena et al. 1998a).

MATERIALS AND METHODS

Samples of about 60 adults of *E. macha* (≥ 15 cm valve length) were taken monthly during 1996 and 1997 for the study of scale and macroscopic indexes. For microscopic indexes, monthly samples of 20 adults were taken during 1996, and monthly samples of 30 adults during 1997. All samples taken during 1996 were selected in the landing zone of Tubul, Golfo de Arauco (37°14'W–73°29'W) and during 1997 were collected on board of artisanal boats in the same Golfo.

E. macha is gonochoric. The males have white–grey gonads with a homogeneous texture, while females show ovaries of a white-cream color and granular texture, especially when they are close to spawning. The sex was always corroborated with microscopic observation of ovary tissue smears.

In the mature razor clams, as in many bivalves, the ovaries extend dorsally over the digestive gland and the anterior adductor muscle, showing a simple way to determine the sex and the development stage. The ovaries invade the ventral zone of the visceral complex, the posterior part of the foot and form a cord in the inner channel of the eatable foot.

To evaluate the mature stage, we applied the following macroscopic indexes:

(i) Somatic Valve Index (SVI)

$$SVI = \frac{DWS*100}{DWV}$$

where DWS is the dry weight of the soft body parts and DWV is the dry weight of the valve.

(ii) Somatic Tissue Index (STI)

$$STI = \frac{DWS*100}{DWT}$$

where DWS is the dry weight of the soft body parts and DWT is the total dry weight.

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(iii) A new Macroscopic Maturity Scale (MMS)

Estimate the covering of the ovary over and around the digestive gland, on a scale of 1 to 4:

1. Ovary covers 1/4 of the digestive gland.
2. Ovary covers 1/2 of the digestive gland.
3. Ovary covers 3/4 of the digestive gland.
4. Ovary covers totally the digestive gland.

Points were also assigned to the progressive development of the gonadic tissue.

1. No development: no observable gonadic tissue or very scarce and transparent.
2. Intermediate developmental stage: average bulk and granulate aspect.
3. Very developed: shows maximum bulk and granulate aspect.

Mixing these two scales, we obtain the mature stages from Table 1 and the monthly average as follows:

$$\overline{MMS} = \frac{\sum_{n}^{i=1} MMS_i}{n}$$

where MMS_i is the value of the scale assigned to the individual i, and n is the total number of individuals counted every month. In addition, the respective variance can be obtained from:

$$VAR\,(MMS) = \frac{\sum_{n}^{i=1}(MMS_i - \overline{MMS})^2}{n(n-1)}$$

(iv) A new Marphometric Index (MI)

The MI is obtained from the measurement of the width of the posterior area of the foot, under the visceral complex, showing the degree of invasion of the gonad tissue. To this purpose, we made a cut in the posterior area of the foot, as shown in Figure 1, to measure its width. The monthly MI is the average of the width of each individual (MIi) on the total number of individual measured (n)

$$\overline{MI} = \frac{\sum_{n}^{i=1} MI_i}{n}$$

and its respective variance:

$$VAR\,(MI) = \frac{\sum_{n}^{i=1}(MI_i - \overline{MI})^2}{n(n-1)}$$

TABLE 1.

Maturity stages of the razor clam: Macroscopic Maturity Scale (MMS).

Covering	Developed	Maturity Stage (MMS)
1	1	2
1	2	3
2	2	4
2	3	5
3	3	6
4	3	7
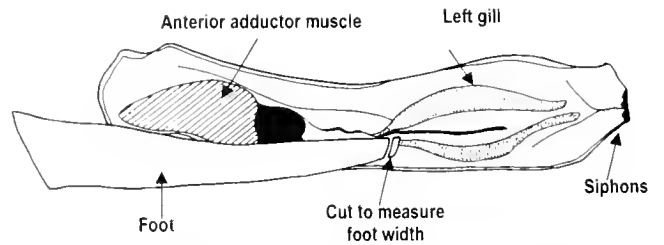
Figure 1. Ventral view of the soft body of *E. macha* showing the cut in the posterior part of the foot where the width measure is made to obtain the Morphometric Index.

Other two microscopic indexes were:

v) Gametic Index (GI)

The GI corresponds to the proportion of ripe gametes in relation to other kinds of cells in the bulk of the unstained ovary tissue smeared on a slide.

The proportion of mature cells for each individual (GI$_i$) was obtained by applying 10 times a microscope integration plate of 100 points to the mass and quantifying:

$$GI_i = \frac{a_i}{m}.$$

where, GI$_i$ is the proportion of ripe gametes for individual i, a_i is the number of ripe oocytes of the total elements in the individual i, and m is the total number of quantified elements. With this, we obtain the monthly average of GI as follows:

$$\overline{GI} = \frac{\sum_{n}^{i=1} GI_i}{n}$$

and its respective variance:

$$VAR\,(GI) = \frac{\sum_{n}^{i=1}(GI_i - \overline{GI})^2}{n(n-1)}$$

(vi) Gametic Histologic Index (GHI)

The GHI is the same prior proportion but on histologic preparations stained with Gallegos embedding in Hystosec, previously embedding in chloroform and mounted in Entellán (Con 1960, modified by Delpin, personal communication). The proportion of matures gametes for each individual (GHI$_i$) was obtained applying four times a integration plate of 100 points on the histologic cuts and quantifying:

$$GHI_i = \frac{a_i}{m}.$$

where GHI$_i$, is the proportion of ripe gametes of each individual, a_i is the number of ripe oocytes of the total elements in the individual i and m is the total number of quantified elements. With this obtain the monthly average of GHI, following:

$$\overline{GHI} = \frac{\sum_{n}^{i=1} GHI_i}{n}$$

and the variance is:

$$VAR\,(GHI) = \frac{\sum_{n}^{i-1}(GHI_i - \overline{GHI})^2}{n(n-1)}$$

SVI and STI were used for the 1996 series on male and female razor clams plus GI only on female clams. MMS and MI were used in the two series of samples, only on female. The GHI was used only in 1997, for female clams.

All these indexes and scales were applied to individuals larger than 15 cm valve length, because Reyes et al. (1995) had previously defined the mean size of first sexual maturity at 14 cm valve length for razor clam of the Region X.

To detect significant differences between the indexes and scales and between males and females for 1996, a Kruskall-Wallis test was carried out ($P > 0.05$). Same statistical test was carried out for the 1997 information and also multiple comparisons test (Least Significant Difference) to validate the macroscopic scale MMS with the GHI index (Aracena et al. 1998b).

To explain the tendencies in the reproductive behavior of razor clam described in this article, complementary oceanography information available (temperature, salinity, density) for the Region VIII was used (Salamanca, 1997).

RESULTS

From a total of 1,502 individuals analyzed during the years 1996 and 1997, the observed ratio of females to males was always slightly smaller with 40.3% and 41.3%, respectively (Table 2), although this difference was obviously never significant.

The Kruskall-Wallis test to detect differences between the indexes and macroscopic scales was applied, among males and females for 1996, but it was not significant ($P < 0.05$). It was therefore possible to compare results of two years, even though in 1997 only females were considered.

Both SVI and STI showed similar tendencies during the year 1996 (Fig. 2c and d), being different to the other indexes and scales applied in that same period. Maximum levels were observed from March to May and low values from June through December. The SVI oscillated between 34.34 and 50.16 with variance between 9.63 and 79.08. The STI oscillated between 25.00 and 33.31, with variance somewhat smaller, between 3.13 and 16.28.

The GI is an index applied directly on gonadic tissues and it is considered together with the GHI, that are the best to describe the evolution of the gametes. From February to June the first index stayed very low (Fig. 2e), with values between 0.02 and 0.04, rising later on to a maximum of 0.21 in October and then down again apparently through January or February of the next year. The variances were always lower than 0.01, except during December of 1995.

The MMS and the MI followed similar tendencies in 1996 (Fig. 2a and b). The MMS fluctuated between 3.36 in March and 8.86 in October, with variances between 0.35 and 4.62. The MI have

TABLE 2.

Sexual proportions of the razor clam: Golfo de Arauco.

	Female (%)	Male (%)	Total (N)
1996	40.3	59.7	834
1997	41.3	58.7	668

values from 3.98 mm (March) and 6.43 mm (September), with variances between 0.39 and 1.48.

During 1997 (Fig. 3), the GHI showed a very similar tendency to the GI of the previous year, but the descent after the maximum of October (0.253) it was very abrupt, falling to 0.017 in December, with equally smaller variances of 0.01. The same might be said of the MMS and MI of that year.

To validate the MMS, the average value of the GHI in 1997, was calculated for each value of the scale, with its variance and confidence intervals (Table 3) and then, the multiple comparisons test (Table 4), indicated that stages 2, 3, and 4 of the MMS have a very similar GHI and thus could only be considered one. The stage 5 and 6 are very similar to each other but stage 7 is different. In this way and for practical effects the MMS could be simplified to the following three states:

1. 0 and ¹⁄₂ of the digestive gland is covered by the ovary; volume and granulation are minimal or intermediate.
2. 1/2 of the digestive gland is covered by the ovary; volume intermediate and granulation are at their maximum.
3. The digestive gland is completely covered by the ovary; volume and granulation are at their maximum.

According to these data, the reproductive cycle of the razor clam in the Golfo de Arauco, is characterized by a resting time between March and June of every year (autumn and early winter), a gradual increase of the maturity of the ovary starting in this last month and reaching a maximum in October (spring), followed by a single spawn period that begins in November and may finish in December or be prolonged until February of the next year.

DISCUSSION AND CONCLUSION

Considering that the index GI and GHI, are the only ones that represent the changes that happen at the level of the gametic tissue, we may conclude that the index SVI and STI are not good to define the reproductive cycle of this specie because through its evaluation the gonadic tissue was not separated from the body tissue. In experiments carried out by Sastry (1968), with *Aequipecten irradians* Lamarck, by Bayne (1975) with several species of bivalve and for Lowe et al. (1982) with *Mytilus edulis L.*, among many other authors, they showed that a nutritious transfer takes place from the digestive gland into the ovary, or since nutritional tissue from mantle toward the gametic tissue lowering the change of volume and weight in the ovary when the total body weight is considered.

However, the index MI and the simplified scale MMS are good descriptors of the reproductive cycle of razor clam because they are obtained from the observation and measurement of the ovary, they are faster, easier and of low cost. Regarding the MMS, even though the ovary cover on the digestive gland is a simple measure to carry out and to be standardized, the gonadal volume and granulation, are very subjective parameters. For this reason, between these two indexes we recommend the MI index as a macroscopic index because is easy to obtain and quantify. The GI is a low cost method as well, although not so simple or quick, but since it is a direct quantification of the gonadic tissue it is more advisable than the macroscopic index.

As observed in Figs. 2 and 3, the reproductive cycle of *E. macha* during 1997 follows a similar tendency to the one observed during 1996, but with a more marked fall between October and December indicating a shorter, and more intense and synchronous spawning than in the previous year. This may be associated with salinity and temperature anomalies that affected the coastal areas
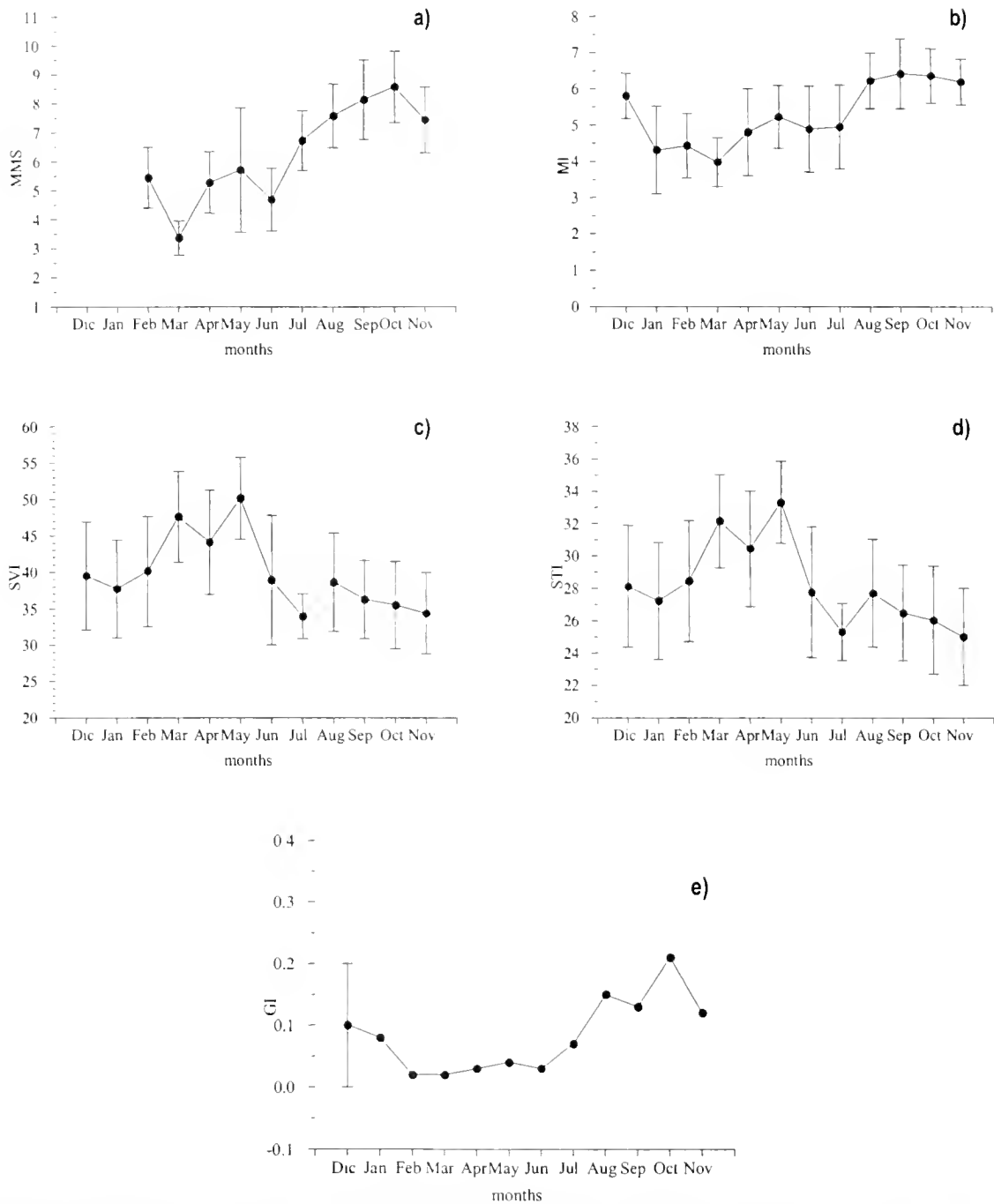
Figure 2. Monthly averages and variances of scale and maturity index of razor clam, Golfo de Arauco, 1995-1996. a, MMS = Macroscopic Maturity Scale; b, MI = Morphometric Index; c, SVI = Somatic/Valve Index; d, STI = Somatic/Total Weight Index; e, GI = Gametic Index in the bulk of gonadal tissue without stain.

of the Region VIII during 1997 (Salamanca, 1997). Table 5 shows that the spring-summer period 1997 was warmer and with less saline water (on the average) than in a "normal" year as the one that was detected in 1981 for the same area (Llancamil 1982). The winter conditions are similar among the two studies.

During the years 1996 and 1997 the razor clam of Golfo de Arauco in the Region VIII, registered a cycle of annual maturity with only one spawning period between November-December corresponding to the late spring early summer of the southern hemisphere and this is very similar to the razor clams of the northern

hemisphere. Thus for *Ensis minor* (Chenu) of the Manfredonia Gulf in Italy Casavola et al. (1994) describe a cycle of annual maturity with a longer resting period between May and October. The gametogenic activity starts in December and finish in March, and spawning between these last months and April, spring of the north hemisphere. Gaspar & Monteiro (1998) point out that *Ensis siliqua* (L.) from the south coast of Portugal has an annual gametic cycle with an extended inactive period from June to October, the gametogenesis activity starting in December with a maturity peak in March. Spawning starts in this last month and shows a maxi-
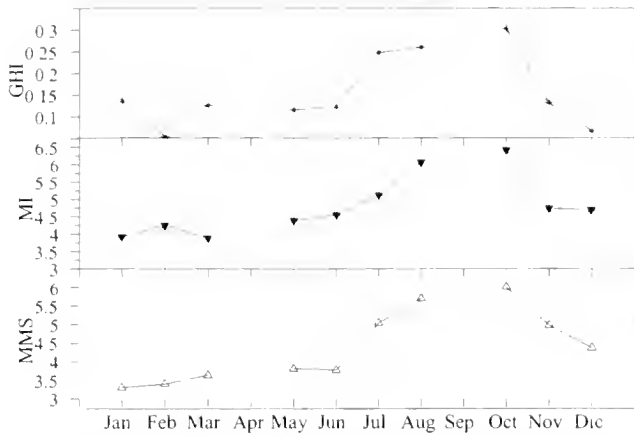
Figure 3. Maturity index and scale of razor clam, Golfo de Arauco, 1997. a, MMS = Macroscopic Maturity Scale; b, MI = Morfometric Index; c, GHI = Gametic Histologic Index.

mum in April, which may be extended through May. They also add that the males and females have a synchronous gonadal development similar to *E. macha*. In the Gormanstow bed *of E. siliqua* in Ireland, Fahy (1999) found that gonadal cycle are fairly similar to the same species off the Portuguese coast, but Ireland clams spawns later in the year.

Reyes et al. (1995) found that the largest evacuations of razor clam gametes take place at the end of September, November, February, and March in the Region X of Chile, which is in late spring and throughout summer. This difference may be related to the oceanographic conditions in the area because the fiords in the Region X are very different to the Golfo de Arauco.

Urban (1996) describe an annual reproductive cycle with a short spawning season in summer for *E. macha*, from Chile at 36° S, very similar to our finding. However, Avellanal et al. (2002), in a study of the reproductive cycle of *E. macha* in the south of Chile, used a very different methodology consisting of the assigning of six stages of gonadic organization to histologic preparations to determine the reproductive cycle for this species at Tubul (Golfo de Arauco) between November of 1996 and 1997. They found that 20% of the females presented ovaries partially spawned in February of 1997 and 100% presented a partial spawning in March and April. Between June and July, there was a quick recovery of the ovaries and 40% of the samples presented partial spawning in August, a percentage that increased to 100% in November and December of the same year.

This apparent difference between the reproductive cycle de-

scribed by Avellanal et al. (2002) and this work, both on the same population and period, probably would be due to different methods for evaluating the state of development of the gametogenesis. In both cases, a massive spawn between November and December of 1997 is described.

Other results by Avellanal et al. (2002) indicate that the spawning of *Ensis macha* in Corral (39°50'S–73°28'W) was similar to that one described by the same authors for Tubul. But, the cycle of this species in Ancud (41°50'S; 73°47'W), was different because the partial spawning started in January of 1997 and reached 100% in April, June, and July. Later on a recovery of the ovary was observed to reach 100% of mature individuals in December of that year. These authors also found in Tubul and Corral a positive correlation between the percentage of mature females and the chlorophyll α and a negative correlation among the percentage spawning and the chlorophyll α. In Ancud, the percentage of mature females was highest when the temperatures were increasing and the spawning reached a maximum when the temperatures were low. According to Avellanal et al. (2002), this relations points to the important influence that temperature and the quantity of food can have in the energy balance, transfer of nutrients and other processes occurring during the gametogenesis.

Finally, we conclude that the quantification method of the stage of maturity of the ovary of the razor clam, not described previously, which closely reflects the gametic cycle of *Ensis macha* are MI and MMS, being easier, fast and of low cost. These two methods have also been used to define the reproductive cycle of *Tagelus dombeii* (Lamarck, 1818) (López et al. 1997b) whose ovaries are also diffuse in the visceral complex and may be adapted for other similar species.

TABLE 3.

Statistics of GHI for each state of Macroscopic Maturity Scale (MMS) of razor clam: Golfo de Arauco, January–December 1997.

Maturity Scale (MMS)	GHI Averaged	GHI Variance	Confidence Interval
2	0.063	0.0004	0.015
3	0.078	0.0010	0.008
4	0.078	0.0015	0.007
5	0.143	0.0075	0.019
6	0.207	0.0031	0.016
7	0.262	0.0010	0.025

TABLE 4.

Multiple comparison tests (LSD) for seven gonadic maturity stages (MMS) of *E. macha* compared with the Gametic Index (GHI) during 1997.

Maturity Stage (MMS)	2	3	4	5	6	7
2		0.40	0.37	0.00	0.00	0.00
3	0.40		0.95	0.00	0.00	0.00
4	0.37	0.95		0.00	0.00	0.00
5	0.00	0.00	0.00		0.12	0.00
6	0.00	0.00	0.00	0.12		0.01
7	0.00	0.00	0.00	0.00	0.01	

TABLE 5.

Comparative chart of oceanography parameters in Coliumo Bay (average conditions).

Place/Water Mass	Temperature C	Salinity ×10⁻³
Subantartic Water (SAW)	>11.0	<34.3
Subsurface Equatorial Water (SSEW)	9–13	>34.4
Coliumo Bay (Elancamil 1982)		
Winter	12.07	32.76
Spring	11.22	34.20
Coliumo Bay (Salamanca 1997)		
Fall-Winter	12.57	33.44
Spring-Summer	14.88	33.69

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POPULATION GENETICS OF TWO BIVALVE SPECIES (*PROTOTHACA STAMINEA* AND *MACOMA BALTHICA*) IN PUGET SOUND, WASHINGTON

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ABSTRACT   Allozyme polymorphisms from individuals of *Protothaca staminea* and *Macoma balthica* were examined electrophoretically and scored at five loci. Both species were sampled at three sites located in different hydrologically defined basins of Puget Sound, Washington. Highly significant differences in allele frequencies among the three *P. staminea* populations were found at all five loci. Significant differences in allele frequencies were detected consistently at only one locus among the *M. balthica* populations. Genetic distances between the three *P. staminea* populations, determined using both Cavalli-Sforza and Edwards (1967) chord distance and Nei's (1972) genetic distance measures, revealed the South Sound population as the genetic outlier. This pattern is consistent with the hydrology of the Puget Sound basins and the mixing that occurs at the sills between basins. Two to four of the allozyme loci demonstrated heterozygote deficiencies in *P. staminea*, depending on population. Only one locus exhibited a heterozygote deficiency in each of the three *M. balthica* populations. Potential contributing factors to the heterozygote deficiencies include a temporal Wahlund effect, selection, and null alleles. When data were corrected for the presence of a putative null allele, conclusions about population differentiation did not change.

KEY WORDS: population, genetics, allozymes, bivalves, *Protothaca staminea*, Puget Sound, *Macoma balthica*

INTRODUCTION

Early genetic studies of marine populations found little evidence for genetic differentiation over large geographic distances. It was generally believed that open aquatic environments permit extensive dispersal of planktonic larvae, resulting in little genetic heterogeneity over wide spatial scales (e.g., Buroker et al. 1979, Crisp 1978, Gooch et al. 1972). This notion was soon challenged, however, by several studies presenting compelling evidence for population structure even along open coastlines (Scheltema 1975, Burton 1983). Increasingly, studies now find that any number of factors can contribute to population differentiation in apparently open systems. Populations may be defined not only by their reproductive mode (Hellberg 1996), but by hydrological forcing (Reeb & Avise 1990), chemical gradients (Koehn et al. 1976, Ma et al. 2000), or changes in source populations (Kordos & Burton 1993). Population subdivision is evident even among the bivalves, whose long-lived planktonic larvae might otherwise be equated with high dispersal potential (e.g., Mariani et al. 2002). Other examples of genetic differentiation in marine populations over both small and large spatial scales are reviewed in Shaklee and Bentzen (1998). Collectively, these studies demonstrate that reproductive and dispersal strategies are not the only determinants of genetic differentiation among marine populations.

In this study, we examined the potential for hydrological forcing to promote differentiation of broadcast spawners with planktotrophic larvae in a small estuarine system. Puget Sound, Washington, is a fjord-like estuary composed of five contiguous basins with constrictions and sills that strongly influence the tidally-driven currents. The basins fall into two categories: well-mixed with rapidly circulating water masses (Admiralty Inlet, Main basin, and Southern basin), or stratified with slow-moving water masses (Hood Canal and Whidbey basin; Fig. 1). Ebbesmeyer et al. (1988) proposed that as much as 50% of the water in each basin

is recirculated back into the basin of origin because of intense mixing at the sills. This recirculation includes the upper layer of the water column (10–30 m deep) where planktonic larvae of marine invertebrates are commonly found, possibly leading to partial restriction of larvae to their basin of origin. Such a barrier to dispersal could create genetically differentiated subpopulations among basins.

Few population genetic studies of marine invertebrates have been conducted in Puget Sound despite the presence of many managed commercial and recreational fisheries. Grant and Utter (1988) examined allele frequencies from two polymorphic loci in the intertidal gastropod *Nucella [Thais] lamellosa* at several sites within Puget Sound, adjacent waters and along the open coasts of Oregon and Washington. They found evidence for population subdivision at various geographic scales, however the differences were attributed primarily to the nonplanktonic life history of this species. A more limited study in Puget Sound involving a species with a planktonic larval stage, the bivalve *Saxidomus giganteus*, found a geographic cline in populations in one of the two allozyme loci examined (Johnson & Utter 1973). Unfortunately, this study did not investigate any differences that might be attributed to separation by the hydrologically defined basins.

Our objective in this study was to test whether the bivalves *Protothaca staminea* (Conrad) and *Macoma balthica* (L.) exhibit evidence of genetic differentiation in Puget Sound consistent with its unique hydrology. Both species broadcast spawn between April and September with planktotrophic larvae that feed for weeks prior to settlement. Given the long planktonic larval phase of the two species and the small length scale of Puget Sound (on the order of 130 km), one might expect genetic homogeneity in the absence of any physical barriers to dispersal. Differentiation of the populations might suggest that recirculation of water masses at the sills contributes to partial isolation of populations in Puget Sound. To determine whether the hydrology of Puget Sound is the principle mechanism for any observed differentiation we chose two species that share similar reproductive and dispersal strategies yet have

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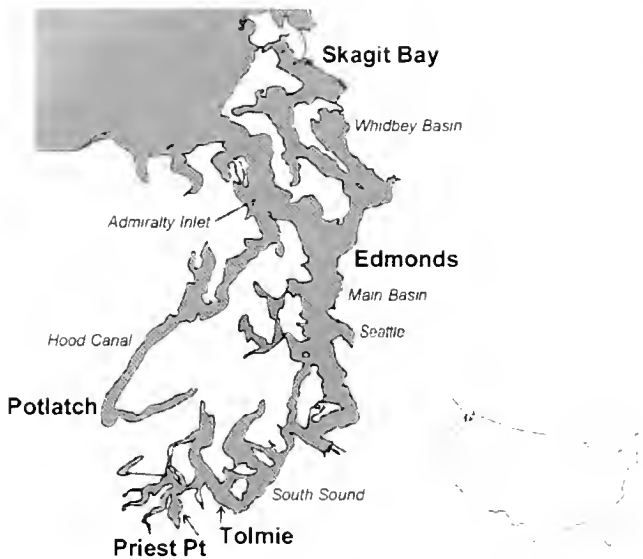*Corresponding author. E-mail: micaela@u.washington.edu

Figure 1. Sampling sites for *Protothaca staminea* (Edmonds, Potlatch, Priest Pt.) and *Macoma balthica* (Skagit, Potlatch, Tolmie) in Puget Sound, Washington.

disparate adult characteristics. *Protothaca staminea* occurs from the Aleutian Islands of Alaska to Baja California; is a suspension feeder preferring coarse sand to gravel substrate; attains a maximum valve length of around 7 cm; and is preyed upon primarily by starfish, moonsnails, and octopuses. *Macoma balthica*, conversely, is circumboreally distributed; may switch between surface-deposit and suspension feeding; prefers muddy substrate; may inhabit brackish waters; in Puget Sound, rarely exceeds 2 cm in length; and is preyed upon primarily by flounder, crabs and sea birds. By examining two species with similar reproductive and dispersal strategies but with different adult characteristics, we hoped to assess the influence hydrology may have on population distributions of different species in this estuary.

We examined allozyme polymorphisms at five presumptive gene loci in each of the two species of intertidal bivalve clams: *Protothaca staminea* and *Macoma balthica*. Genotype and allele frequencies from each species were then compared among three of the hydrologically defined basins of Puget Sound, Washington.

METHODS

Field Sampling

Protothaca staminea were collected at Potlatch (Hood Canal Basin; $n = 94$), Priest Point (Southern Basin; $n = 114$), and Edmonds (Main Basin; $n = 114$) between March 1 and Sept. 7, 1998. *Macoma balthica* were collected from Potlatch ($n = 113$), Tolmie Park at Big Slough (Southern Basin; $n = 116$), and Skagit Bay (Whidbey Basin; $n = 132$) between March 2, 1998 and June 29, 1999 (Fig. 1). All samples were obtained during low low tide along 100- to 500-m transects running parallel to the shore. Care was taken to sample individuals from the full extent of their range in the intertidal zone as well as across size classes. The length of the right valve of *P. staminea* specimens sampled ranged from 9 mm to 57 mm and for *M. balthica* from 4.5 mm to 17 mm. *M. balthica* specimens included the white, pale pink, and dark pink color morphologies. The clams were transferred live in ambient seawater to the laboratory. Immediately upon arrival, foot muscle, ctenidium, digestive gland, mantle, and adductor muscle were dis-

sected from each *P. staminea*. The tissues from each clam were then combined in a single test tube. Because of the small size of the *Macoma* clams, they were stored whole (minus shell) in individual test tubes. All samples were stored at $-80°C$ for subsequent electrophoretic analysis.

Electrophoresis

Following the methods of LeClair and Phelps (1994), tissue samples were homogenized in TC-1 gel buffer (Shaw & Prasad 1970) and centrifuged at 1,000 g for 5 min. Supernatants were absorbed with filter-paper wicks (Schleicher & Schuell no. 470) and used for starch gel electrophoresis. Details of the electrophoretic method are described in Aebersold et al. (1987) and Harris & Hopkinson (1976). Gels were run in a refrigerator at 8°C. Enzyme and gene nomenclature follow the guidelines of Shaklee et al. (1990). Both species were assayed for allozyme polymorphisms on four different buffer systems: CAME 6.8 (LeClair & Phelps 1994, modified from Clayton & Tretiak 1972); LiOH-RW (Ridgeway et al. 1970), TRIS-GLY (Holmes & Masters 1970); and TC-4 (buffer "a" of Schaal & Anderson 1974). The following enzyme/buffer combinations were tested: aspartate aminotransferase (AAT), isocitrate dehydrogenase (IDHP), malate dehydrogenase (MDH), malic enzyme (MEP), phosphogluconate dehydrogenase (PGDH), and phosphoglycerate kinase (PGK) on CAME 6.8; esterase-D (ESTD), formaldehyde dehydrogenase (FDHG), nucleoside-triphosphate pyrophosphatase (NTP), octopine dehydrogenase (OPDH), and strombine dehydrogenase (STDH) on LiOH-RW; alanine aminotransferase (ALAT), arginine kinase (ARGK), ESTD, glucose-6-phosphate isomerase (GPI), lactate dehydrogenase (LDH), mannose-6-phosphate dehydrogenase (MPI), cytosol nonspecific dipeptidase (PEPA), tripeptide aminopeptidase (PEPB), peptidase-S (PEPS), phosphoglucomutase (PGM), STDH, and triose-phosphate isomerase (TPI) on TRIS-GLY; adenosine deaminase (ADA), aconitate hydratase (AH), glyceraldehyde-3-phosphate dehydrogenase (GAPDH), PEPA, and proline dipeptidase (PEPD) on TC-4.

Of the 25 enzymes assayed, activity of six (AAT, ESTD, GPI, IDHP, PGDH, PGM) were well resolved and indicated encoding by polymorphic loci (more than one allelic form detected). These enzymes were subsequently screened in all clams except AAT, which was screened only in *P. staminea*, and IDHP, which was screened only in *M. balthica*. Allelic variants are designated by their electrophoretic mobility relative to the most frequent variant encountered during the initial screening. Variants preceded by a minus sign indicate cathodal migration.

Data Analysis

The population genetics software GENEPOP version 1.2 (Raymond & Rousset 1995a) was used to run analyses of population differentiation and heterozygote deficiency or excess relative to Hardy-Weinberg equilibrium. For testing population differentiation, both "genic" and "genotypic" tests were run. The genic test is used to determine whether allelic distributions are identical across populations. Contingency tables for each locus were tested using the R x C Fisher test to arrive at an unbiased estimate of the P value (Raymond & Rousset 1995b). The genotypic test is used to determine whether genotypic distributions are identical across populations. Although less powerful, the genotypic test is more appropriate when alleles within individuals are not independent, which may occur when there is nonrandom mating (Goudet et al.

1996). For this test, an unbiased estimate of the P value is achieved by using the G-based test (Goudet et al. 1996) on contingency tables for each locus. Tests for both heterozygote deficiency and excess are concerned with the same H_0, random union of gametes. For both tests, the unbiased P value was estimated using the score test (U test; Rousset & Raymond 1995). Because of the presence of rare alleles, defined as having frequencies <0.005 (Hartl & Clark 1997), the exact tests used by GENEPOP are more appropriate than the commonly used χ^2 test because the results will not be biased by rare alleles (Guo & Thompson 1992). Expected heterozygosities (H_E), fixation indices (F_{IS}) and the extent of population divergence (F_{ST}) were also calculated for each locus in each population using GENEPOP. The F-statistics used by GENEPOP follow Weir & Cockerham (1984). GENEPOP was also used to test for genotypic linkage disequilibria. The program BIOSYS-1 (Swofford & Selander 1981) was used to determine Cavalli-Sforza and Edwards (1967) chord distances and Nei's (1972) genetic distances. Finally, when individuals without a banding pattern are observed, yet are not conclusively null homozygotes, the frequency of a putative null allele can be estimated using ($H_E - H_O)/(H_E + H_O)$, where H_E and H_O refer to the expected and observed heterozygosities, respectively (Brookfield 1996). Using this algorithm allele frequencies for the populations of both species were corrected for the presence of a null allele.

RESULTS

Stains for GPI and PGM were most successful on the Tris-Gly buffer system; PGDH, AAT, and IDHP on CAME 6.8; ESTD on LiOH-RW. In each species, two private alleles (alleles only detected in one population) were found: $GPI*-17$ (P. staminea, Potlatch), $AAT*-1500$ (P. staminea, Edmonds), $IDHP*150$ (M. balthica, Skagit), $PGDH*114$ (M. balthica, Skagit). Four rare alleles occurred in P. staminea populations and eight in M. balthica populations (Table 1).

Expected heterozygosities (H_E) and fixation indices (F_{IS}) varied widely in both species depending on the locus (Table 1). Notably, F_{IS} values for the P. staminea population at Edmonds were consistently higher than values for the population at Potlatch or, with most loci, at Priest Point suggesting strong heterozygote deficiencies in this population. The tests for Hardy-Weinberg equilibrium revealed significant heterozygote deficiencies ($P < 0.05$) in up to four of the five loci in the P. staminea populations (Table 2). Only at the $ESTD*$ locus was a significant heterozygote deficiency detected in the M. balthica populations ($P < 0.001$; Table 2). In neither species was a heterozygote excess detected.

For both species, locus pairs were also tested for genotypic linkage disequilibrium within each population. A significant linkage disequilibrium suggests the genotypes at different loci are not independent. Linked loci may be an indication of inbreeding. After applying a sequential Bonferroni correction (Ury, 1976), only one population (P. staminea, Edmonds) had loci with significant linkage disequilibria. The two locus pairs demonstrating a significant disequilibrium were: $GPI*$ and $AAT*$ ($P < 0.001$) and $AAT*$ and $ESTD-2*$ ($P < 0.005$).

With both the genic and genotypic tests, we found strong evidence for population differentiation among all three P. staminea populations at all loci ($P < 0.001$; Table 3). Both chord and Nei's distances indicated that the populations from Edmonds and Potlatch are more closely related than either is to the Priest Point

population (Table 4). When distances were determined locus by locus, four of five loci were in agreement with this pattern. F_{ST} values for the P. staminea populations ranged from 0.07 ($PGM*$) to 0.13 ($ESTD*$).

In the M. balthica populations, both the genic and genotypic tests demonstrated differentiation at one of the five loci ($PGDH*$ Table 3). The genic test revealed an additional differentiation at the $ESTD*$ locus (Table 3). F_{ST} values for M. balthica ranged from −0.002 ($PGM*$) to 0.009 ($ESTD*$). Because of the lack of differentiation among M. balthica populations at most loci, distance measures were not significant (data not shown).

To determine whether heterozygote deficiencies had any effect on the population differentiation tests, the allele frequencies were recalculated to account for the potential presence of a null allele. An indication of null alleles is a null homozygote demonstrating no banding pattern. In the P. staminea samples, absence of enzymatic activity occurred with only one individual from Priest Pt. when stained for GPI and two individuals from Edmonds when stained for ESTD and PGM. In the M. balthica samples, absence of enzymatic activity occurred in three individuals from Skagit Bay (all using the stain for IDHP, one additionally did not stain for ESTD) and four individuals from Potlatch (all using the stain for ESTD, one additionally did not stain for PGDH). Because this absence of activity could also have been caused by tissue degradation, staining inconsistencies, or tissue samples that are too small (for M. balthica), we could not conclusively assign these individuals as null homozygotes. It is possible to estimate the frequency of a putative null allele based on the heterozygote deficiency in a population. Following Brookfield (1996), allele frequencies were corrected in each population to account for the presence of a null allele and the genic and genotypic tests re-run. The level of population differentiation observed did not decline for either species. On the contrary, both the chord and Nei's genetic distances increased slightly with the addition of the null allele (between 1 and 30% increase, data not shown).

DISCUSSION

Evidence for Distinct Populations of P. staminea But Not M. balthica

Both *Protothaca staminea* and *Macoma balthica* are free-spawning bivalves, with feeding larvae that spend about 3–4 wk in the plankton. These larvae are the dispersal propagules, largely at the mercy of local horizontal currents. Given the similar reproductive and dispersal strategies of P. staminea and M. balthica, one might expect consistency in the level of population differentiation of these species when exposed to the same estuarine currents. The population structure of these two species, however, is very different in the complex estuarine system of Puget Sound, Washington. Populations of P. staminea were found to be highly differentiated at all loci surveyed, whereas the M. balthica populations were significantly different at only one locus using both the genic and genotypic tests. While it is possible that allozymes are not variable enough to detect differences between the populations of M. balthica, it is likely that species-specific selective pressures also play a role in structuring these populations.

Protothaca staminea and *Macoma balthica* occupy very different ecological niches. It is possible that these two species experience different selective pressures in Puget Sound from the physical environment or from local predators, including humans (van der Veer et al. 1998, Ejdung & Elmgren 1998, Chew & Ma 1987). P

TABLE 1.

Allele frequencies at loci for *Protothaca staminea* and *Macoma balthica* individuals from three locations in Puget Sound, WA

Locus, allele	*Protothaca staminea*			Locus, allele	*Macoma balthica*		
	Potlatch	Edmonds	Priest Pt.		Potlach	Skagit	Tolmie
GPI							
−17	0.011	0.000	0.000	−22	0.004*	0.004*	0.000
14	0.074	0.039	0.138	8	0.009	0.019	0.022
36	0.420	0.237	0.170	38	0.434	0.481	0.457
58	0.351	0.202	0.589	66	0.128	0.092	0.116
77	0.112	0.167	0.085	100	0.376	0.385	0.353
100	0.032	0.285	0.013	130	0.049	0.019	0.052
127	0.000	0.070	0.004*	(N)	(113)	(130)	(116)
(N)	(94)	(114)	(112)	H_E	0.654	0.614	0.652
H_E	0.685	0.791	0.600	F_{IS}	−0.014	−0.027	−0.097
F_{IS}	0.177	0.358	−0.012				
PGM							
66	0.101	0.108	0.090	38	0.018	0.008	0.030
85	0.261	0.171	0.232	62	0.159	0.129	0.156
100	0.314	0.230	0.602	86	0.053	0.057	0.065
112	0.245	0.387	0.076	100	0.611	0.621	0.593
132	0.080	0.104	0.000	124	0.124	0.159	0.113
(N)	(94)	(111)	(105)	154	0.035	0.027	0.043
H_E	0.761	0.750	0.574	(N)	(113)	(132)	(115)
F_{IS}	0.148	0.267	0.238	H_I	0.585	0.570	0.609
				F_{IS}	0.107	0.097	0.000
AAT							
−1500	0.000	0.004*	0.000	70	0.004*	0.000	0.004*
−700	0.293	0.180	0.009	82	0.062	0.036	0.039
−100	0.670	0.798	0.947	94	0.013	0.008	0.000
500	0.021	0.004	0.044	100	0.903	0.933	0.953
900	0.016	0.013	0.000	124	0.018	0.020	0.004*
(N)	(94)	(114)	(114)	150	0.000	0.004*	0.000
H_E	0.467	0.332	0.101	(N)	(113)	(126)	(116)
F_{IS}	0.112	0.207	−0.043	H_I	0.182	0.129	0.091
				F_{IS}	0.124	0.017	0.152
PGDH							
−1100	0.048	0.024	0.253	62	0.004*	0.004*	0.000
−600	0.425	0.524	0.552	74	0.022	0.068	0.030
−100	0.495	0.423	0.155	82	0.157	0.209	0.129
500	0.011	0.014	0.041	90	0.511	0.392	0.500
1000	0.022	0.014	0.000	100	0.305	0.316	0.341
(N)	(93)	(104)	(97)	114	0.000	0.011	0.000
H_E	0.575	0.548	0.599	(N)	(111)	(131)	(116)
F_{IS}	0.178	0.299	0.333	H_I	0.625	0.700	0.619
				F_{IS}	0.063	0.052	0.025
ESTD-2							
75	0.000	0.045	0.004*	90	0.081	0.116	0.078
84	0.202	0.134	0.425	100	0.720	0.633	0.759
92	0.069	0.290	0.294	106	0.199	0.251	0.164
100	0.723	0.513	0.276	(N)	(105)	(129)	(116)
111	0.005	0.018	0.000	H_I	0.443	0.526	0.393
(N)	(94)	(112)	(114)	F_{IS}	0.448	0.485	0.519
H_E	0.433	0.635	0.659				
F_{IS}	0.166	0.270	0.002				

N = the number of clams scored in each collection. Frequencies in bold indicate private alleles. Asterisks (*) indicate rare alleles (frequencies <0.005). H_I = expected heterozygosities; F_{IS} = fixation index for individuals within each population.

staminea has a larger adult size and often occupies much more sandy substrates than *M. balthica*. Sanchez-Salazar et al. (1987a, 1987b) demonstrated the influence both tidal elevation and shore crabs can have on the population structure of the bivalve, *Ceras-toderma edule*. The recreational harvest of *P. staminea* in Puget Sound may also contribute to selective pressures in this species. In addition, harvesting of *P. staminea* may reduce its effective population size (N_e), contributing to differentiation of populations

TABLE 2.

Probability values for the test of heterozygote deficiency relative to Hardy-Weinberg expectations at each locus for each population

Locus	Protothaca staminea			Locus	Macoma balthica		
	Potlach	Edmonds	Priest Pt.		Potlach	Skagit	Tolmie
GPI	0.096	<0.001*	0.347	GPI	0.333	0.835	0.942
PGM	0.084	<0.001*	0.035*	PGM	0.100	0.403	0.173
PGDH	<0.001*	0.057	<0.001*	PGDH	0.123	0.097	0.474
AAT	0.019*	0.030*	1.000	IDHP	0.087	0.447	0.149
ESTD-2	0.008*	<0.001*	0.383	ESTD	<0.001*	<0.001*	<0.001*

Asterisks (*) indicate significant heterozygote deficiencies (P < 0.05).

through genetic drift. *M. balthica* is too small to attract recreational or commercial interest and may therefore also have a much larger N_e. Additionally, neither selective pressures nor genetic drift may be strong enough to drive population differentiation of *M. balthica* if there are sufficient migrants to homogenize the populations (Hartl & Clark 1997).

Exchange of individuals between populations may be facilitated by larval behavior. The planktonic larvae of many estuarine invertebrates do not behave as strictly passive particles, instead exhibiting selective transport in horizontal currents mediated by vertical migration (Morgan 1995). Although the most extensive research has focused on crustaceans (e.g., Sandifer 1975, Cronin 1982, Forward et al. 1995), a few studies have confirmed selective larval transport among bivalves (Wood & Hargis 1971, Manuel et al. 1997). It is possible that *P. staminea* and *M. balthica* larvae exhibit divergent swimming behaviors that could affect their transport out of their respective estuarine basins of origin in Puget Sound. Unfortunately, there have not been any studies investigating vertical migration behavior of *P. staminea* larvae. Work by Roegner (2000) suggests that the larvae of *M. balthica* are passively distributed. However, there is evidence for selective post-metamorphic drifting of *M. balthica* juveniles (Beukema & de Vlas 1989). Byssal threads attached to these post-larvae provide drag and lift allowing transport on horizontal flow. A recent study of *Macoma* spp. post-larval distributions in the York River estuary of the Chesapeake Bay strongly suggests that this life-history stage exerts a behavioral control over position in the water column (Garrison & Morgan 1999). Because byssal thread-drifting has not been demonstrated in *P. staminea*, one possibility is that *M. balthica* populations in Puget Sound are less differentiated due to selective thread-drifting of the post-metamorphic juveniles.

TABLE 3.

Probability values for the genic and genotypic tests for population differentiation of three *Protothaca staminea* and three *Macoma balthica* populations

Locus	Protothaca staminea		Locus	Macoma balthica	
	Genic test	Genotypic test		Genic test	Genotypic test
GPI	<0.001*	<0.001*	GPI	0.467	0.524
PGM	<0.001*	<0.001*	PGM	0.589	0.681
AAT	<0.001*	<0.001*	IDHP	0.273	0.320
PGDH	<0.001*	<0.001*	PGDH	0.011*	0.007*
ESTD-2	<0.001*	<0.001*	ESTD	0.043*	0.140

Asterisks (*) indicate significant differences (P < 0.05).

P. staminea *Populations May Be Constrained by Puget Sound Hydrology*

Because we found substantial differentiation among *Protothaca staminea* populations, we hypothesize that gene flow between these populations may indeed be restricted. The chord distances as well as Nei's genetic distances suggest that the populations of *P. staminea* in Hood Canal and the Main Basin are more similar to each other than either is to the South Sound population (Table 4). Hydrology of the Puget Sound estuary supports the hypothesis of South Sound isolation. Cokelet et al. (1991) determined that as much as 52% of the water entering Admiralty Inlet from Puget Sound is recycled back through mixing at the sill (Fig. 1). This refluxing coupled with their proximity suggests a large potential for exchange between Hood Canal and the Main Basin. Cokelet et al. (1991) also estimated that the longest residence times in Puget Sound are for waters originating in the southernmost reaches of the Sound. Populations from the South Sound and Main Basin might therefore be restricted in their ability to exchange individuals. In fact, there are two minor sills and one major sill (at Tacoma Narrows) between the Priest Point population in South Sound and the Edmonds population in the Main Basin. Recently, the slow flushing times of South Sound have been implicated in the die-off of a number of benthic species, perhaps due to pollutant retention (Ebbesmeyer et al., 1998). It remains to be seen whether the refluxing of South Sound waters is directly preventing dispersal of *P. staminea* larvae. There is, however, a correlation between the observed genetic pattern and the expected circulation pattern of Puget Sound.

Deviations from Hardy–Weinberg Equilibrium

Heterozygote deficiencies are commonly found with a variety of molecular methods, especially in marine bivalve populations (Raymond et al. 1997, Gaffney 1994, Zouros & Foltz 1984, Singh

TABLE 4.

Genetic distance measures for the three *Protothaca staminea* populations (Edm = Edmonds; Pot = Potlatch; PPt = Priest Pt.)

	Protothaca staminea, all loci		
	Edm-Pot	Edm-PPt	Pot-PPt
CSE	0.1997	0.3141	0.2858
NEI	0.0723	0.1947	0.2085

CSE = Cavalli-Sforza and Edwards (1967) chord distance; NEI = Nei's distance from Nei (1972).

& Green 1984). Often, heterozygote deficiencies are indicative of reproductive isolation resulting in inbreeding. Additional causes ascribed to heterozygote deficiencies are wide ranging but may include aneuploidy, molecular imprinting, genotype-dependent spawning, selection, population mixing, null alleles, scoring bias, and tissue degradation. Aneuploidy, molecular imprinting, and genotype-dependent spawning have not been reported for either of these species and there is little evidence to support these phenomena in bivalves. Heterozygote deficiencies resulting from spatial population mixing do not seem likely either, given the large sampling area (100- to 500-m transects), high abundances, and long pelagic phases of these two species in Puget Sound.

However, it is possible that we encountered temporal population mixing since we likely sampled over several generations by sampling over a wide range of sizes. It has been hypothesized that the chance reproductive success of free-spawners may lead to large variances in the genetic composition of each successive generation due to random drift (Hedgecock 1994). The result is a small number of individuals contributing disproportionately to the next generation. Sampling across these generations may lead to temporal population mixing, also know as a temporal Wahlund effect. To maintain differences between year-classes, selection and/or assortative mating may also be occurring (Hartl & Clark 1997). Similar to Ruzzante et al. (1996), we investigated the effect of pooled age-classes on Hardy-Weinberg equilibrium by dividing the *P. staminea* individuals into large- and small-size classes and re-testing for heterozygote deficiencies. For all populations, the number of loci with heterozygote deficiencies decreased in both size classes except one (small class, Potlatch) compared with populations that had both size classes pooled (data not shown). This suggests that pooling the size classes may have contributed to the observed heterozygote deficiencies.

Selection may also act to reduce the number of heterozygotes in a population. An ongoing debate in bivalve genetics is the apparent paradox between observations of hybrid vigor and heterozygote deficiencies. Individuals in both the laboratory and natural field populations demonstrate strong correlations between heterozygosity and fitness-related traits, e.g., size, growth rate, and reproductive capacity (Hedgecock et al. 1996, Zouros 1987). Yet field populations of many bivalve species are heterozygote deficient. One possible explanation is genotype-dependent larval mortality (Singh & Green 1984, Zouros & Foltz, 1984). Investigating the timing of the heterozygote deficit, Fairbrother and Beaumont (1993) found heterozygote deficiencies in a cohort of newly settled mussel (*Mytilus edulis*) spat, concluding that the loss of heterozygotes must have occurred during the larval stage or early settlement. Singh (1982) suggested that selection might act against the more heterozygous, faster-growing larvae because of their increased food requirements during the critical period of larval development. If plankton abundances are not high during this period,

these larvae face a greater mortality. This phenomenon has yet to be investigated in either *P. staminea* or *M. balthica*.

Finally, the presence of null alleles may also contribute to the observed deficiencies. It is possible that either true null alleles or artifacts, such as insufficient tissue or staining inconsistencies, caused the deficiency in the one locus (*ESTD*) across all *Macoma balthica* populations. However, heterozygote deficiencies occurred in most loci and in all populations of *Protothaca staminea*, suggesting null alleles are not sufficient to explain the observed deficiencies in this species. For these populations, selection and inbreeding due to partial reproductive isolation could explain the deficiencies we observed. In addition, it is possible that we encountered a temporal Wahlund effect in the *P. staminea* populations. Importantly, when all other allele frequencies were corrected for the presence of a null allele and the analytical tests re-run, the population differentiation conclusions did not change.

CONCLUSIONS

Many factors may contribute to population differentiation of marine invertebrates in Puget Sound. To prevent genetic homogeneity over such a small geographic scale, however, selective forces must be strong, gene flow must be restricted, and/or temporal variance of the populations must be extreme. Environmental fluctuations can be dramatic in the estuarine ecosystem. Extremes of salinity and temperature can be found over small spatial scales. In such a heterogeneous environment, selection may take the form of both physical and biological constraints. They may act in concert to vary pressures on adult clams or the recruiting larvae. Populations may vary from generation to generation simply due to pulsed recruitment or sweepstakes sampling from the previous generation. Factors that might limit gene flow between populations in this estuary include large-scale reflux via mixing at sills, larval behavior, or small-scale circulation patterns such as nearshore eddies. We have demonstrated a correlation between the population differentiation of *P. staminea* and the circulation pattern of Puget Sound warranting further study of the effects of Puget Sound hydrology on larval dispersal. The hydrology of Puget Sound, however, does not ensure differentiation in every species. In stark contrast to *P. staminea*, we have shown that *M. balthica* populations reveal little differentiation among the same basins. The amount of differentiation between sites is highly species dependent, and therefore population dynamics should not be generalized based on reproductive characters alone.

ACKNOWLEDGMENTS

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SHELL REPAIR OF MECHANICALLY INDUCED FRACTURES IN *MERCENARIA MERCENARIA* UNDER EXPERIMENTALLY SUBOPTIMUM CONDITIONS

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ABSTRACT Sixty hand-tonged, harvested specimens of *Mercenaria mercenaria* from wild stock in Raritan Bay, New Jersey, measuring 34 to 43 mm in dorsal ventral length, were apportioned among buckets of sediments submerged in predator-excluded flow-through tanks. Experimental sediments simulate substrata found native to hard clams and included: (1) well-sorted sand, (2) pure mud, (3), an admixture of equal volume of shell-free sand and mud, (4) an admixture of 75% sand and 25% shell hash, and (5) an admixture of 75% mud and 25% shell hash. Hand-excavated clams reburrowed monthly for one year. Progressively dysoxic interstitial pore water beneath the sediment interface mediated burrowing conditions. Shells of live specimens in progressively blackened sands became chalky in appearance with ornamentation completely abraded and/or etched away. Upon sacrifice, 30 (50%) specimens revealed fractures in the valve interior that radiated from the ventral (24), posterior (four), and anterior (two) margins, whereas only five of 36 (14%) specimens in the unburrowed "control" group showed anthropogenically (harvesting and machine-sorting) induced microfractures at the ventral margin. Mean annual dorsal-ventral shell accretion was negligible under these experimentally suboptimal conditions. Distribution of fractured specimens among the five experimental substrata is statistically random, although, paradoxically, more clams that reburrowed in mud than sand-shell hash had internally repaired valves. Severity of fractures is evidenced by stuccoed cracks that encroached within a cm of the dorsal hinge and others that bifurcated and deflected though the adductor muscle scars. Converged fractures in one reburrowed specimen removed a large triangular wedge of shell that proved lethal. Nevertheless, repaired fractures did not fail under the strain of repeated re-burrowing.

KEY WORDS: *Mercenaria mercenaria*, burrowing, fracture, repair, abrasion

INTRODUCTION

Lethal and sublethal shell fractures in *Mercenaria mercenaria* have been primarily attributed to durophagous predators. The toll these molluscivores inflict on this commercially valuable species has been reviewed by Krauter (2001). Dredging activity also may sublethally fracture shells of commercially valuable clams as observed in commercially harvested *Glycymeris glycymeris* (Ramsey et al. 2000), *Ensis siliqua* (Gaspar et al. 1994), *Solen* sp. (Bergman & Hup 1992), and *Arctica islandica* (Witbaard & Klein 1994). Scar frequencies have been used to attempt reconstruction of the history of past shellfishing pressure. Another possible non-predatory cause of shell fracture in bivalves is burrowing, although such shell-fracturing mechanical processes have been infrequently investigated experimentally. Checa (1993) illustrated specimens of the thin-shelled deep bivalves *Lutraria lutraria*, *Panopea gylcymeris*, and *Solecurtus strigalatus* with scars of repaired cracks induced by reburrowing by individuals that were prone to excavation by winter storm waves.

However, repair of burrowing-induced fractures and its frequency has not been documented in a shallow-burrowing, thicker-shelled, and commercially harvested clam, such as *Mercenaria mercenaria*. The extent to which such mechanically induced fractures can be repaired is unreported. Appreciable abrasion of the ventral margin of *M. mercenaria* has been documented in transplant experiments (Pannella & MacClintock 1968, Rhoads & Panella 1970, Kennish 1978). Repeated burrowing may chip the commissure margin of some young adults of *M. mercenaria*, thereby providing a site for initiation of dorsally propagated fractures. Conceivably, sediment texture and cohesiveness could influence both sediment loading against the valves (Checa 1993) and/or the likelihood that shell shards become occluded between the valves during the repeatedly opening and adduction of the valves. Reburrowing may provide the additional stress on valves marginally chipped by the commercial excavation, handling, and

sorting processes. Raked specimens, jostling against each other in transport and sorted by conveyor-belt into bags of commercially graded sizes may bear very slightly chipped margins that could become the initiation sites of fractures if the clams are afforded an opportunity to reburrow. Conceivably, sediment texture may be causally related to frequencies of (1) anthropogenically induced microfractures that are propagated through the valve during reburrowing, and/or (2) burrowing-induced microfractures that are further expressed during repeated penetration of the sediment. Sediment texture may also influence interstitial water chemistry mediated by sediment porosity and permeability. Substrates of different mean grain sizes and degree of sorting have different porosity and permeability properties. Suboptimum interstitial conditions beneath the sediment surface where the clam burrows also may influence both shell fracture propagation and the ability of the mantle to repair cracks.

Accordingly, this investigation experimentally focuses on the repair of nonpredatory shell fractures in young adults of *M. mercenaria* that repeatedly burrow into various textured sediments. The testable, refutable null hypotheses are (1) that microfractures possibly initiated by anthropogenic excavation and handling are repaired prior to or during reburrowing activity, (2) that the burrowing process also initiates microfractures that are repairable, (3) repaired fractures withstand the strain induced by reburrowing, (4) that no significant difference in the frequency of fractures results from reburrowing in different textured sediments, and (5) that no significant change in valve thickness and external ornamentation resulted from re-burrowing in different textured sediments.

METHODS AND MATERIALS

Within Raritan Bay, New Jersey–New York, commercial shellfish beds, some situated in depths above effective storm wave base, include sediments characterized as mud, shell, gravel, sand, and sand-mud that host varying densities of *M. mercenaria*. To test

the effect that sediment texture has on shell abrasion, chipping, and fracture-initiation or propagation in *M. mercenaria*, five 12-L buckets of sediment were submerged in each of two 690-L flow through tanks at the NOAA Laboratory at Sandy Hook, New Jersey, which pumps in water from Raritan Bay. Each bucket was filled with a substratum to a 14 cm depth, resulting in the sediment surface recessed about 4 cm form the top of the bucket. Enclosed substrate included one of five types of sediments and shell hash native to Raritan Bay to simulate the various substrata naturally occupied by *M. mercenaria*. The five sediment categories included (1) sieved, intertidal sand void of any gravel size grains and shell fragments, (2) pure mud, (3), an admixture of shell-free intertidal sand (50% by volume) and mud (50% by volume), (4) an admixture of 75% by volume of beach sand and 25% by volume of shell hash, and (5) an admixture of 75% by volume of mud and 25% by volume shell hash. Shell hash included shards of razor clams (*Ensis directus*), blue mussels (*Mytilus edulis*), surfclams (*Spisula solidissima*), and hard clams (*M. mercenaria*) created by mortar and pestol. The longest dimension of any shell shard did not exceed 4 mm. Admixtures of sediment types were thoroughly mixed with a trowel to homogenize the substrates. Two replicates of each substratum were created, one for each flow through tank.

Sixty hand-raked, machine-sorted, specimens of *M. mercenaria* obtained from a depuration plant operating in Raritan Bay were measured dorsal-ventrally (= shell length), and perpendicular to the hinge line at the point of maximum curvature or maximum cross-sectional height (= shell height) to the nearest 0. 1 mm by means of electronic vernier calipers. All specimens ranged from 34 to 43 mm in dorsal-ventral length. Initial scrutiny of the specimens revealed no hairline fractures expressed on the valve exteriors. A separate batch of 36 machine-sorted specimens from the depuration plant, measuring 34–41 mm in DV length, were held in an aquarium without sediment for four weeks and then sacrificed to determine if commercial harvesting and handling could have initiated any interior fractures in the shells prior to reburrowing. Among the 60 experimental clams, six specimens were assigned to each of the 10 buckets of substrata and placed reclining on one valve in a clockwork arrangement (12, 3, 6, and 9 o'clock with two specimens at the center) on the sediment in May 1998. Acclimation to the conditions in the tanks occurred during the ensuing summer months. Monitoring of changes in the shell dimensions and external surface appearance commenced in October 1998 and lasted through October 1999.

The flow of water discharged into each tank was maintained at nearly 20 cm/s. Discharge occurred from eight 3-mm diameter perforations along the length of 30-mm diameter pipe that jetted water into the tank. These perforations are too narrow to allow metamorphosed clam predators to enter the tanks. Nevertheless, tanks were checked monthly for incidental invasions. None were found. Water exited the tanks from two vertical oriented, overflow drains at each end of the 70-cm deep tank. Twice a month the dissolved oxygen, salinity, temperature, and pH were recorded for each tank by means of a portable hydrolab. A Marsh McBirney current meter checked the flow velocity jetting from perforations in the tube in the tank twice a month. The tanks were not dosed with any algal extract to enhance clam growth during the experiments.

The clams were excavated by hand from their buckets monthly, and their shell length, and height recorded after any adhering sediment was washed off from the valve exteriors. This procedure was followed monthly from October 1998 until October 1999 when the clams were sacrificed. No data were collected in May 1999. Dead specimens were cleaned and examined for abrasion, fracture, and repair. No specimen showed infestation with the boring sponge *Cliona* sp.

Fractures and repairs among *M. mercenaria* at the end of experimental interval were described and categorized as to (1) fracture expression (crack visible on interior or exterior of valve, or both), (2) valves affected by fracture (right, left, or both), (3) number of fractures per valve, (4) length of fractures, (5) fracture initiation site at, or very near the valve margin (ventral, posterior, anterior), (6) fracture propagation inward from the valve margin (diagonal, curved, right angled deflections, merging and/or bifurcating), and (7) state of fracture repair (internally stuccoed cracks or unrepaired). A Goodness of fit test determines (1) if sublethal fractures occur randomly among specimens in different textured sediments, (2) if fractures occur randomly around the shell margin (posteriorly, ventrally, or anteriorly), and (3) if fractures propagate in a restricted pathway. A t test determined (1) if fractured and unfractured specimens differ according to valve thickness at the ventral margin and (2) if repair condition (stuccoed vs. unrepaired) differs according to fracture length.

Additionally, at the conclusion of the 12-month monitoring period, the valve surface of each surviving clam was examined under magnification and the degree of shell abrasion and/or surficial etching categorized according to the relief of the concentric lamellae as (1) abrasion-negligible, (2) abrasion/etching—slight; wear restricted to ventral area, (3) abrasion/etching—moderate; shiny, bare patches over central and ventral valve area, and (4) abrasion/etching—extensive; obliteration of concentric lamellae over most of valve surface area. It should be noted that abrasion and etching must be distinguished from ontogenetic changes in shell micro-ornament over the valve surface. A swath of the central valve of *M. mercenaria* inherently lacks micro-ornamentation in adulthood, although the entire valve surface of many juveniles to young adults possess fine concentric ribbing.

RESULTS AND ANALYSIS

Among the 36 "control" specimens held in an aquarium and sacrificed after 4 weeks, five showed microfractures radiating dorsally from the ventral margin that were most probably induced anthropogenically during raking, transport, and/or machine-sorting. None showed any signs of repair.

The pH in the experimental tanks holding the sixty specimens fluctuated from 7.0 to 8.0 during the 16 mo interval (Fig. 1). The dissolved oxygen ranged from 3.4 to 6.7 mg/L over the same time frame (Fig. 1). Temperature changed seasonally, peaking in the summer at 25°C, and dropping to a low of 8°C in the winter months (Fig. 1). Salinity fluctuated (sub) parallel with temperature, ranging from a high of 28 ppt in November 98 to a low of 22 ppt in March 99 (Fig. 1). Current velocity from the pipe perforations ranged between 16 cm/s and 24 cm/s over the 16-month interval (Fig. 1)

Of the sixty experimental specimens, 30 were fractured sublethally (Fig. 2A–G) and one lethally (Fig. 2H). Of those fractured sublethally, the crack was visible on the valve interior exclusively in 20 specimens. For 10 specimens, the fracture was evident on both the valve interior and, faintly, on the exterior (Fig. 2A; Table 1). In 12 specimens, the fracture occurred in both opposing valves (Table 1; Fig. 2A). Eleven specimens had a crack in the right valve only and seven had a fracture in the left valve only (Table 1).
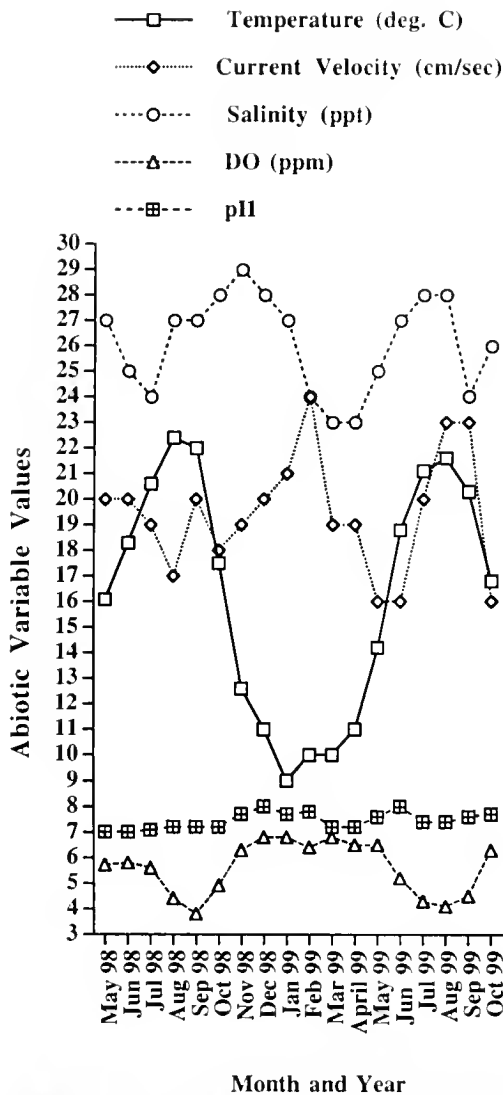
Month and Year

Figure 1. Monthly fluctuations in monitored abiotic variables in flow through tanks with *M. mercenaria* at NOAA laboratory, Sandy Hook, New Jersey.

Fracture length varied from 4 mm to 38 mm. Very long, stuccoed fractures propagated to within 5 mm of the dorsal margin of the clam (Fig. 2B). One specimen had three fractures in one valve and five specimens had two fractures in one valve. Short cracks (5–10 mm) that extended dorsally from or near the ventral margin to the pallial line, but not beyond, were unrepaired (= unstuccoed; Table 1). However, cracks longer than 15mm, extending dorsally beyond the pallial line, were repaired by the mantle (Table 1).

Fractures were initiated at or very near the ventral margin (Fig. 2A and B) in 24 specimens, at the posterior margin (Fig. 2C) in four specimens, and the anterior margin in two specimens. Goodness of fit test revealed that this distribution is nonrandom (Table 1). Thirteen fractures radiated inward on a diagonal from the ventral valve margin, slightly oblique to the dorsal-ventral axis (Fig. 2D; Table 1). Six fractures curved and three had sharp right angle deflections cutting through the edge of the adductor muscle scars in two instances (Fig. 2E–F; Table 1). Two fractures bifurcate near the center of the valve (Fig. 2G). Two cracks converge inward from the ventral margin. One convergence of cracks resulted in a lethal fracture (Fig. 2H).

Thirty-six specimens (62%) have chipped ventral margins. Nevertheless, only 13 of the 30 fractures radiated dorsally from a chipped point on a valve margin (Fig. 3A and B). Resecretion of a small, v-shaped wedge to fill in the chipped ventral margin accompanied mortaring of the fracture in one specimen (Fig. 3C). Seventeen fractures became fainter between the pallial line and the ventral margin, and cannot be traced to the very edge of the shell.

Although the highest frequency of sublethal shell breakage occurred in clams burrowing into pure mud (9 of 12), the distribution of fractures is statistically random (Fig. 4). Ventral margin thickness had no bearing on which valves fractured (Table 1). Fractures were just as likely to be confined to one valve as to be mirrored in both valves (Table 1). Furthermore, valve fractures occurred mostly in mud-burrowing specimens (Fig. 4), and appears to be to be independent of the degree of external valve abrasion, which is most severe in sand-shell burrowing specimens (Fig. 5). Two thirds of the specimens that burrowed in sand had the concentric lamellae obliterated on all areas of the valves (Fig. 3D), whereas 18 specimens of the 24 that burrowed into mud and mud-shell had only slight ventral abrasion (Fig. 5).

Accretion along the ventral margin was suppressed under these experimental conditions. Mean annual increase in dorsal-ventral shell length varied from only 0.45 mm in clams kept in sand to 1.3 mm for clams kept in mud (Fig. 6). Clams reared in sand and shell-sand showed an annual decrease (<0.05 mm) in cross-sectional shell height whereas clams reared in mud and shell-mud showed an annual increase of ~0.3 mm in cross-sectional shell height (Fig. 7).

DISCUSSION

These experiments indicate that monthly reburrowing by young adults increases the risk of either self-induced shell-breakage or the propagation of fractures induced anthropogenically. The fact that 50% of the experimental clams had fractures when sacrificed but only 14% of the control group had fractures indicates that the burrowing process is responsible for initiation and propagation for many, if not the majority of fractures. These experiments do not, however, indicate a threshold of reburrowing frequencies at which fracturing is likely to be initiated or expressed.

The rate of repair of the fractures also cannot be precisely established, although repair of fractures induced by burrowing possibly occurred between monthly reburrowing episodes. In a separate study, seed of *M. mercenaria* 15-25 mm in dorsal-ventral length were able to resecrete 2- to 3-mm long notches beveled by a high-speed Dremel in the anterior, posterior, and ventral valve margins within 2 weeks while living caged on an intertidal flat in North Carolina (Fig. 8; Alexander & Dietl, in preparation). Serrations or contiguous scallops in the valve posterior (Fig. 3E) may be lethally inflicted on young adult, intertidal *M. mercenaria* by wading birds (Krauter 2001). Conditions on the North Carolina mudflat subjected to tidal flushing facilitated rapid repair with preclusion of predators. The shell repair processes may have been retarded under suboptimum laboratory conditions at Sandy Hook, New Jersey. Furthermore, internal fractures may be stuccoed (Fig. 2) at a different rate than notches of the valve margin are filled in by resecreted shell (Fig. 8). Nevertheless, the repair of the internal fractures within one month. i.e., between reburrowing sequences, is a realistic estimate given the much shorter time it takes to repair notches around the valve margin.

Regardless of the timing of initiation of the fractures, or their
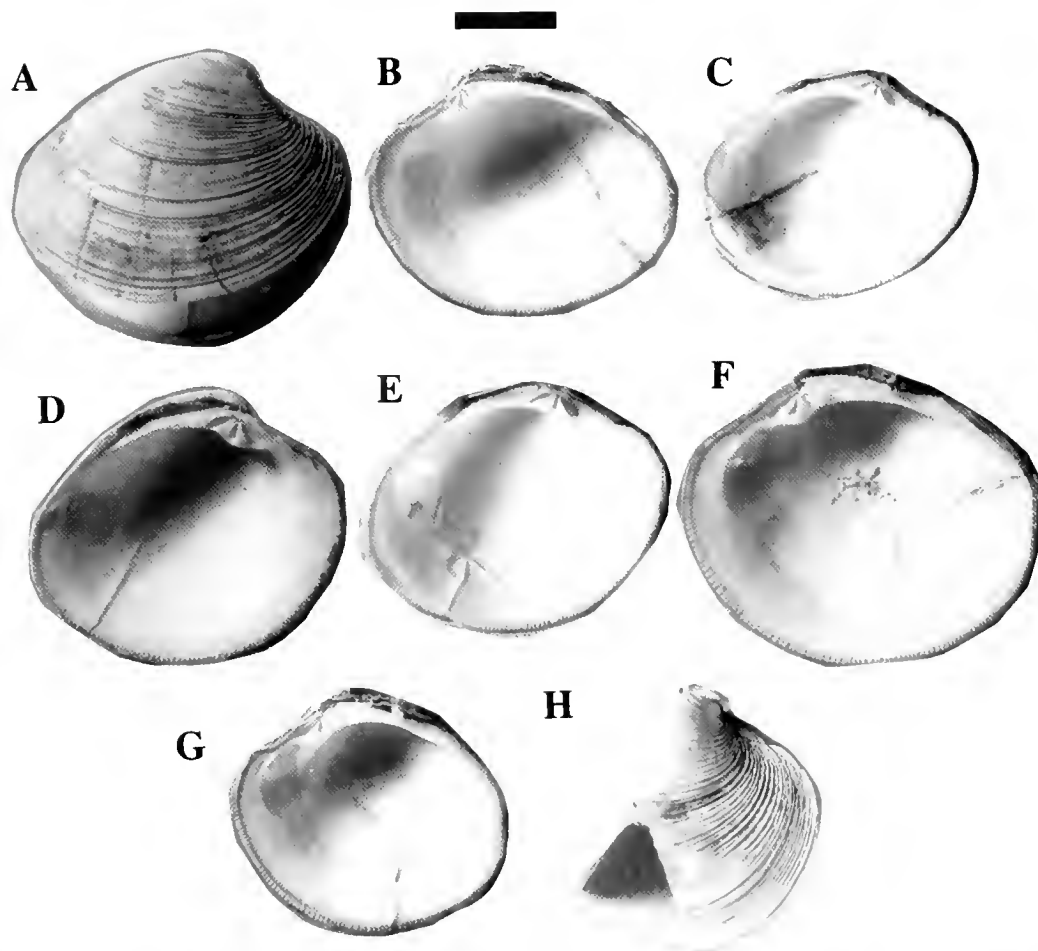
Figure 2. Expression of internal, stuccoed, sublethal fractures, and lethal breakage in valves of *M. mercenaria* that repeatedly rehurrowed in sediment. Width of bar = one cm. A, External and internal expression of sublethal fractures in opposing valves. B, Stuccoed fracture radiating from posterior-ventral margin to within 5 mm of dorsal hinge. C, Stuccoed fracture radiating from posterior margin. D, Stuccoed linear fracture from ventral margin. E, Stuccoed ventral fractures that merge and deflect through edge of adductor muscle scar. F, Stuccoed fracture that makes right angle deflection through adductor muscle scar. G, Bifurcating, stuccoed fracture radiating from ventral margin. H, Lethal fractures that merged, resulting in removal of large triangular piece of valve.

propagation, during the months of reburrowing, these experiments complement the experimental results of Checa (1993) who demonstrated that reburrowing only once fractured the valves of the deep-burrowing *Solecurtus strigalatus*. However, fractures in the shallow-burrowing *M. mercenaria* were not necessarily invariably induced by sediment-loading against the hardclam valve exteriors as advocated by Checa (1993) for *S. strigalatus*. First, the experimental hard clam specimens never burrowed deeper than 10 cm (maximum sediment depth 14 cm) in contrast to the deep burrowing (>40 mm beneath the sediment surface), thin shelled clams studied by Checa (1993). Second, three times as many fractures are visible on the in the interior of the valve rather than the exterior of *M. mercenaria*. Yet all of Checa's (1993) illustrated examples show external expression of the fractures. Eschewing those specimens fractured by anthropogenic handling, these observations are congruent with the argument that closure of the valves on sediment grains or shell shards introduced between the valves fractured the ventral margin and valve interior of many if not most of the specimens.

The high percentage of cracks (67%) that did not propagate from the valve interior to be expressed on the valve exterior indicates that fracture propagation was halted at the annual growth increment discontinuities in the shell microstructure of *M. mercenaria*. The valve microstructure consists of overlapping layers of crossed lamellar aragonite (Boggild 1930) bounded by organic films (Pannella & Maclintock 1968, Rhoads & Pannella 1970, Kennish 1980). Although all but six of the fractures were initiated near the ventral margin, the fact that 17 of the 30 cracks did not radiate from a chipped point on the valve margins, but instead disappear within 1 to 2 mm of ventral margin, suggests that chipping of the margin is not invariably the progenitor of fractures. The faint expression of the fractures in the area between the pallial line and the ventral margin coincides with the thicker part of the shell relative to shell thickness dorsal to the pallial line. Fractures may have originated dorsal to the pallial line, dissipating before cracking the entire thicker area between the pallial line and the ventral margin.

Although contrasting sediment textures did not statistically significantly differentiate the frequency of fractures among this sample of *M. mercenaria* (Fig. 4), the greater frequency of sublethal fractures among clams that burrowed in mud (nine) vs. sand (three) and shell-mud (six) is counterintuitive. If adduction of the valves upon clasts introduced between the valves during burrowing caused the fractures, the probability of encountering shell

TABLE 1.

Distribution and morphology of fractures induced by burrowing of 60 specimens of *Mercenaria mercenaria*.

Mean ventral valve thickness of fractured vs. unfractured shells			Fractures = 1.5 mm	Unfractured = 1.5 mm		t test; P = 0.71; Accept Ho (means are equal)
Mean length for stuccoed vs. unplastered shell fractures			Stuccoed = 20 mm	Unplastered = 12 mm		t test; $P < 0.001$; Reject Ho (means are unequal)
Location of fracture initiation on valve margin		Posterior margin = 4	Anterior margin = 2	Ventral margin = 24		Goodness of Fit; χ^2 = 29.6 with 2 df; Reject Ho at P = 0.01 (nonrandom distribution)
Expression of fracture	Valve interior only = 20	Valve exterior only = 0	Both sides of valve = 10			Goodness of Fit; χ^2 = 20.0 with 2 df; Reject Ho at P = 0.01 (nonrandom distribution)
Fracture-affected valves	Right = 11	Left = 7	Both = 12			Goodness of Fit; χ^2 = 1.2 with 2 df; Accept Ho (distribution random)
Propagation of fracture	Dorsal-ventrally straight = 5	Dorsal-ventrally curved = 7	Rt. angle deflection = 3*	Merging and branching = 4	Diagonal to dorsal-ventral axis = 13	Goodness of Fit; χ^2 = 10.22 with 4 df; Reject Ho at P = 0.05 (nonrandom distribution)

* Two stuccoed fractures cut across muscle scar area.

shards during burrowing would be highest in the sediment admixtures with 25% by volume shell hash. Furthermore, burrowing in sand increased external shell abrasion, including the ventral margin, (Fig. 5), but any ensuing chipping of the ventral margin did not increase the frequency of fractures propagated dorsally. As previously noted, only 13 of the 30 fractures can be traced from a chipped point on the posterior-ventral margin. One possible explanation is that the initial commercial excavation and handling of the specimens induced the fractures, and more specimens with microfractures were fortuitously placed on the muddy versus the sandy susbstrata. Given the probability of the low percentage (14%) of specimens with fractures induced before the burrowing

experiment commenced, based on extrapolation from the control group, it is unlikely that a preponderance of the few clams fractured before commencement of the experiments were experimentally placed on mud.

It should be noted that the interstitial water in the sand and sand-shell hash had become blackened during the experiments with accumulated fecal matter in the sediment interstices a few cm beneath the sediment surface before the conclusion of the experiments. This accumulation of organic matter occurred despite hand-tilling of these sediments each month during excavation of the specimens. Valve surfaces became slightly chalky in appearance, but if the valve skeletal microstructure was altered and mechani-
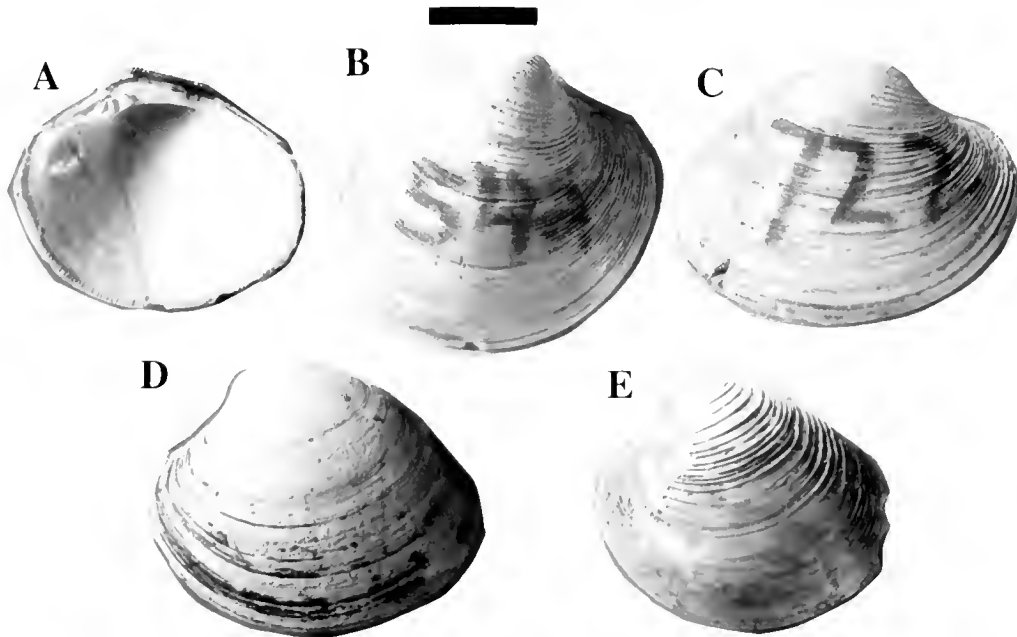
Figure 3. Valves of *M. mercenaria* with chipped margins, resecreted valve wedges, and degree of abrasion following monthly reburrowing into sediment. Width of bar = one cm. A, Specimen with chipped ventral margin from which crack radiates dorsally. B, Ventrally chipped margin with faint expression of dorsally radiating fracture. Note also abrasion of concentric micro-ornament limited to ventral margin of specimen. C, Specimen with secreted wedge at ventral margin filling in small triangular piece of shell removed by cracks. Internally, fractures are stuccoed. D, Complete obliteration of micro-ornament on valve exterior of specimen that monthly reburrowed into sand. E, Predator-induced, contiguous divots at posterior shell margin

Reburrowed *Mercenaria   mercenaria*

- ⊡ Repaired Fracture; mean valve thickness = 1.5 mm
- ▨ Lethal Fracture; mean valve thickness = 1.5 mm
- ■ Unfractured; mean valve thickness = 1.5 mm
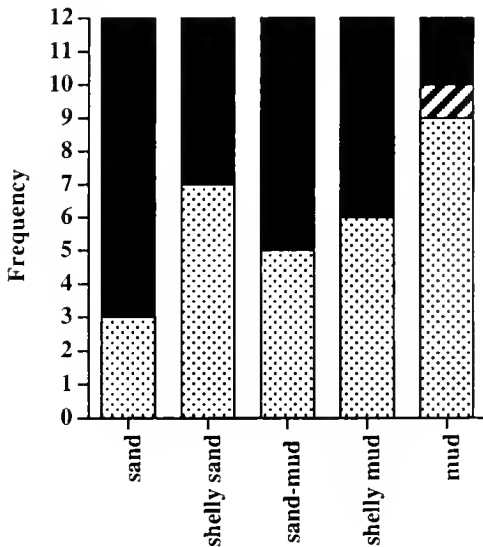
Experimental Sediment Substratum

Figure 4. Frequency of fractures among specimens of *M. mercenaria* that reburrowed monthly in various sediment textures. Distribution is random according to Goodness of Fit test ($\chi^2 = 4.52$ with 4 df).

cally weakened by the change in interstitial water chemistry, it didn't facilitate the initiation of more fractures than specimens that reburrowed in muds (Fig. 4). Clams that repeatedly reburrowed in mud did not show the same degree of loss of surface ornament (Fig. 5). Reburrowing in abrasive sand, accompanied by etching of the shell exterior by the interstitial water did significantly retard the expected annual increase in cross-sectional shell height relative to that shown by clams burrowed in mud and mud-shell hash (Fig. 7).

A valve thickness threshold may exist at which shell fracture due to burrowing does not occur (Table 1), but it could not be unequivocally established by this investigation. All of the specimens in this investigation that cracked had a valve margin thickness along the dorsal-ventral axis of less than 2.0 mm just ventral to the pallial line. The four specimens with a ventral margin valve thickness greater than 2.0 mm did not bear fractures. This investigation deliberately used similar size young adults (mean DV length 37 mm; std dev. 4 mm) to minimize ontogenetic (age) effects on experimental results. Expanded experiments should use a wide range of hard clam sizes to determine if a size threshold for burrowing-induced fracture exists.

The question can be raised as to whether young adult (30–40 mm in dorsal-ventral length) hard clams show such reburrowing-induced fractures *naturally* in their native substrata, or if the frequency of repair in the experimental clams is merely an artifact of shell fatigue under suboptimum conditions in sediments in holding tanks where they reburrowed monthly. A specimen of *M. mercenaria* collected from the field shows very similar internal fractures to Figure 3A, but this is only one individual out of 500 specimens re-examined from a collection analyzed for repair scars from

Tuckerton NJ (Alexander & Dietl, 2001). Several specimens have fractures similar to those in Figure 2, but they lack the stuccoed thread-like ridge over the crack. Without the stuccoed repair ridge, it cannot be determined if the crack occurred during the life of the clam or during its post-mortem, transportational history. Greg Dietl (personal communication) forwarded a photograph of a farm-raised hard clam from North Carolina that has an internal fracture and repair in both valves similar to Figure 2B. These anecdotal occurrences of internal, stuccoed fractures from field collections belie the high frequency of fracture and repair found in the experiments. The disparity suggests that the anthropogenic handling of the specimens and/or the strain induced by monthly reburrowing contributed to internal fracturing of the shell in the experiments.

Regardless, these artificial experimental stresses did not preclude repair of the fractures by the mantle tissue. Given that many repairs were probably followed by re-burrowing episodes, the stuccoed repair process is sufficiently strong to enable the overwhelming majority of clams to repeatedly stress the valves during reburrowing without a repaired fracture failing lethally. Whatever the percentage of fractures induced anthropogenically before the reburrowing experiments commenced, which based on the control group could be approximately 14%, or 7 specimens, the repairs withstood the repeated strain in the shell due to reburrowing as many as 12 times. Thirty specimens had fractures in the valves, but only one specimen fatally cracked its valves during monthly reburrowing over a 12-month period (Fig. 2H). The dysoxic pore water beneath the sediment surface, and diminished supply of plankton flowing through the holding tanks may have contributed to the severely retarded acccretionary growth (Fig. 6), but these

DEGREE OF VALVE ABRASION

- ⊡ negligible
- ▨ slight - ventral
- ⊞ moderate - ventral & central
- ■ extensive - all surface area
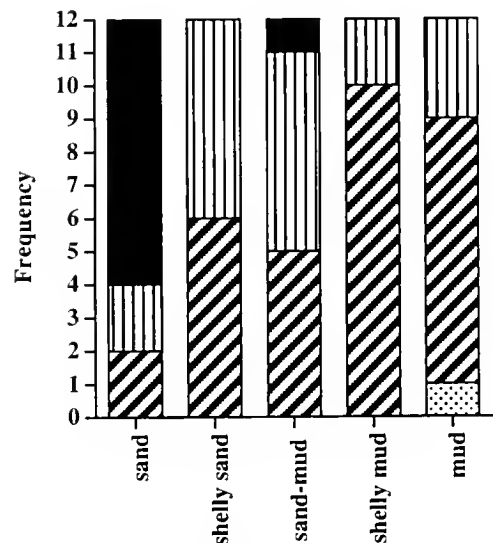
Experimental Sediment Substratum

Figure 5. Frequency of various degrees of valve abrasion for specimens that reburrowed monthly into various sediment textures.

Mercenaria mercenaria

One Way ANOVA

p < 0.079
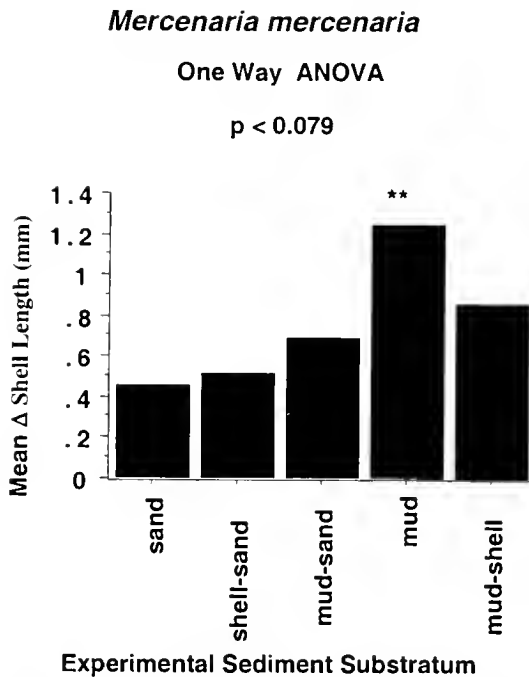
Figure 6. Mean increase (delta mm) in dorsal–ventral length (mm) of specimens of *M. mercenaria* that reburrowed monthly in various textured sediments. **Mean increase is significantly greater than value for sand ($P = 0.01$) and sand-shell ($P = 0.02$) according to Fisher's PSLD test. Mean ventral margin thicknesses are not significantly different among specimens reburrowing into different sediments based on analysis of variance (ANOVA; $P = 0.067$). Sample size = 60.

Mercenaria mercenaria
One way ANOVA
P < 0.0018

Figure 7. Mean net change (delta mm) in shell height in cross-sectional, lateral profile for *M. mercenaria* that reburrowed monthly in different textured sediments. **Mean value significantly greater vs. sand and sand-shell, ($P < 0.01$), as well as mud-sand ($P = 0.025$) according to Fisher's PSLD test. ##Mean values significantly greater vs. sand, shell-sand, and mud-sand ($P =$ or < 0.01) according to Fisher's PSLD test. Sample sizes = 60.

suboptimum conditions did not prevent the mantle from stuccoing the fractures.

This investigation on hardshell clams also shows that Checa's (1993) investigation on burrowing-induced fractures and repair is not necessarily a phenomenon restricted to thin-valved, deep-burrowing clams, although the frequency of reburrowing necessary to fracture the valves may be an order of magnitude higher for thick shelled clams and less likely to occur naturally in their native habitats. This investigation should prompt bivalve functional morphologists interested in shell biomechanics to search for internally stuccoed fractures in field surveys of shells of a variety of venerids, not just *M. mercenaria*. Just as external shell repairs in commercially valuable clams may be an indicator of shellfishing pressure (Bergman & Hup 1992, Gaspar et al. 1994, Withaard & Klein 1994, Ramsey et al. 2000), frequency of stuccoed micro-fractures expressed on the interior of valves may indicate the history of both naturally and anthropogenically caused excavations and reburrowing episodes experienced by a clam population. Repair frequencies also may reflect the physiochemical conditions beneath the sediment surface in which commercially valuable clams reburrowed. Fracture repair may have impact on accretionary growth rates of hardclams yet to reach harvestable sizes.

CONCLUSIONS

Reburrowing into the substrata by *M. mercenaria* may either induce sublethal shell fractures, or further propagate fractures induced by anthropogenic excavation and handling processes. Texture of the sediment (sand, mud, sandy mud, shelly sand, shelly mud) may not necessarily differentiate frequencies of burrowing-

propagated fractures. Nevertheless, adduction of valves on sediment grains and shell shards can induce the strain that initiates or propagates fractures; sediment-loading against the valve exterior is not the likely culprit of fracture propagation in the shallow-burrowing *Mercenaria mercenaria*. Shell surface micro-ornament may be completely abraded and/or corroded away by repeated reburrowing in organic-rich sands with dysoxic pore water conditions beneath the sediment surface. Nevertheless, such abraded and etched shells are no more susceptible to fractures than shells of clams that repeatedly reburrowed in muds.

Fractures are most often initiated at or very near the ventral margin, rather than shell posterior or anterior margin. Fractures that do not extend dorsal to the pallial line are not likely to be repaired. Fractures may extend beneath the adductor muscle and be
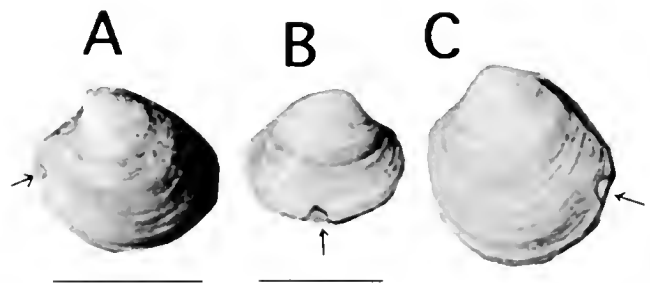
Figure 8. Resecreted shell in notched anterior (A), ventral (B), and posterior (C) margins of seed of *M. mercenaria* after 2 weeks while kept caged on a muddy sand tidal flat near Masonboro Inlet, North Carolina. Notches created by a Dremel in early October 2001. Width of line is 2 cm.

repaired. Fractures are mostly likely repaired (stuccoed) between monthly burrowing episodes, given rates of shell regeneration in marginally notched specimens. These repaired fractures withstand the strain induced by repeated burrowing as evidenced by the fact that only one of 30 fractures failed lethally.

ACKNOWLEDGMENTS

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IDENTIFICATION AND INCORPORATION OF GROWTH AND SURVIVAL BOTTLENECKS IN ECONOMIC MODELS OF NORTHERN QUAHOG (HARD CLAM), *MERCENARIA MERCENARIA* MARICULTURE

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ABSTRACT Research that identifies potential bottlenecks in survival and growth penalties during the different phases of clam grow-out is necessary to maximize the profitability of clam aquaculture and reduce pressure on already threatened wild stocks along the Atlantic coast of the Eastern United States. In this study, initial planting density (489, 729, and 972 clams m^{-2}) did not affect survival (64.8–77.5%) during the first year of clam grow-out. Clams planted at the lowest density outgrew (greater final shell length, SL, and individual clam volume) those planted at higher densities; therefore, clam growth was density dependent during the first year of grow-out. In the second experiment, size of clams (26.2, 32.5, 37.7, and 42.0 mm SL) planted after year one did not affect survivorship (92.3–96.6%) or growth (36.2, 41.7, 45.1, and 49.2 mm SL, respectively). Evaluations of the economic feasibility of clam culture demonstrated that clams planted at intermediate densities would result in the greatest return on the initial investment. To increase the robustness of our economic feasibility analysis to interannual variations in clam survivorship and growth during the initial year of grow-out, we performed an identical analysis with data from an earlier study. Taken together, these studies bracket a realistic range of survivorship and growth during the initial year of clam grow-out: low survivorship and growth (this study) and high survivorship and growth (earlier study). Based on this range, the estimated expected profitability ranged from $4893 to $7717 per 100,000 seed clams. In contrast to aquaculture of other bivalve species (e.g., oysters), our analysis demonstrates that the profitability of clam aquaculture is fairly robust to substantial variations in market prices primarily as a result of the development of methods over the last decade that enable relatively high survivorship with moderate growth penalties.

KEY WORDS: *Mercenaria mercenaria,* northern quahog, hard clam, aquaculture, survivorship, growth, density dependence, economic feasibility

INTRODUCTION

Bivalve aquaculture holds great promise in contributing to the goal of sustainable and dependable production of seafood. Whereas aquaculture of some marine species, primarily fish and shrimp, is associated with a host of negative environmental effects (e.g., increased biological oxygen demand as a result of fecal production (Silvert & Sowles 1996, Paez-Osuna et al. 1998, Tovar et al. 2000), habitat loss associated with construction of shoreline aquaculture facilities (Hopkins et al. 1995, Paez-Osuna 2001), and introduction and propagation of pathogens (Harvell et al. 1999)), negative environmental effects of bottom or near-bottom culture of bivalves are relatively minor (Kaiser et al. 1998, Naylor et al. 2000). In fact, aquaculture of bivalves may contribute positively to the local environment. Removal of phytoplankton as a result of filter feeding may improve water clarity in coastal areas, thus promoting the growth of sea grasses, which serve as essential habitat for fish and crabs. Because many coastal estuaries have experienced increased eutrophication in recent decades (Paerl et al. 1998), bivalve aquaculture could assist wild populations of filter feeders remove excess nutrient loading.

Despite a relatively reliable market for northern quahog (hard clam), *Mercenaria mercenaria* and the minimal environmental effects of bivalve aquaculture, hard clam aquaculture in many areas, including North Carolina, has yet to reach its potential (Diaby 1997). Two of the primary obstacles hindering establishment and

expansion of economically viable hard clam aquaculture are (1) restrictive regulations by states and (2) low and/or unpredictable yields of clams on leases. The latter obstacle largely results from heavy predation of seed clams (Carriker 1959, Castagna & Kraeuter 1981, Peterson et al. 1995, Kraeuter et al. 1998), lower growth rates of clams associated with many practices adopted to exclude predators (Summerson et al. 1995, Grabowski et al. 2000), mortality induced by clam diseases, variation in the quality of lease sites for clam growth, and the frequency of natural perturbations (e.g., hurricanes and floods). Further hindering the development of successful aquaculture initiatives is the relative paucity of economic feasibility models that couple relevant biological information with economic assessments. Specifically, bioeconomic models that identify and incorporate major survival and/or growth bottlenecks during the entire grow-out phase while allowing for fluctuations in market price are of critical importance in developing an industry that is competitive to wild harvest.

Profitable clam culture requires planting clams at densities far above those found under natural conditions. If not mitigated, such aggregations of potential prey items can greatly increase predator efficiencies, resulting in severely reduced clam survival (Carriker 1959, Eldridge et al. 1976). Methods to reduce clam mortality rates have involved identifying threshold seed sizes for planting and appropriate times to plant seed clams in the field (Menzel et al. 1976, Whetstone and Eversole 1978, Manzi et al. 1986, Peterson et al. 1995, Marelli & Arnold 1996, Grabowski et al. 2000). Further reductions in predation have been achieved by planting clams in gravel, nylon-mesh bags, or cages and possibly by using biological controls (Castagna & Kraeuter 1977, Eldridge et al. 1979, Walker 1984, Bisker & Castagna 1989, Summerson et al. 1995, Kraeuter et al. 1998, Fernandez et al. 1999). Because most of these protec-

tive measures typically reduce clam growth (Grabowski et al. 2000), effective grow-out requires balancing increased survivorship with subsequent growth penalties. In a previous study, we quantified clam growth and survivorship in bottom beds versus tented bags and determined that tented bags increased survivorship but reduced growth rates of clams (Grabowski et al. 2000). We also determined that expected additional revenue from increasing survivorship should more than compensate for potential lost revenue as a consequence of slower growth rates during the first year in tented bags. Eldridge et al. (1979) noted that survival rates were greater for clams planted at higher initial densities. Eldridge et al. (1979) also found that clams planted at higher densities can take up to an extra 12 mo to achieve legal size in South Carolina, which could jeopardize the economic feasibility of clam aquaculture. Yet it is uncertain whether increasing planting density during the first year will affect survivorship or growth (i.e., if these processes are density dependent) enough to counterbalance associated reductions in costs of clam grow-out.

Aquaculture research has traditionally focused on the early stages of clam grow-out (Peterson et al. 1995). Although clam mortality in the wild and in culture operations is typically greatest during postlarval and early juvenile life history stages, survivorship and growth rates of larger clams may be size or density dependent (Eldridge et al. 1979). Further empirical tests are necessary to determine whether the size of larger clams will affect growth rates when planted at intermediate densities. Culture studies often assume mortality is extremely low after the initial stages and use estimated mortality rates for the final stages when evaluating the profitability of differing types of clam grow-out. Quantifying survivorship and growth during the later stages of clam grow-out is necessary to evaluate whether these assumptions are valid and to enhance the reliability of economic models that project the profitability of clam culture. Even if mortality is relatively minor after the initial hatchery phase, small differences in survivorship and growth at later stages may be critical in determining profitability under marginal market conditions. Consequently, effective crop management requires identifying culture techniques during each phase of grow-out that increase revenues relative to costs.

In this study, we examined potential growth penalties and/or survival bottlenecks within the first two years of clam grow-out. Included in this effort were experiments designed to quantify the relationships between seed clam planting density and growth when using methods that offer substantial predator protection. In particular, we tested whether clam planting density during the first year of grow-out in nylon bags, a widely used predator exclusion technique in bivalve aquaculture, influences clam survivorship, individual growth, and total yield. Further, we examined whether differences in growth after one year of grow-out are propagated throughout the second year or if compensatory growth reduces size variation in older clams (Peterson 1979). Finally, results from both of these experiments were incorporated into a cost-benefit analysis designed to examine the profitability of manipulating planting densities within a range of empirically derived survivorship and growth conditions under varying market conditions.

MATERIALS AND METHODS

Experimental Grow-Out

In August 2000, seed clams (4–6 mm) were obtained from Atlantic Farms, Inc., Charleston, South Carolina, and placed into a nursery system on the premises of Hooper Family Seafood, Smyrna, North Carolina. In October 2002, seed clams were sieved on a 10 mm screen to obtain clams of mean 13.7 mm shell length (SL), with SL being the maximum measurement along the anterior–posterior axis. Seed clams were planted at three densities (700, 1050, and 1400 clams per bag) in three sets of 10 nylon bags, mesh size 9.4 mm (stretch) and measuring 1.2×1.2 m. Each of the three sets of nylon bags corresponded to one of the three densities of clams. We planted seed clams in nylon-mesh bags because this method resulted in greater survivorship and was more viable economically than bottom beds (Grabowski et al. 2000). A random sample of 50 clams was measured for SL from four of the 10 nylon bags of each initial density at the inception of the experiment. A one-factor ANOVA confirmed that the initial SL of the three density classes was not significantly different ($F_{2,9} = 1.2$; $P = 0.35$). Nylon bags were interdispersed randomly on North Carolina shellfish lease 570 D in Midden's Creek, Smyrna, North Carolina. Each nylon bag was sealed with a cable tie, staked down on each corner, and raised in the center with a 30-cm-long PVC stake that projected 20 cm above the substrate surface. In January of 2001, the center stake in each nylon bag was removed.

In October of 2000, one-year-old clams grown out under similar methods and at the same lease site as described in the previous paragraph were collected. Clams were graded by shell thickness using slotted graders (1.5, 1.9, 2.2, and 2.5 cm bar spacing) into four distinct size classes (small, mean SL = 26.2 mm; medium, mean SL = 32.5 mm; large, mean SL = 37.7 mm; and extra large, mean SL = 42.0 mm). We then planted six sets of 500 clams of each size class in 1.2×1.2 m bottom beds (24 total beds) and covered the beds with 7 mm polypropylene mesh. Random samples of 50 clams were measured for SL from three of the six bottom beds for each size class. A one-factor ANOVA confirmed that the initial SL of the four size classes were significantly different ($F_{3,8} = 129.2$; $P < 0.0001$). The clams were planted in shallow water (<1 m below mean low water [MLW]), sandy substrate. The 24 bottom beds were interdispersed on a subplot of North Carolina shellfish lease 9102 near the premises of Hooper Family Seafood.

In October of 2001, all nylon bags and bottom beds were harvested. Bottom beds were raked and then checked by hand to ensure that all surviving clams were harvested. Every bag and bed was sampled by counting all surviving clams, measuring a random sample of 50 clams for length, and grading the clams using the slotted grading system mentioned previously. The number of clams in each grade was counted, and the displaced water volume of a random sample of 50 clams from each graded size class was quantified to estimate the entire volume of each replicate.

Statistical Analyses

Data were analyzed using separate one-factor ANOVAs for clam survivorship and size of seed clams (density experiment) and one-year-old clams (size class experiment). A one-factor ANOVA was conducted to assess whether initial planting density influenced clam survivorship. One-factor ANOVAs were also used to determine the effect of initial planting density on the following size parameters: individual SL, individual volume of clams, and total volume of clams. A second set of one-factor ANOVAs were conducted to determine the effect of initial clam size of planted one-year-old clams on percent survivorship and all three size parameters. Prior to any of these analyses, data were tested for homo-

geneity of variance using Cochran's test (Underwood 1981). The analysis of the effects of planting density during the first year of grow-out on the individual volume of clams required square-root transformation to remove the heterogeneity of variance. *Post hoc* contrasts were performed on all significant effects detected by the ANOVAs using Fisher's protected least significant difference (PLSD) test (Day & Quinn 1989).

Economic Analyses

Cost-benefit analysis was conducted to assess the economic implications of differing culture methods used in our study. We first evaluated whether reduced revenues from any survival bottle-necks or growth penalties from planting seed at higher density during the first year of clam culture outweigh the reduced cost created by planting clams at higher density. Projection of revenues from this size of operation was achieved using the results of the first two years of clam grow-out to estimate the number of clams that would survive to be harvested in subsequent years. From data collected in the first experiment, we determined the proportion of clams that grew to each size grade (<1.5 cm, 1.5–1.9 cm, 1.9–2.2 cm, and 2.2–2.5 cm shell thickness) after one year for all three planting densities. From data collected in the second experiment, we could then determine the proportion of clams from each of these size categories that attained legal size (>2.5 cm shell thickness) after one additional year of grow-out. For those clams that did not attain legal size after two years, we estimated the time to legal size by (1) determining which size category they grew into after the second year of growth and (2) projecting future growth by determining the proportion of two-year-old clams in each of the size classes that would grow to legal size after one or more additional years of grow-out. Using this series of calculations, we were able to project the time duration of clam grow-out and the timeline of harvests for clams planted at each density during the first year of grow-out (75% legal after 48 months). Clams in North Carolina typically grow to legal size in two to four years depending on several physical and biological factors associated with grow-out location. Clams that achieved legal size in each projected year of grow-out were multiplied by a price of 18c per clam, the average market price in North Carolina for clams at or just above the legal size over 1998–2001, and discounted at an annual rate of 3%.

The costs (i.e., labor, disposable supplies, equipment, bottom-water lease, electricity, and seed clams) of planting 100,000 seeds were estimated from records of Mark Hooper's clam culture operations over the past half-decade. Based on informal surveys of other clam culturists in North Carolina, we are confident that Hooper's operations are representative of hard clam culture in the region. Costs of equipment such as nylon bags and bottom-bed materials were factored in under two scenarios: (1) actual, all costs incurred and (2) annualized, equipment costs projected over a five-year lifespan (i.e., equipment would be used for future crops). To evaluate the robustness of hard clam aquaculture to fluctuation in market price, we calculated the break-even clam price at which revenues still could meet or exceed expected costs given the projected streamline of clam harvests. The break-even price (P_b) was calculated as follows:

$$P_b = \frac{\text{Costs}}{\sum H_i * 1/(1 + d)^i} \quad (1)$$

where H_i is the number of clams harvested in year i, d is the discount rate (3%), and costs are as mentioned previously.

We next compared results from the first year of grow-out to those from our previous study (Grabowski et al. 2000) to determine how variability in growth and survival in the first year of grow-out influences the profitability of clam aquaculture. In 1999, seed clams were planted at a density of 700 clams per bag using a similar range of seed sizes (Grabowski et al. 2000); therefore, we compared economic estimates derived from survivorship and growth parameters of the first year of grow-out in 1999 to those in 2000 (this study). For each year, actual and annualized costs were subtracted from revenues, which were calculated using a price of 18c and a discount rate of 3%. Finally, we compared the profitability and break-even price of 1999 versus 2000 operations.

RESULTS

Experimental Grow-Out

Initial planting density did not affect percent survivorship among the three density treatments ($F_{2,27} = 1.6$, $P = 0.23$), and survivorship ranged from 71.2% (low density) to 77.5% (medium density) and 64.8% (high density). Initial planting density did affect SL after one year of grow-out (Fig. 1; one-factor ANOVA $F_{2,27} = 8.7$, $P = 0.001$). Shell length in low-density bags was significantly greater than SL for both medium- and high-density clam bags ($P < 0.05$ for both comparisons), but medium- and high-density treatments did not differ ($P = 0.59$). Initial planting density also influenced individual clam volume (one-factor ANOVA $F_{2,27} = 6.9$, $P = 0.004$), which was also significantly greater in low-density bags than in either medium- or high-density clam bags ($P < 0.05$ for both comparisons). Individual clam volume for medium- and high-density clam bags did not significantly differ ($P = 0.26$). Finally, initial planting density did not affect the total clam volume per sample (one-factor ANOVA $F_{2,27} = 1.5$, $P = 0.25$), which ranged from 2034 mL/replicate (low density) to 2500 mL/replicate (medium density) and 2559 mL/replicate (high density).

In the second experiment, we tested whether clam planting size after one year of growth affects survivorship and growth during the second year of clam grow-out. Clam planting size marginally affected survivorship after the second year of grow-out (Fig. 2; one-factor ANOVA $F_{3,20} = 2.8$, $P = 0.06$). *Post hoc* comparisons indicated that the small size class had significantly lower survival
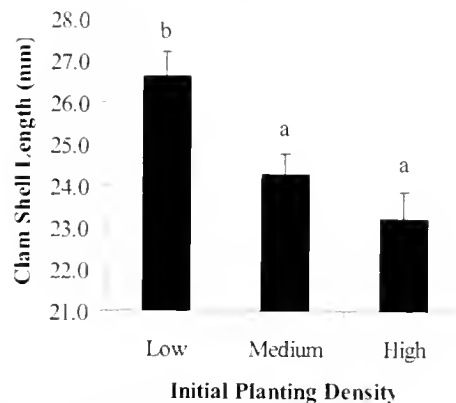

Figure 1. Final clam shell length after 1 y of grow-out in nylon bags: low density, 26.6 mm; medium density, 24.3 mm; high density, 23.2 mm. Error bars are +1 SE ($n = 10$ for each planting density). Letters above bars signify *post hoc* results (different letters denote significant differences at $P < 0.05$, Fisher's PLSD).
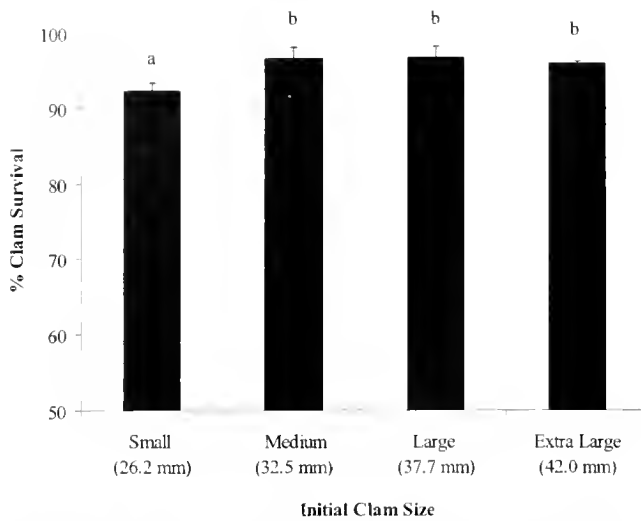
Figure 2. Clam survivorship after the second year of clam grow-out: small, 92.3%; medium, 96.5%; large, 96.6%; extra large, 95.9%. Error bars are +1 SE (*n* = 6 for each clam size). Letters above bars signify *post hoc* results (different letters denote significant differences at *P* < 0.05, Fisher's PLSD).

Figure 3. Final clam size after the second year of clam grow-out: small, 36.2 mm; medium, 41.7 mm; large, 49.2 mm; extra large, 45.1 mm. Error bars are +1 SE (*n* = 6 for each clam size). Letters above bars signify *post hoc* results (different letters denote significant differences at *P* < 0.05, Fisher's PLSD).

than the other three size classes (*P* < 0.05 for all three comparisons), and that the three larger sizes did not differ from each other (*P* > 0.05 for all three comparisons). Clam planting size significantly affected clam SL after the second year of grow-out (Fig. 3; one-factor ANOVA $F_{3,20}$ = 160.1, *P* < 0.0001). The ranking of final SL was consistent with differences in initial clam planting size (*P* < 0.0001 for all comparisons). The results of both volume measurements were consistent with the results from the analysis of final clam size (SL). Clam planting size also influenced individual volume per surviving clam (one-factor ANOVA $F_{3,20}$ = 160.6, *P* < 0.0001) and total volume of surviving clams per replicate (one-factor ANOVA $F_{3,20}$ = 139.3, *P* < 0.0001).

Economic Analyses

Cost-benefit analysis determined that planting clams at an intermediate density of 1050 clams per bag during the first year of grow-out resulted in the greatest projected return on the investment. Clams planted at the intermediate density were 25.1% and 33.2% more profitable than clams planted at the high (1400 clams per bag) and low (700 clams per bag) densities, respectively (Table 1). Annualizing equipment expenses over a more realistic time period of five years increased overall profits by an average of 18.9% in 2000. After annualizing equipment costs, cost-benefit analysis again determined that profits were greatest from clams planted at the intermediate density (Table 1). Under this scenario, profits from clams raised at low densities were slightly greater than profits of clams at high densities (Table 1). The break-even price ranged from 11.0¢ (low) to 9.2¢ (medium) on the actual expenses and 9.2¢ (low) to 7.9¢ (medium) when expenses were annualized (Table 1), which was substantially lower than the price (18¢) used to calculate projected revenues. Projected profits after the initial year of grow-out in 1999 (high survivorship and growth) were 72.3% higher for actual expenses and 57.7% higher for annualized expenses than profits based on data from 2000 (poor survivorship and growth).
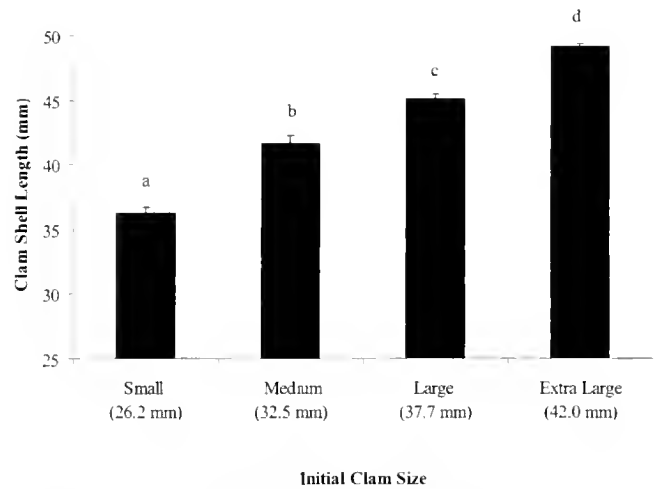
DISCUSSION

Empirical assessments of clam aquaculture have attempted to identify the magnitude and scope of survival bottlenecks and growth penalties associated with differing culture methods and techniques. Unfortunately, methods that increase survivorship often are associated with subsequent growth penalties (Grabowski et al. 2000). Assessment of the economic consequences of survival bottlenecks and growth penalties associated with each culture method is necessary to maximize the profitability of hard clam culture ventures and to determine the price levels where revenues exceed costs. In this study, we determined the degree to which survivorship and growth were affected by (1) seed planting density during the first year of grow-out and (2) size of 1-y old clams in the second year of grow-out. Both of these experiments were incorporated into cost-benefit analysis of hard clam culture to identify if any reductions in survivorship or growth penalties associated with planting density during the first year of grow-out would impact profitability.

Although survivorship did not vary statistically with planting density, planting clams at the higher density (1400 clams per bag) reduced survivorship by 12.7% and 6.4% in comparison to medium- (1050) and low-density (700) bags, respectively. Similar results (i.e., clam survivorship is unaffected by planting density during the first year of grow-out if clams are protected from predation) have been shown by other studies (Summerson et al. 1995, Fernandez et al. 1999). Eldridge et al. (1979) found in South Carolina that seed clams (13.0 mm SL) planted at low densities experienced higher mortality rates, which they attributed to predation rather than competitive exclusion. These studies suggest that if survival bottlenecks in clam culture exist as a consequence of clam density, they occur at smaller sizes and during the hatchery or nursery phases of grow-out. Furthermore, choosing an appropriate method of grow-out that protects clams against local predators might be more important to patterns of survivorship than initial planting density (Summerson et al. 1995). It is important to note that although these relatively small differences in survivorship rarely meet a formal statistical threshold for detecting differ-

TABLE 1.

Economic evaluation of (A) planting clams at different densities and (B) variation in the first year of grow-out (1999 vs. 2000 results).

(A) Planting Clams at Different Densities in the First Year of Grow-out (700, 1050, and 1400 per bag)

Planting Density:[b]	Actual			Annualized[a]		
	Low ($)	Medium ($)	High ($)	Low ($)	Medium ($)	High ($)
Cost of labor ($10/h)	3,453	3,145	2,561	3,453	3,145	2,561
Supplies	243	195	171	243	195	171
Equipment	1,236	983	849	247	197	170
Electricity	150	150	150	150	150	150
Lease price	10	10	10	10	10	10
Clam seed	1,000	1,000	1,000	1,000	1,000	1,000
Total costs	6,092	5,483	4,741	5,103	4,697	4,062
Projected revenues	9,995	10,683	8,897	9,995	10,683	8,897
Net present value (NPV)	3,904	5,200	4,156	4,893	5,987	4,835
Break-even price	0.110	0.092	0.096	0.092	0.079	0.082

(B) Comparison of Projected Range of Earnings from 1999 and 2000 Data

Grow-Out Year	Growth and Survivorship Comparison	
	1999	2000
Initial planting size (SL)	13.9 mm	13.9 mm
Survivorship after 1 y	90.1%	71.2%
Mean size (SL) after 1 y	32.4 mm	26.6 mm

	Actual		Annualized[a]	
	1999 ($)	2000 ($)	1999 ($)	2000 ($)
Total costs	6,599	6,092	5,610	5,103
Projected revenue[c]	13,326	9,995	13,326	9,995
Net present value (NPV)	6,728	3,904	7,717	4,893
Break-even price	0.089	0.110	0.076	0.092

[a] Annualized costs accounts for equipment expenditures that were spread over 5 y.

[b] Clams were planted at 700 per bag in 1999; therefore, 1999 results are compared to the low density treatment in 2000.

[c] Revenues were estimated at a price of $0.18/clam and 3% annual discount rate.

ences, the economic impact of these declines still affect profitability of clam culture, particularly in years of poor growth and survivorship.

Increasing clam density did negatively impact clam growth (both in terms of SL and volume): clams planted at the low (700 clams per bag or 489 clams m^{-2}) density grew larger than clams planted at either of the higher (1050 and 1400 clams per bag or 729 and 972 clams m^{-2}) densities. Total clam yield at the end of the first year of grow-out did not differ among the three treatments because clams at the low density grew larger than clams at either of the higher densities, thus providing further evidence that clam growth was density dependent. Eldridge et al. (1979) showed in South Carolina that seed clams planted in oyster trays protected with 9.0 mm mesh cloth at a density of 290 clams m^{-2} were significantly larger than similar-size clams planted at 869 or 1159 clams m^{-2}. Fernandez et al. (1999) planted larger (21.1 mm) seed clams in 10.5 mm nylon mesh bags (1.2 × 1.2 m) at Oak Hill, Florida, at densities of 520, 694, and 866 clams m^{-2} and found no difference in SL among the density treatments after nine months of growth. However, they did find a greater proportion of legal-size clams in the low-density treatment than in the medium and high densities. Differences in findings between Fernandez et al. (1999) and our study could be explained by their use of a narrower density range, larger initial seed size, or protective-mesh size. More plausibly, differences between the quality of grow-out conditions (e.g.,

physical/chemical variables, phytoplankton supply, abundance of fouling organisms) between areas used by Fernandez et al. (1999) and our study explain the dichotomy in findings. In areas that experience marginal growth conditions, clam cultures would experience more pronounced density-dependent growth penalties (Powers & Peterson 2000).

Our findings demonstrate that methods which effectively reduce clam predation not only reduce clam growth but also result in more pronounced growth penalties at higher densities where food limitation is presumed to be more intense. If risk of clam loss to theft or from hurricane damage is great, the interest rates and thus the rate of inflation are high, or volatility in clam prices is considerable, growth penalties associated with planting clams at high densities may decrease overall profitability and add substantially more risk to grow-out success.

Results from the second experiment suggest that survival is very high and largely independent of size during the second year of clam grow-out. Eldridge et al. (1979) also reported high survivorship of clams after the initial six months of grow-out. Thus, clam mortality is predominately an issue for clam growers during the nursery phase and the first year of grow-out. Differences in individual clam size and total clam yield in volume after two years of grow-out were in direct proportion to differences after one year of growth. Therefore, growth penalties resulting from culture techniques in year 1 are propagated unmodified through the additional

years of grow-out. The results of our study as well as that of Eldridge et al. (1979) demonstrate that investigation of the temporal sequence of growth penalties and survivorship can produce more precise crop management and further the profitability of aquaculture endeavors.

Economic analyses suggest that planting clams at intermediate (729 clams/m^{-2}) densities should increase the profitability of clam culture operations. Therefore, the reductions in operating costs associated with planting at higher densities are initially greater than the lost revenues from growth penalties and lower clam survivorship. However, increasing clam densities even further (972 clams/m^{-2}) during the first year of growth eventually results in reduced profit margins as a consequence of survivorship and growth penalties. If market demand for clams does not meet recent increases in clam production from several southern coastal states within the eastern United States, clam prices could continue to fall and threaten the viability of aquaculture operations. Planting clams at intermediate densities should reduce the threat of clam prices dropping beneath the break-even price, which should be of great concern to potential clam growers.

Given the fairly modest value of our calculated break-even price (7.6 to 9.2e per clam with annualized costs), our analysis demonstrates that profitability of clam aquaculture could be robust to substantial variations in market prices. The relatively low cost per clam produced results primarily from the development of methods over the last decade that enables relatively high survivorship with moderate growth penalties. Further identification of and reductions in the magnitude of growth penalties and survival bottlenecks in clam aquaculture may lead to additional increases in the profitability of these operations. These enhancements coupled with realistic economic models for clam aquaculture operations should continue to stimulate expansion of bivalve mariculture. In turn, further expansions may indirectly reduce harvest pressure and thus provide greater opportunity for natural recovery of wild stocks.

ACKNOWLEDGMENTS

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A STUDY OF THE NOAH'S ARK SHELL (*ARCA NOAE* LINNAEUS 1758) IN MALI STON BAY, ADRIATIC SEA

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ABSTRACT A study of the Noah's Arks (*Arca noae*) was conducted in Mali Ston Bay, between November 2001 and November 2002. Noah's Arks were collected monthly for the analysis of the condition index (CI), and every 2 mo for biometric measurements. CI was related to seawater temperature, salinity, and chlorophyll *a* levels, which were measured every 2 wk. Throughout the study, 39% of the *A. noae* were >50 mm in size. Based on length frequency distribution, a modified Von Bertalanffy growth equation was constructed: $L_t = 79.19 [1-e^{-0.340(t-t_0)}]$. Using the modal sizes estimated from the length frequency distributions, the estimated population growth rates of the shell were greater than the individual growth rates estimated from shell sections. Low values for the CI were recorded in December and January, and also in the period from July to October. The highest condition values were recorded from April until June.

KEY WORDS: Bivalvia, *Arca noae*, biometrics, condition index

INTRODUCTION

In recent years, an increase in the collection and aquaculture of bivalves from the family Arcidae has occurred (Food and Agriculture Organization 2002a, Food and Agriculture Organization 2002b). In 1991, a total of 69,700 metric tons (MT) of bivalves from the *Arcidae* family were collected from natural populations around the world, while in 2000 94,518 MT were landed in Cuba, Venezuela, Korea, Mexico, Japan, Indonesia, Fiji, and the Philippines (Food and Agriculture Organization 2002a). In addition, >330,000 MT of *Scapharca broughtonii* and *Anadara granulosa* was cultured in 2000, mostly in China, Malaysia, Thailand, and Korea (Food and Agriculture Organization 2002b). *Arcidae* species are also fast becoming important fished species in some new regions, such as along the eastern coast of the United States (McGraw et al. 2001, Power & Walker 2002).

The Noah's Ark shell (*Arca noae* Linnaeus 1758) is a commercially important bivalve that is distributed in the eastern Atlantic Ocean, the Mediterranean Sea, the Black Sea, and the West Indies (Nordsieck 1969). It lives attached with a solid byssus on rocks or shells, and is widely distributed and locally common in the Adriatic Sea (Hrs-Brenko & Legac 1996). The species is commercially exploited and, until the end of the Second World War, constituted an important component of the diet of local populations (Hrs-Brenko 1979, Zavodnik 1997). In the late 1940s, due to a catastrophic mortality caused by an unknown agent, the *A. noae* fishery in the Adriatic Sea collapsed (Hrs-Brenko 1980). Although the fishery has never returned to the annual catch rate of >600 MT of the 1940s (Hrs-Brenko 1980), it is still one of four major commercially exploited bivalves in the eastern Adriatic (Benović 1997).

Due to an increasing number of tourists and a subsequent increase in demand for seafood products, the *A. noae* fishery reported in this article has recently intensified in the Croatian part of the Adriatic. In a recent study of *A. noae* shell sections (Peharda et al. 2002), it was found to be a slow-growing bivalve. *A. noae* can live for >16 y, a feature that makes it potentially susceptible to overfishing. However, the research conducted by Peharda et al.

(2002) investigated only the growth of the shell, but did not investigate the population structure of this species. The research undertaken in this article had the objective of gaining a better understanding of seasonal changes in the *A. noae* population structure and condition index (CI), data that are crucial for monitoring the sustainability of the *A. noae* fishery in the Adriatic.

MATERIALS AND METHODS

Mali Ston Bay is an extended bay located in the southeastern Adriatic Sea (Fig. 1). It is characterized by strong marine currents, underwater freshwater springs, and abundant and constant sedimentation that influences the formation of soft-mud sediments (Šimunović 1981). The concentration of nutrients is high due to the high freshwater input (Vukadin 1981, Carić et al. 1992). Analysis of phytoplankton abundance and zooplankton community structure indicate that the bay is a naturally moderately eutrophic ecosystem (Viličić 1989, Lučić & Kršinić 1998). The sampling station for the study was located in part of Mali Ston Bay called Bistrina.

The study was based at Bistrina marine station between November 2001 and November 2002. Noah's Arks were collected from the seabed by scuba divers at depths of between 2 and 4 m. Sampling was conducted once a month for the analysis of CI ($n = 30$) and every 2 mo for biometric measurements.

The following parameters were measured for each specimen: length (L), height (H), and width (Wd) in millimeters; and dry flesh weight and wet weight of shell in grams. Flesh was dried at 60°C to constant weight, and the following CI was calculated according to the method of Davenport and Chen (1987):

$$C.I. = Dry\ flesh\ weight/Shell\ weight \times 100$$

Temperature and salinity were measured at a depth of 2 m twice a month with a WTW (Ft. Myers, FL) multiline hydrographic probe. Seawater samples for chlorophyll *a* (Chl *a*) analysis were collected twice a month at the same depth using Niskin (General Oceanics, Miami, FL) water bottles. Samples of 0.5 dm^3 were filtered using Whatman (Kent, U.K.) GF/F glass-fiber filters and were subsequently stored at −20°C. The Chl *a* level was determined fluorometrically using a Turner TD-700 Laboratory Fluorometer (Sunnyvale, CA), and was calibrated with pure Chl *a* (Sigma Chemical, St. Louis, MO) after homogenization and 90% acetone

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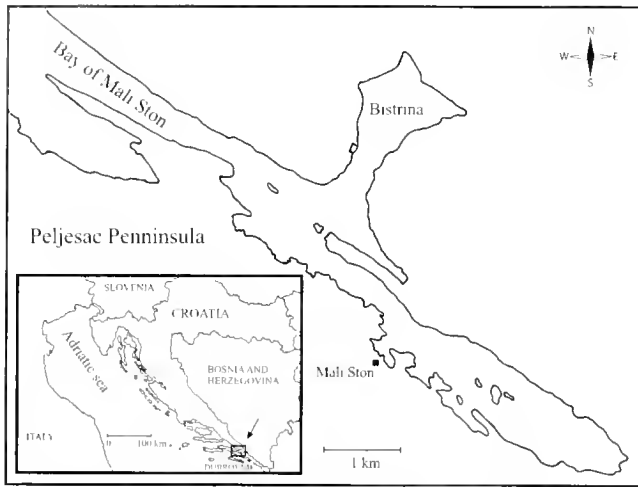*Corresponding author. E-mail: melita@izor.hr

Figure 1. Location of Mali Ston Bay and Bistrina.

extraction (24 h at room temperature) of filters, following the method of Strickland and Parsons (1972).

Spearman's correlation analysis and regression were applied to describe the biometric characteristics of the shell and body tissue, and to determine the degree of association with the CI and environmental conditions. A nonparametric Kruskal-Wallis test was used to examine monthly changes in CI. Length frequency data were analyzed using the FiSAT statistical package (Food and Agriculture Organization-The International Center for Living Aquatic Resources Management (ICLARM), Rome, Italy). Data were smoothed using the running average of three classes, and the Powell-Wetherall method (Wetherall 1986) was applied to estimate asymptotic length (L_∞). The method of Bhattacharya (1967) was used to separate a composite distribution into separate cohorts, while the method of the sum of squared errors was used to determine the curvature parameter (k) of the modified von Bertalanffy growth equation $L_t = L_\infty [1-e^{-t(t-t_0)}]$ (Sparre & Venema 1992).

RESULTS

The seawater temperatures ranged from 7.2°C (January 2002) to 25.8°C (June 2002). Seawater temperatures >20°C were recorded between June and mid-September (Fig. 2). The lowest salinity values were recorded during sampling in July [26.9 practical salinity units (psu)] and October 2002 (28.8 psu). The highest
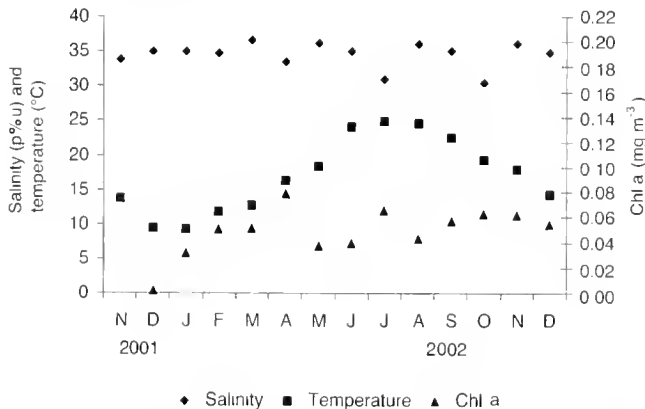
salinity value recorded in this study was 37.1 psu (March 2002). Chl a values ranged from 0 mg m^{-3} (December 2001) to 0.094 mg m^{-3} (April 2002).

The minimum shell length recorded during the 1-y study was 6 mm, while the maximum was 80 mm [mean (±SD) length 45.04 ± 13.68]. Only 1% ($n = 14$) of measured individuals were longer than 70 mm, 6% ($n = 96$) were longer than 60 mm, and 39% ($n = 589$) were longer than 50 mm. Shell height values ranged from 3 to 44 mm (mean 23.17 ± 6.59 mm), and shell width ranged from 3 to 51 mm (24.81 ± 7.24 mm). Length frequency histograms, according to sampling months, are shown in Figure 3. The polynomial type of length distribution is visible in all the presented graphs, indicating the presence of several age classes.

Using the length frequency distributions, up to eight cohorts were separated according to the method of Bhattacharya (1967) (Table 1). The asymptotic length (L_∞) of A. noae was estimated at 79.91 mm, while the calculated curvature parameter (k) was 0.342 y^{-1} ($r^2 = 0.992$). According to the von Bertalanffy growth equation obtained, A. noae reaches a length of 60 mm in its 5th year of life, while it takes over 10 y to grow to its asymptotic length (Fig. 4).

The relationship between length and height could be described using the following equation: H = 4.33 + 0.418 L ($n = 1531$; $r^2 = 0.75$; $P < 0.001$), while the equation Wd = 3.32 + 0.477 L ($n = 1531$; $r^2 = 0.82$; $P < 0.001$) described the relationship between shell L and Wd. The calculated values of r^2 indicate the degree of variation in the shape of the shells. The relationship between shell weight and length could be described using the following equation W = 0.01* L$^{1.92}$ ($n = 390$; $r^2 = 0.79$; $P < 0.001$). A. noae has negative allometric growth, meaning it grows proportionally more in length than in H, Wd, total weight, or flesh weight with increase in age (Table 2.)

Seasonal differences in body CI are shown in Figure 5. Low mean ratios of dry flesh weight and shell weight (<9) were recorded in December and January, and also in the period from July to October. The highest ratio values (~11) were recorded from April until June. A sharp decrease in CI was noted between June and July. Observed monthly changes were statistically significant (Kruskal Wallis H = 126.95; $P < 0.001$). There were no statistically significant correlation between CI and temperature (r = −0.369; $P = 0.468$) and Chl a (r = 0.036; $P = 0.477$), while a negative correlation was found between CI and salinity (r = 0.137; $P = 0.007$).

DISCUSSION

The Bay of Mali Ston is the largest bivalve aquaculture area in the eastern Adriatic Sea, with a long tradition of collecting marine organisms and their aquaculture over several centuries, and, according to some authors, even from the time of the Roman Empire (Basioli 1968). Although A. noae is one of the main bivalve species traditionally collected in this area, there are no data on its biometry, population structure, or seasonal changes in CI at this location. The current study confirms previous observations that A. noae is variable in shape (Valli & Parovel, 1981, Poppe & Goto 2000, Peharda et al. 2002). Negative allometric growth noted for A. noae in the Gulf of Trieste (Valli & Parovel 1981) also was confirmed in this study.

According to the literature, A. noae can grow up to 90 mm, but usually it grows up to 70 mm in size (Parenzan 1974, Hrs-Brenko 1980, Poppe & Goto 2000, Peharda et al. 2002). In this study, the

Figure 2. Seasonal variation in the salinity (psu), temperature (°C), and Chl a levels (mg m^{-3}) in Mali Ston Bay.
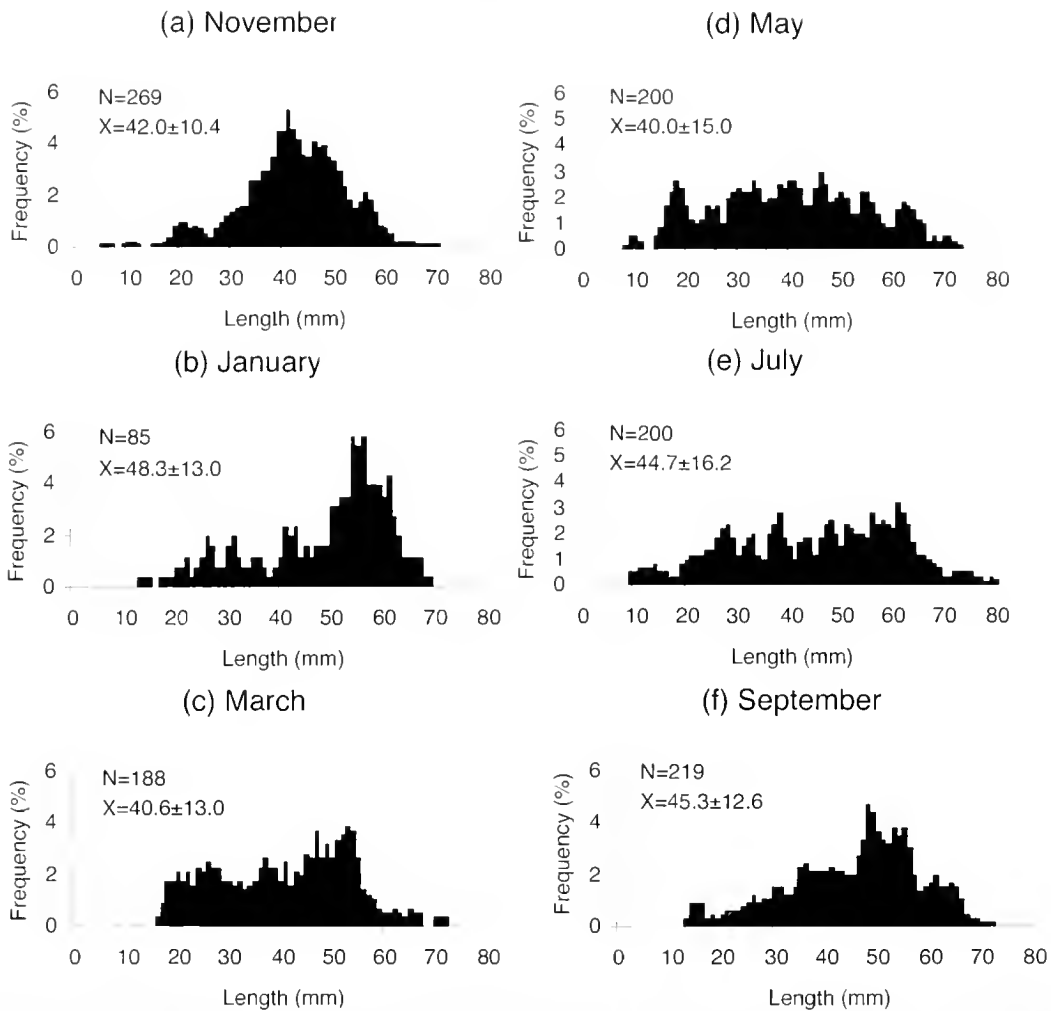
Figure 3. Length-frequency histograms for *A. noae* collected in (a) November 2001, (b) January 2002, (c) March 2002, (d) May 2002, (e) July 2002, and (f) September 2002.

largest individual had a length of 80 mm, but very few animals longer than 70 mm were recorded. Length frequency distributions recorded in 1977 and 1978 on the west coast of the Istria peninsula (Hrs-Brenko 1980) are similar to that recorded in this study. The relatively high percentage of *A. noae* longer than 50 mm indicates good survival, a stable population, and a potentially good spawning stock (Hrs-Brenko 1980).

The parameters of the von Bertalanffy growth equation obtained in this study show that it takes >10 y for *A. noae* to grow to its asymptotic length. Mistri et al. (1988) found similar results for a related species, *Scapharca inaequivalvis*, which was introduced by ballast water into the northern Adriatic. *S. inaequivalvis* grows slowly and needs >10 y to reach its maximum theoretical length (Mistri et al. 1988). A slow growth rate was also shown for the ark

TABLE 1.

Cohorts split using Battacharya's method, according to sampling months.

Cohort	November	January	March	May	July	September
1st	10.89 (4.474)	16.09 (3.323)	20.43 (2.829)	10.00 (3.101)	13.19 (3.385)	16.34 (2.447)
2nd	21.37 (3.122)	25.95 (4.085)	28.28 (3.711)	18.67 (2.717)	22.87 (2.997)	24.96 (2.980)
3rd	31.78 (3.585)	33.45 (2.085)	38.68 (3.640)	28.50 (3.926)	30.25 (3.694)	34.63 (4.276)
4th	41.31 (4.243)	42.93 (4.052)	47.06 (2.959)	39.68 (5.951)	39.70 (4.639)	44.50 (5.219)
5th	50.33 (3.358)	53.18 (2.812)	53.47 (2.898)	49.38 (3.510)	48.95 (2.104)	52.95 (5.639)
6th	57.54 (2.686)	58.27 (2.190)	62.63 (3.921)	56.79 (4.219)	58.71 (5.702)	64.36 (2.579)
7th	64.65 (3.934)	63.38 (3.157)		64.81 (3.256)	74.94 (3.448)	
8th				71.75 (1.779)		

Values given as mean length (SD).
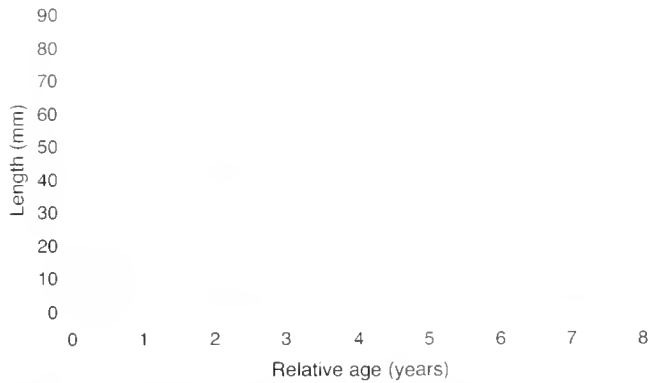
Figure 4. Growth curve for *A. noae* fitted using the von Bertalanffy growth equation $L_t = 79.19 [1-e^{-0.34(t-t_0)}]$.

shell *Noetia ponderosa*, which was shown to attain a market size when it is 8+ years old and could live for 15 y (McGraw et al. 2001).

The presented results in this study indicate that *A. noae* might be a somewhat faster growing species than previously found using shell sections (Peharda et al. 2002). According to this study, the asymptotic length of *A. noae* is larger (80 mm) than the asymptotic length that was obtained from the analysis of shell sections (65 mm). Similarly, the calculated curvature parameter (k) was also larger (0.34), than the k value (0.17) in Peharda et al. (2002), which indicates a faster growth rate. However, it is important to keep in mind that the Bhattacharya method applied in this study is useful for splitting a composite distribution into separate normal distributions in cases in which there are several age groups (cohorts) and is less reliable for longer living species (Gayanilo & Pauly 1997). On the other hand, the study of shell sections is usually limited to a smaller number of specimens (Richardson 2001), and it is possible that the earlier study by Peharda et al.

TABLE 2.

Parameters of log_{10} regressions of biometric parameters.

Dependent Variable	Independent Variable	a	b	r^2	*n*
H	L	−0.04	0.85 ± 0.010 [*]	0.830	1531
Wd	L	−0.09	0.90 ± 0.009 [*]	0.864	1531
Total weight	L	−2.04	1.92 ± 0.050 [*]	0.794	390
Flesh weight	L	−2.61	1.94 ± 0.065 [*]	0.695	390

[*] Values given as mean ± SE (departure from isometry at $P < 0.01$).

(2002) was somewhat influenced by sample size. Further research should compare these two methods and examine the possibility of determining growth parameters using mark-recapture techniques.

The results obtained for the CI show that there are seasonal changes in body weight. The maximum values were recorded during the period from April until June. The minimum values of CIs were recorded in December and January, at the end of the summer, and at the beginning of the fall. The first minimum value is probably related to temperature stress and a period of reduced feeding, as it is a period when we also recorded the lowest seawater temperatures. The later minimal value might be attributed to a period following spawning. Reduction in body CI in September and October was attributed to a summer spawning in related species. *S. broughtonii* (Park et al. 2001) and *S. inaequivalvis* (Mistri et al. 1988), respectively.

According to Graeffe (1903) and Vatova (1928, 1949), the spawning of *A. noae* in the Adriatic Sea occurs in May and June. In the Gulf of Trieste, this species has a prolonged spawning season, with peaks occurring in March and September (Valli & Parovel 1981). Our data on seasonal changes in body weight indicate that the spawning activity of *A. noae* in Mali Ston Bay might be the most intense in June. This is further supported by the observation of small individuals in the November samples.

2002
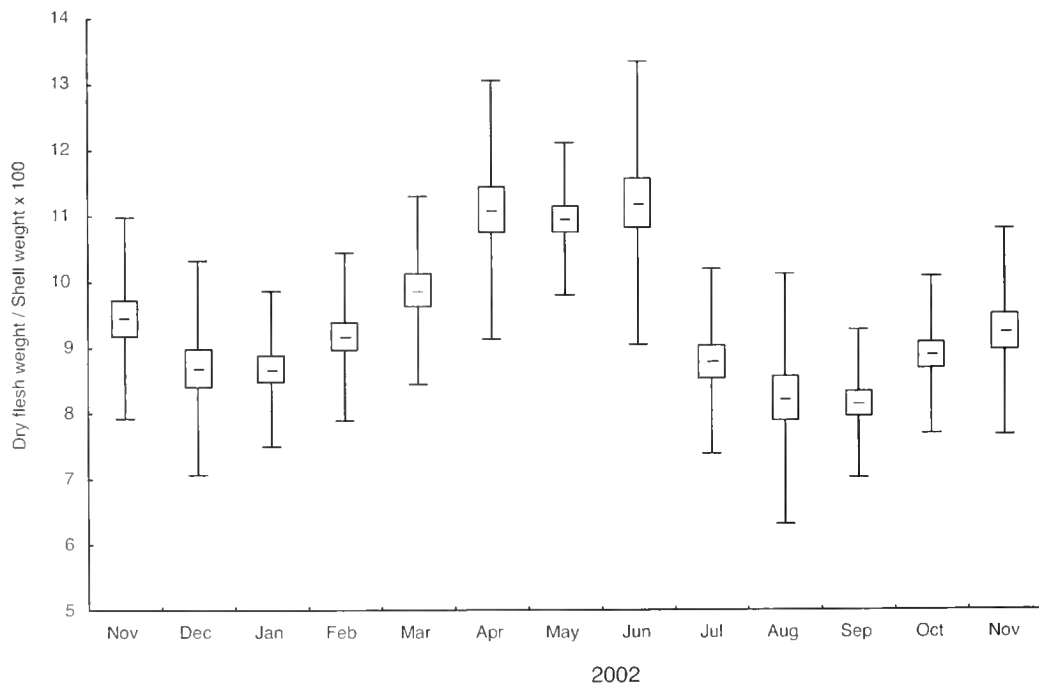
Figure 5. Monthly variations in CI of *A. noae*. CI = ratio between dry flesh weight and shell weight, with values given as percentages.

According to our results, salinity is the only environmental factor that correlates with CI throughout the year. This result is similar to the findings of Park et al. (2001), who did not record a correlation between the CI of *S. broughtonii* and temperature and Chl *a* level, and have recorded a positive correlation between CI and salinity. Although during our study period Chl *a* concentrations were about 10 times lower than the lowest values recorded by Jasprica and Carić (1997), previously established patterns of seasonal variation of phytoplankton in the semi-closed bays along the eastern Adriatic Sea and our values are in agreement with the presented data (Viličić & Stojanoski 1987). Increased temperature values, in addition to available nutrients (Carić pers. comm.), favored phytoplankton development in April, when an increase in *A. noae* CI was registered. According to the thermohaline conditions and Chl *a* levels analyzed, Mali Ston Bay may be considered to be an ecologically stable location suitable for the growth of *A. noae*.

ACKNOWLEDGMENT

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PRESENCE OF GIANT POLYMORPHIC CELLS IN *CRASSOSTREA GIGAS* CULTURED IN BAHIA FALSA, BAJA CALIFORNIA NW MEXICO

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ABSTRACT The culture of the Japanese oyster *Crassostrea gigas* is a successful commercial activity in Northwest Mexico. Since 1997, high mortality outbreaks have occurred in the area without apparent reasons. In this study we found gill erosions during clinical observations, hemocyte infiltration into the tissues at the histopathological level, and in some cases we detected the presence of giant polymorphic cells. In general, conditions mentioned above, including the presence of *Trichodina* sp. and especially the presence of giant polymorphic cells matches with the signs of the gill disease caused by an icosahedral DNA virus (Gill Necrosis Virus infection) first recorded in the Portuguese oyster *Crassostrea angulata* and in the Japanese oyster *C. gigas* in Europe. However, the Transmission Electron Microscopy (TEM) analysis of damaged tissues did not reveal the presence of viral particles.

KEY WORDS: Crassostrea gigas, giant polymorphic cells, mortality, gill necrosis virus infection (GNV), trichodines.

INTRODUCTION

The Japanese oyster *Crassostrea gigas* is one of the most widely cultured mollusks in the world. This species has been introduced from its original area in Japan, to countries such as Australia, France, Holland, Spain, Portugal, Thailand, to the Pacific coast of the United Sates, and United Kingdom (Bardach et al., 1982; Edwards, 1997). In 1973, *C. gigas* was introduced in several coastal lagoons in the states of Sonora, Baja California Sur, and Baja California, in Northwest México, including Bahía Falsa, B.C. The oyster seed was obtained from The Laboratory of the Lumi Indians in Marietta, Washington U.S.A. (Islas Olivares, 1975). In Bahía Falsa, this species was cultured in floating rafts. At present, the culture is produced in racks, bags, and occasionally some stocks are maintained directly on the bottom. Currently, the annual production in the region is around 1,622 metric tons with a value of 2.4 million dollars. Around 1,800 workers are involved in this activity and Bahía Falsa contributes 16% of this figure (Anuario Estadístico de Pesca, 2001). The industry depends on the importation of oyster spat from Oregon, Washington, and California, USA.

Since 1997, several high mortality outbreaks of *C. gigas*, including seed, juveniles, and adults, have occurred in Sonora and Baja California Sur. In April 1998, mortality outbreaks began to be recorded in Bahía Falsa, B.C. Unusual mortality has remained in the region. Several causes have been attributed to these mortalities: 1) unusual high temperatures and conditions produced by El Niño in 1997 and 1998, 2) the presence of toxins in the environment produced by microalgae or other organisms, 3) pollution, 4) the quality and quantity of food (phytoplankton), and 5) pathogens, or synergic conditions by the joint action of two or more of the above factors (Cáceres-Martínez 2000, Hoyos 2000). This work presents the results of a clinical and histopathological survey of *C. gigas* cultured in Bahía Falsa, B. C. and the possible relation of the observations and parasites with mortality outbreaks present in the bay.

MATERIALS AND METHODS

The study was conducted in Bahía Falsa, Baja California, México from July 1997 to June 1998 (monthly samplings). Two localities, Agromarinos, in the middle area of the bay, and Alfonsos, in the inner area of the bay, were sampled (Fig. 1). Rack and bag cultures are used in Agromarinos, whereas bottom culture is used in Alfonsos. In each locality and culture condition, 30 commercial size oysters were collected (mean total shell length, 12.45 cm ± 5.5 in the Agromarinos' racks, mean total shell length 10.46 cm ± 5.5 in the Agromarinos' bags and Alfonsos'). In all three culture conditions, oysters are exposed to air during low tide. Live oysters were transported to the laboratory and all fouling organisms were removed with a brush and a stream of seawater. Oysters were placed in a Petri dish, opened, and the intervalvar water and oyster flesh were examined for the presence of parasites under a dissecting microscope. The soft body of the oysters were removed from the shell and fixed whole in Davidson's fixative (Shaw & Battle 1957) for at least 24 h An anterior transverse section including the digestive gland, mantle, and gills was taken. Tissue samples were embedded in paraffin wax and were sectioned and stained with hematoxylin and eosin (Shaw & Battle 1957). Tissue analysis and measurements were made with a micrometer placed in an optical microscope. Prevalence of parasites and lesions were considered as the number of infested or wounded oysters/number of oysters examined ×100. Intensity was considered as the number of lesions or parasites per damaged or infected oysters in the
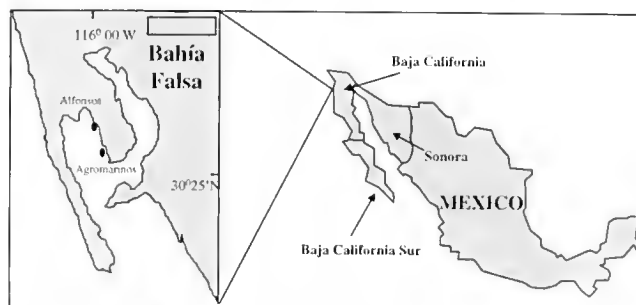
Figure 1. Map showing the region, where *Crassostrea gigas* culture is conducted in North Western México, and the Bahía Falsa, where the study was performed. Black dots indicate the sampling sites.

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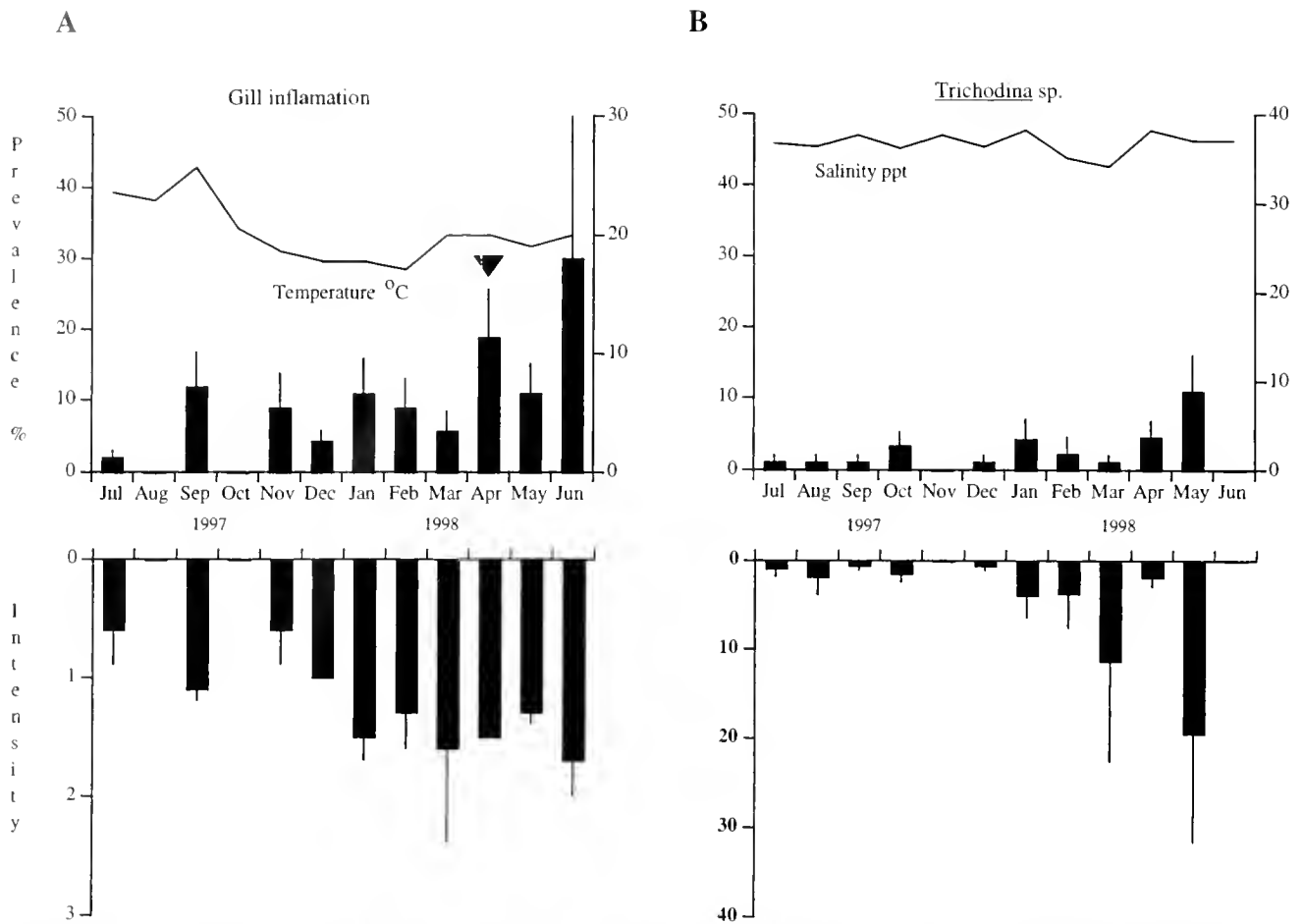
Figure 2. A, The combined mean values and standard error of prevalence and intensity of gill inflammation of *C. gigas* from all three cultures conditions during the study period. The dark triangle indicates when the oyster culturist noted the mortality outbreak. B, Mean values and standard error of prevalence and intensity of *Trichodina* sp. in *C. gigas* from the three cultures conditions during the study. Temperature and salinity values are shown as a line on the top of A and B, respectively, and their scale values are placed in the right side of the graphics.
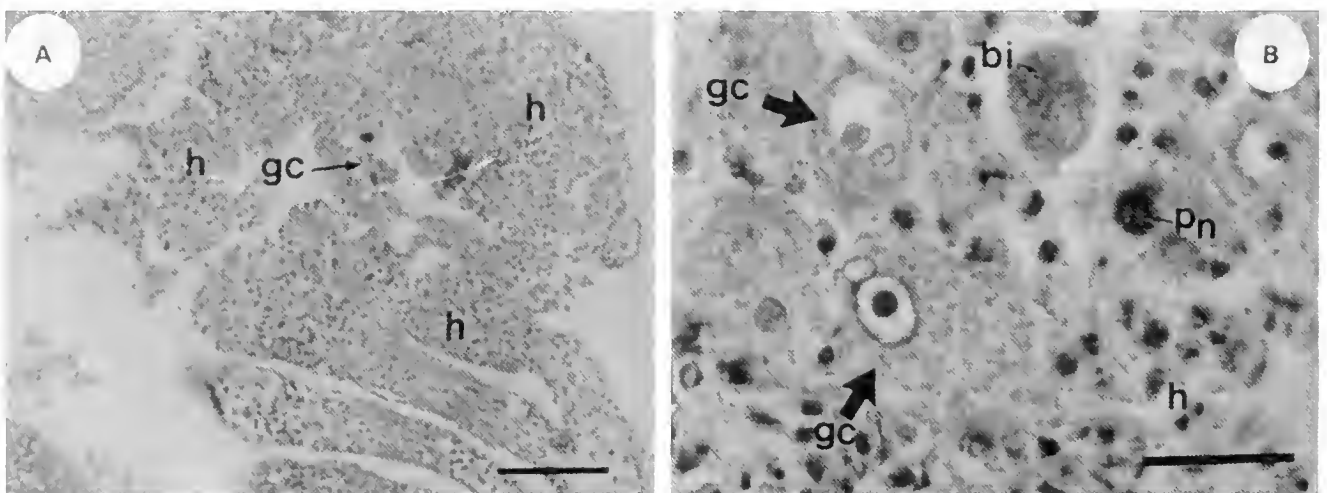
Figure 3. A, Strong gill inflammation in *C. gigas* with destruction of gill filaments, tissue rupture with massive infiltration of hemocytes (h) and the presence of some giant polymorphic hypertrophic cells (gc), scale bar = 100 µm. B, Detail of a gill lesion showing the polymorphic hypertrophic cells (gc) with basophilic inclusion (bi), picnotic nucleus (pn) and hemocytes around the lesion (h). Haematoxylin and eosin, scale bar = 20 µm.

sample. For the estimation of the gill lesions the following scale was used: (1) light gill inflammation, low infiltration of hemocytes and the gill structure unaltered; (2) medium gill inflammation, infiltration of hemocytes within the gill filaments and interlamellar septum; (3) Strong gill inflammation, massive infiltration of hemocytes, swelling of gill filaments and necrosis of the gill tissue.

After this study period, two additional samplings were carried out, one after a mortality episode occurred in the Bahia Falsa, in June 2000. During this sampling 30 surviving oysters were collected, opened in the field, and the condition of the gills was observed. Photographs of the gills were taken with a camera placed on a stereomicroscope and the degree of gill damage was determinate. All gills were fixed in Davidson's fluid and processed for histopathological analysis as mentioned above. The last sampling was carried out in November 2000. Ten oysters from an area of the bay where mortalities were common were reviewed and those showing symptoms of gill erosion were separated and a small pieces from the eroded area of the gill was removed and fixed in 3% glutaraldehyde in 0.1 M sodium cacodylate buffer, pH 7.8, for 4 h at 4°C. Fixed tissues were washed for 12 h at 4°C in the same buffer and cut into 1 mm^3 pieces. These pieces were then postfixed in buffered 1% OsO$_4$ for 4 h at the same temperature, dehydrated through a series of ethanols, and embedded in Epon. Sections (90 nm thickness) were cut and stained with 5% uranyl acetate for 30 min and lead citrate for 2 min and observed with a Transmission Electron Microscope (TEM) operated at 75 kV at the Instituto de Investigaciones Marinas, Vigo, Spain.

RESULTS

Histologic slides showed from light to strong gill inflammation. There were no differences among the prevalence and intensity of gill inflammation of oysters collected from different culture conditions (Kruskal–Wallis test, H = 2.42, P = 0.29 and H = 0.97, P = 0.61, respectively). The combined mean values of prevalence and intensity of gill inflammation from the three culture conditions are shown in Figure 2. There was a positive correlation between the prevalence and intensity of gill inflammation (r = 0.72, P = 0.007) and there was a general increase in the prevalence and intensity of gill inflammation from the beginning to the end of the study period (Fig. 2). In two cases, April in the Alfonsos' and June in the Agromarinos' rack, we detected cellular lesions reminiscent of the gill necrosis virus infection (GNV) caused by an icosahedral cytoplasmic deoxyribovirus (Comps, 1988): occurrence of giant polymorphic cells (ranging from 25 to 30 μm in diameter) containing basophilic granules and some oval hypertrophied nucleus and hemocytes accumulation in the lesion (Fig. 3). Also, *Trichodina* sp. were detected in the mantle cavity and gills of *C. gigas*. Their prevalence and intensity was similar in oysters taken from the three different culture conditions (Kruskal-Wallis test, H = 0.9, P = 0.63 and H = 0.77, P = 0.67, respectively). The combined mean values of prevalence and intensity of *Trichodina* sp. from the three culture conditions are shown in Fig. 2. There was a positive correlation between the prevalence and intensity of *Trichodina* sp. (r = 0.77, P = 0.003) and there was a general increase in the prevalence and intensity of *Trichodina* sp. from the beginning to the end of the study period (Fig. 2). Temperature and salinity remained between the tolerance limits of this oyster species (Pauley et al. 1988; Fig. 2). There was no statistical correlation between gill lesions and *Trichodina* sp. presence; however, there was a trend of an increase in gill lesions and *Trichodina* sp. presence from the beginning to the end of the study period (Fig. 2).

Figure 4 shows a varying degree of gill erosion in surviving oysters sampled in June 2000, from normal appearance (0 degrees), to very eroded appearance (4 degrees). Histologic analysis revealed the presence of picnotic nuclei in cells at the distal edge of the gill filaments where erosion occurred but no Trichodine presence. Curiously, no evidence of necrotic areas was observed in the eroded areas of gill filaments; however, deformations of the distal edge of the gill filaments were evident (Fig. 4). The prevalence of the lesions was 100% and its intensity value was medium. These lesions showed inflammation of tissue and cicatrisation The TEM study did not reveal the presence of giant polymorphic cells and viral particles.

DISCUSSION

According to Comps (1988), virus infection has been associated with major diseases of oysters of the genus *Crassostrea*. These infections include the GNV affecting the Portuguese oyster and, to a lesser degree, the Japanese oyster cultured in Europe.
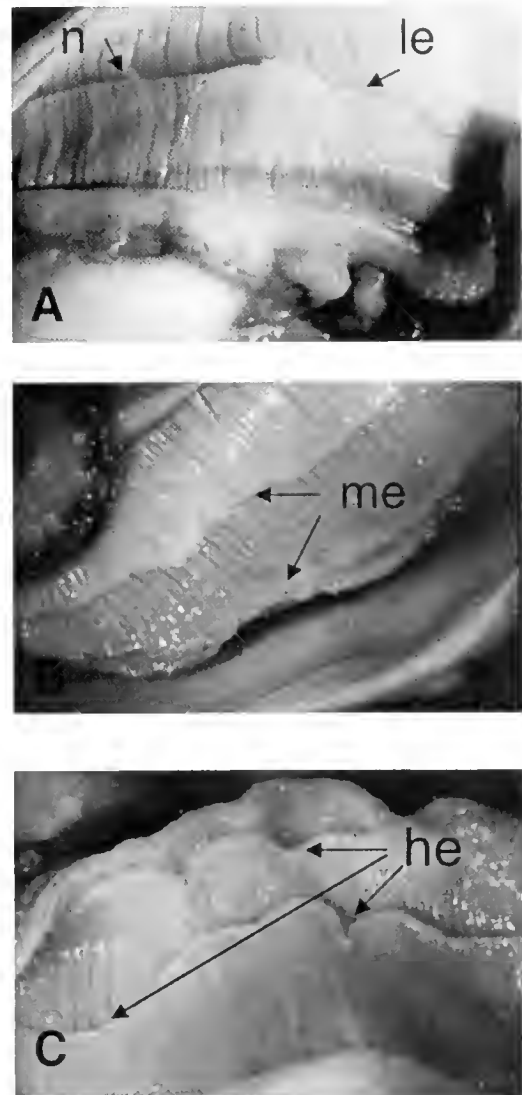

Figure 4. Different degrees of gill erosion in surviving *C. gigas*. A, Normal appearance (n), light eroded appearance (le). B, Medium eroded appearance (me). C, Highly eroded appearance (he).

This author remarked that the gill necrosis virus causes, mainly in the Portuguese oyster, an evolutive ulceration of the gills, including cellular hypertrophy and severe hemocyte infiltration. Mortalities have been observed in the most serious cases. In this study, we found clear histologic evidence of giant polymorphic cells, which have been associated with GNV infection; however, we did not detect characteristic damage of GNV on the gills because we failed to look for it. It is important to mention two points: 1) this study was concluded three months after an unusual mortality in the bay was recorded, and 2) no moribund oysters were sampled. However, in the second sampling (June 2000) when we went specifically looking for gross signs of the GNV infection; we detected the erosion of the gill filaments, which is the characteristic gross sign of the GNV infection. In spite of the failure to reveal viral particles by TEM possibly because of the sample process, the status of the gill tissues of surviving oysters (cicatrisation), and the difficulties in finding those particles in fixed tissues, evidence of giant polymorphic cells in *C. gigas* cultured in the bay and region showed an obligated line of research and the possibility that a virus may be involved in oyster mortality outbreaks. It is known that herpes-like viruses in several oyster species have been associated with high mortality rates (Le Deuff & Renault 1999). However, the presence of *Trichodina* sp. was observed in a similar condition when the unusual mortality of *C. angulata* occurred in France. On that occasion, it was thought that *Trichodina* sp. could be the cause of oyster gill lesions (Besse 1968). Afterwards, it was found that *Trichodina* sp. might have been a secondary invader of oysters that were suffering from virus-caused gill necrosis (Bower et al. 1994). The trend of the increase in prevalence and intensity of *Trichodina* sp., from the beginning to the end of the study period, is consistent with the increase of gill lesion observed and a secondary invasion of the protistan. Oyster culturists from Bahía Falsa first reported a mortality outbreak in April 1998, and gill inflammation increased from November 1997 to the end of the study period. It is possible that the presence of giant polymorphic cells or the gill inflammation condition could have been present in the oysters of the bay earlier; however, an unknown factor may favor the increase of gill lesions at the end of the study period. Temperature and salinity remained between the tolerance limits of the oyster species and the culture technique seems to be independent of gill lesions, and the parasite prevalence and intensity observed. Further studies using molecular tools are being conducted to confirm the presence of virus in oysters from the region and its possible relationship with mortality outbreaks.

ACKNOWLEDGMENTS

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IN VIVO AND *IN VITRO* APPROACHES TO THE ANALYSIS OF GLYCOGEN METABOLISM IN THE PACIFIC OYSTER, *CRASSOSTREA GIGAS*

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ABSTRACT Seasonal variations of glycogen and protein metabolism in the Pacific oyster *Crassostrea gigas* were investigated *in vivo* using a radiolabeled glucose injection technique and were compared with *in vitro* experiments on vesicular cells. Protein metabolism appeared stable during a gametogenetic cycle, whereas glycogen metabolism *in toto* was found to be clearly dependent on the sexual cycle, with decreasing incorporation during gonadal tubule development. The *in vivo* results correlated well with data from *in vitro* experiments on vesicular cells, which correspond to the animal's glycogen storage compartment.

KEY WORDS: Pacific oyster, *Crassostrea gigas*, gametogenesis, glycogen, storage tissue, *in vivo*, bioassay

INTRODUCTION

In the Pacific oyster, *Crassostrea gigas*, as in most bivalves, glycogen is one of the major energetic fuels for gametogenesis (Bayne et al. 1982, Gabbott & Whittle 1985, Ruiz et al. 1992, Mathieu & Lubet 1993). On the west coast of Europe, gametogenesis in *C. gigas* follows an annual cycle: gonial mitosis occurs in autumn and early winter in the gonadal tubules; the gonad develops in winter and spring; and in summer, the ripe gonad is ready for sequential spawning in July or August, depending on the rearing site. The biochemical composition of the whole animal and isolated organs was previously studied, and glycogen levels were determined (Walne & Mann 1975, Robert et al. 1993, Berthelin et al. 2000b). Glycogen storage and mobilization activities were tightly correlated to the reproductive cycle. Histologic studies showed a seasonal inverse relationship between the increase of the gonadal tubules and the regression of the storage tissue in the gonadal area. Moreover, glycogen was stored during autumn and early winter while gonadal tubules regressed, and was subsequently mobilized during active gametogenesis (Berthelin et al. 2000a, 2000b).

In the oyster, the biochemical mechanisms of glycogen storage and mobilization in relation to reproductive activity remain poorly documented in comparison with other models like the marine mussel, *Mytilus edulis* (Houtteville 1974, Pipe 1987, Lenoir 1989). Recently, an *in vitro* bioassay was developed for *C. gigas* to measure glucose incorporation into glycogen in vesicular cells, the glycogen storage compartment (Berthelin et al. 2000a). The *in vitro* approach provided some valuable information on the cellular mechanisms of glycogen metabolism in vesicular cells, but it may not reflect the true metabolism of reserve cells *in vivo*. First, cellular dissociation could damage receptor protein structure leading to glucose uptake modifications, compared with physiologic conditions in the whole animal. Second, the incubation conditions may not reflect the seasonal variations in the natural environment.

For these reasons, the glycogen metabolism of cupped oysters was investigated using an *in vivo* approach based on injections of ^{14}C-labeled glucose into the adductor muscle. After defining the optimal experimental conditions, glucose incorporation was studied in the whole animal in relation to the annual sexual cycle. Finally, *in vivo* results were matched with *in vitro* data obtained from isolated vesicular cells.

MATERIAL AND METHODS

Animals

Pacific oysters (*C. gigas*, 3 years old) were obtained from a commercial oyster farm in Saint-Vaast-La-Hougue, Normandy, France. The animals were kept in aerated seawater at 13°C throughout each experiment. The animals were starved for 24 h prior to all *in vivo* experiments.

In vivo *Bioassay*

Conditions of Injection

Two days before injection, both valves of the oyster were notched beside the adductor muscle, paying attention not to damage the muscle. A preliminary experiment was conducted to evaluate the diffusion of the injected solution into the animal tissues: 200 µL of neutral red in sterile seawater was injected into the adductor muscle. The oysters, kept in 0.5 L tanks, were dissected 45 min or 3.5 h after injection. Seawater coloration in each tank was controlled, and neutral red diffusion was observed in each animal.

[U-14C] Glucose Injection

For labeling experiments, the injected solution included 50 µL of [U-14C] glucose and 150 µL of D-glucose (13 mM), resulting in a final glucose concentration increase of approximately 1 mM in the hemolymph, according to the standard of Livingstone and Clarke (1983). For each condition, six animals were injected and were kept in aerated seawater at 13°C. Control animals were analyzed just after injection to estimate nonspecific radioactivity. After incubation, soft parts were separated from shells that had been individually collected in 50-mL tubes and blended (ultra-turrax, Labosi, France). Animal tissues were stored at −20°C before sample treatment. For each animal, the blended tissue sample volume was adjusted to 40 mL with sodium hydroxide (0.006 N) and thoroughly homogenized.

Different [U-14C] glucose doses were tested (0.5, 1, 5, and 10 µCi). Kinetic measurements of incorporation in protein and gly

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cogen also were performed (after 0, 7.5, 16, 24, 48, and 72 h of incubation). Seasonal variations of incorporation were studied.

Total Radioactivity Determination

Five hundred microliters of potassium hydroxide (0.3 N) was mixed with 500 μL of a blended tissue sample, and 250 μL of this mixture was diluted in 4 mL of scintillation fluid (Optiphase, Hisafe II 2*51 Wallac, France, EG and G division instruments) were analyzed for ^{14}C activity (Packard scintillation counter, France).

[U-^{14}C] Glycogen Content

Five hundred microliters of a blended tissue sample was mixed with 500 μL of 10% trichloroacetic acid, and precipitated proteins were discarded by centrifugation (8000 g, 10 min, 4°C). Seven hundred microliters of the supernatant was transferred to a 5-mL tube containing 10 mg of unlabeled oyster glycogen (Sigma-Aldrich, France) as a carrier and 4 mL of absolute ethanol. After overnight precipitation at 4°C, glycogen was collected by centrifugation (2500 g, 10 min, 4°C), and the pellets were washed three times with absolute ethanol containing D-glucose (0.1 M). Glycogen pellets then were dried and resuspended in 500 μL of potassium hydroxide (0.3 N). Radioactivity was determined in 250 μL of glycogen suspension diluted in 4 mL of scintillation fluid.

[U-^{14}C] Protein Content

Protein content also was determined in each tissue sample as follows: 500 μL of oyster extract was mixed with 1 mL of potassium hydroxide (0.3 N) and 3 mL of 10% trichloroacetic acid. After overnight precipitation at 4°C, protein pellets were collected by centrifugation (3000 g, 10 min, 4°C), were washed three times in trichloroacetic acid, and were resuspended in potassium hydroxide (1 mL, 0.3 N). Radioactivity was determined in 250 μL of protein suspension diluted in 4 mL of scintillation fluid.

In Vitro Approach

Preparation of Vesicular Cell Suspension

Oysters were maintained on ice during the dissection, were opened by sectioning the adductor muscle, and were rinsed thoroughly with sterile seawater. The labial palps were dissected, rinsed three times in sterile sea water, and decontaminated for one night in 50 mL of Leibovitz culture medium [Leibovitz L15; NaCl 340 mM, KCl 50 mM, Hepes 20 mM (pH 7.4), 1100 mOsm, filtered on a Millipore 0.22-μm filter] supplemented with antibiotics (streptomycin 100 mg L^{-1}, penicillin 60 mg L^{-1}, gentamycin 50 mg L^{-1}, and nystatin 8.2 mg L^{-1}).

Vesicular cell isolation was performed as previously described by Berthelin et al. (2000a). Dissociated cells were diluted in Leibovitz culture medium to obtain 3×10^6 cells mL^{-1} and were distributed into 24-well culture plates. Significant survival was evaluated with the MTT [3-(5,5- dimethyl-thiazol-2-yl)-2,5-diphenyl tetrazolium bromide] reduction assay (Mosmann 1983, Coulon 1993).

[U-^{14}C] Glucose Incorporation into Glycogen by Vesicular Cells

Glucose incorporation measurement was derived from Berthelin et al. (2000a). In each well, 500 μL of vesicular cell suspension (3×10^6 cells mL^{-1}) was mixed with 50 μL of [U-^{14}C] glucose (0.5 μCi, specific activity: 150–260 mCi mmol^{-1}) and 50 μL of unlabeled D-glucose (1.5 mM). After incubation (7 h, 15°C), the

well contents were transferred into 1.5-mL microtubes and centrifuged (8000 g, 10 min, 4°C), resulting in cell disruption. Three hundred microliters of supernatant was transferred into a 5-mL tube containing unlabeled oyster glycogen (10 mg) and then was mixed with 4 mL of cold absolute ethanol. Glycogen was precipitated overnight and rinsed three times with a solution of D-glucose in absolute ethanol (0.1 M). Glycogen pellets were dried and diluted in 500 μL of distilled water. An analysis of radioactivity was performed on 200 μL of glycogen suspension diluted in 4 mL of scintillation fluid.

Blanks without cells or without radioactive labeled glucose were tested, and control samples were prepared by stopping the incubation immediately after [U-^{14}C] glucose addition.

Data Analysis

Results were expressed as the mean ± SD. Each value is the mean of six replicates. A nonparametric test (Kruskall-Wallis test) followed by a multiple comparison test (Newman-Keuls test) also was performed to determine the significant differences between samples (Scherrer 1984).

RESULTS

Injection Into the Adductor Muscle

A preliminary experiment using neutral red as a visual tracer led to an estimate of its diffusion in seawater. After injection, no seawater coloration was observed. Moreover, after 45 min, the digestive cardiac sinus, the adductor muscle, and the gills were stained red, whereas the palps and mantle appeared colorless. Three hours after injection, the gills were still stained, and the palps and mantle also appeared red. However, the digestive cardiac sinus was faded.

Recovered Radioactivity

It represented 35 to 85% of injected radioactivity with large individual variations due to injection efficiency. Because of these variations, [U-^{14}C] glucose incorporation into protein and glycogen were expressed as the percentage of recovered radioactivity for each animal.

[U-^{14}C] Glucose Incorporation

Four doses of [U-^{14}C] glucose were tested: 0.5 μCi (0.0185 MBq) and 1.0 μCi (0.037 MBq) values were chosen by reference to previous in vitro experiments (Berthelin et al. 2000a); and 5 μCi (0.185 MBq) and 10 μCi (0.37 MBq) were tested considering the potential dilution of radioactive material in the whole animal. After 24 h of incubation, irrespective of the amount of injected labeled glucose, 8 to 10% of recovered radioactive carbon was found in the protein fraction, and about 2% was incorporated into glycogen (Fig. 1). Ten microcuries (0.37 MBq) of radioactivity was used routinely in all subsequent experiments to keep sensitivity as high as possible, since low levels of labeled glycogen were expected at certain periods of the year.

The kinetics of radioactive carbon incorporation into proteins is presented in Fig. 2a. The incorporation rate was maximal during the first 16 h of incubation. After 48 and 72 h, 11% and 12.2%, respectively, of radioactive carbon was incorporated into proteins.

For the same animals, glucose incorporation into glycogen increased linearly during the first 24 h of incubation (Fig. 2b) and reached a maximal value of 1.6% of recovered radioactive carbon
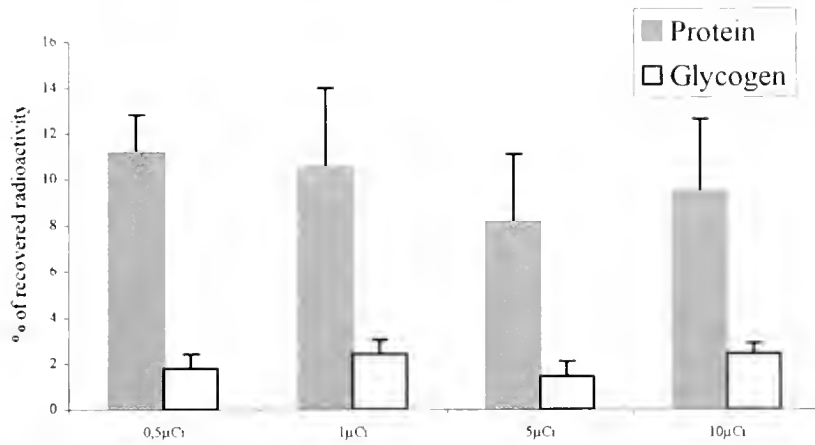
Figure 1. Fraction of recovered radioactivity measured in protein and glycogen depending on the quantity of radioactivity used (µCi). Results are expressed as the percentage of recovered radioactivity in oyster ±SD.

after 48 h (this incubation time was chosen for further experiments). This maximal value varied in the range of 1.6 to 2% for the eight experiments performed.

Seasonal Variations of *In Vivo* Carbon Incorporation into Proteins

The radioactive carbon fraction incorporated into proteins was measured over the annual cycle with an incubation time of 48 h. The radiolabeled protein fraction was found to represent 7 to 11% of recovered radioactivity according to month (Fig. 3). With re-

spect to protein metabolism, two statistically different groups were observed: one from January 1 to October 1; and the second, with reduced metabolism, from November 1 to March 2 [$P < 0.05$ (Kruskall-Wallis and Newman-Keuls tests)].

In Vivo and *In Vitro* Glucose Incorporation into Glycogen

In vivo results (Fig. 4) showed that glycogen represented between 0.6% and 1.9% of recovered glucose incorporation in the oyster, depending on the season (injected amount 0.37 MBq; in-
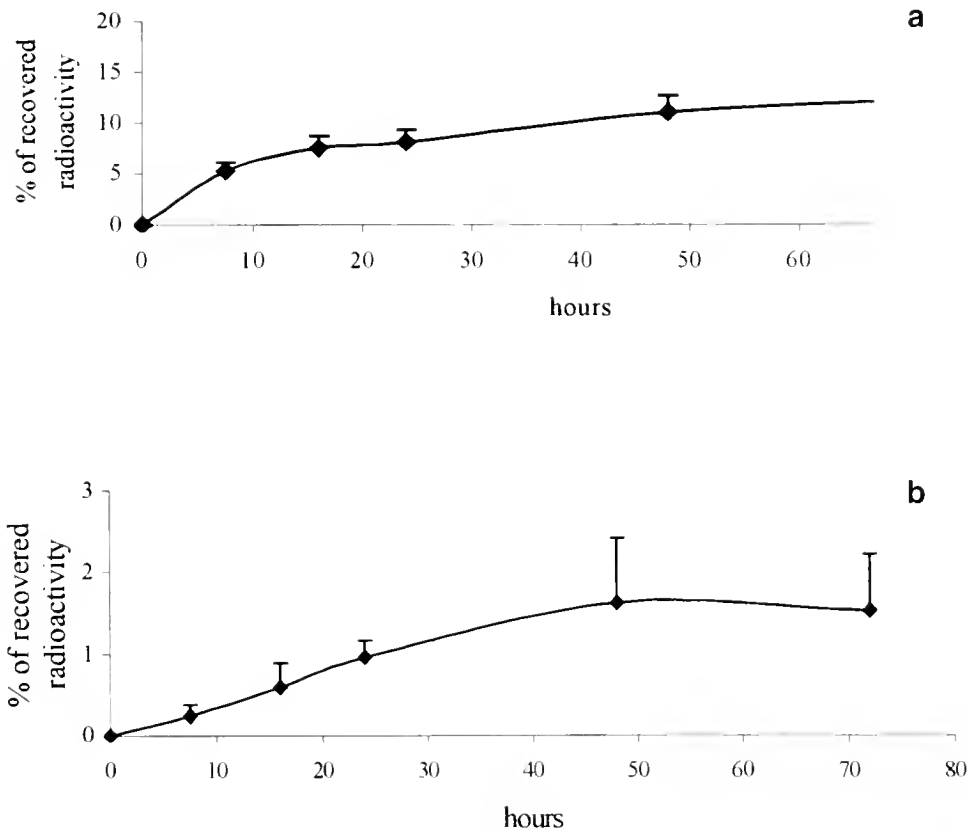
Figure 2. Kinetics of ^{14}C incorporation. (a) Incorporation of ^{14}C into proteins. (b) Incorporation of ^{14}C into glycogen. Results are expressed as the percentage of recovered radioactivity ±SD.
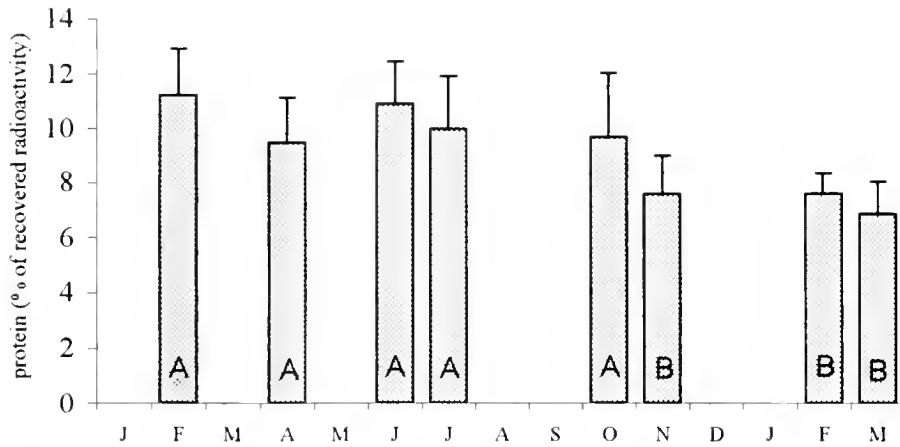
Figure 3. Seasonal variation of carbon incorporation in protein fraction. Results are expressed as the percentage of recovered radioactivity ±SD. Groups A and B are statistically different.

cubation time 48 h). In the first year, labeling was maximal in February (1.9%), decreasing progressively to 0.6% in July, before increasing during the autumn and returning to the maximal value the following March (with a lower value in February of the second year) ($P < 0.05$). The stages of development of the gonadal and storage tissues in the gonadal area (Heude Berthelin et al. 2001) are overlaid on Fig. 4.

In vitro measurements were performed on vesicular cells from February 1 to October 1 (Fig. 5). These values show that in vitro

incorporation was maximal in February (1.5 nmol per 1.5×10^6 cells), decreasing to an undetectable level in July and August, and finally rising again during the following autumn ($P < 0.05$).

DISCUSSION

The investigation of different metabolic pathways in bivalves has been based mainly on in vitro techniques, due to the anatomic characteristics of these animals. These in vitro approaches have
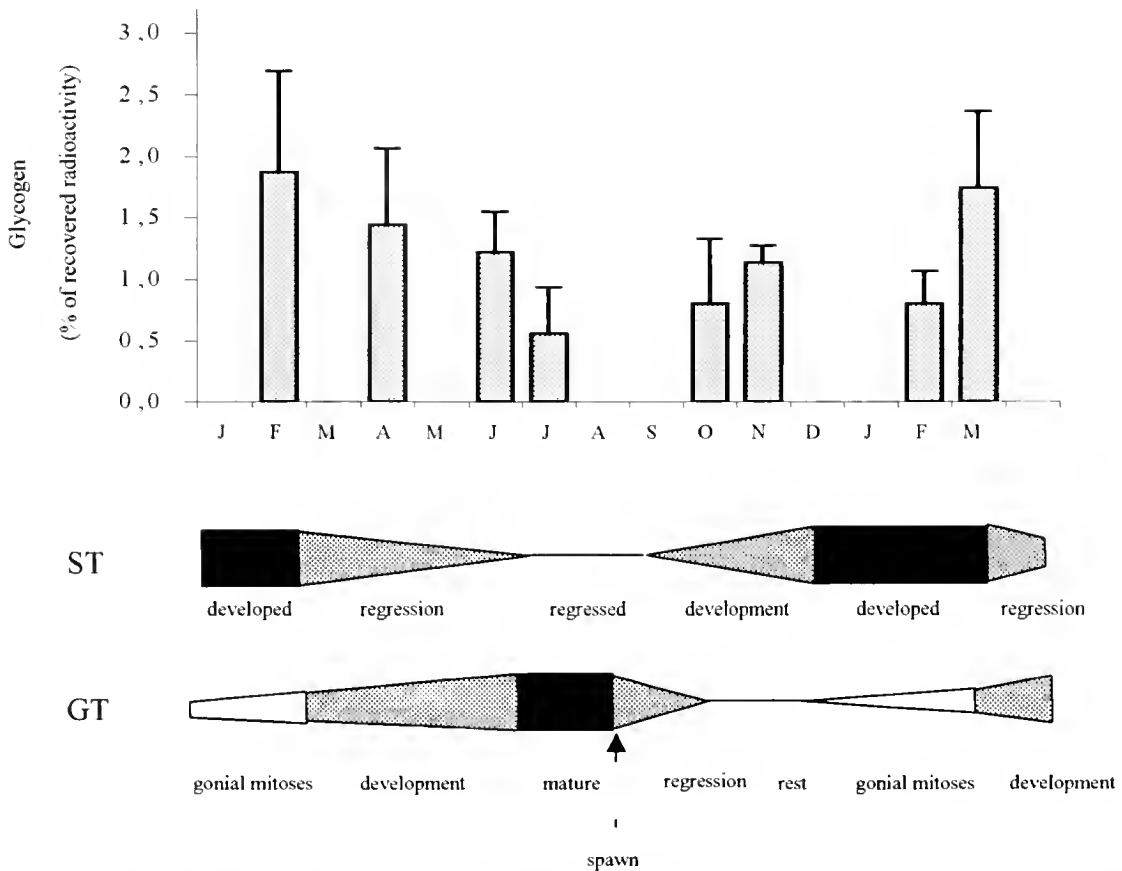
Figure 4. (top) Seasonal variation of carbon incorporation in glycogen fraction. Results are expressed as the percentage of recovered radioactivity ±SD. (bottom) Gametogenesis and storage tissue development in oysters (Saint Vaast la Hougue, France) [redrawn from histologic data published in Heude Berthelin et al. (2001)].
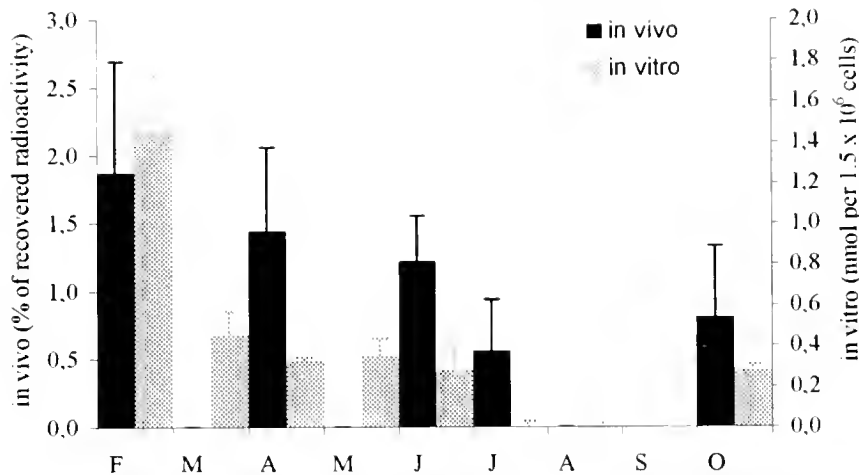
Figure 5. Changes of [U-^{14}C] glucose incorporation into glycogen measured both *in vitro* and *in vivo*. The results are expressed in nanomoles of glucose incorporated per 1.5×10^6 cells for *in vitro* experiments, and as a percentage of recovered radioactivity for *in vivo* experiments (mean $\pm$ SD; $n = 6$).

revealed specific information about metabolism at the scale of isolated cells or specialized tissue samples maintained in strictly controlled conditions. However, tissue or cell preparation steps associated with the chosen artificial conditions may disturb the metabolic activity of the sample relative to its true state in the whole animal.

The present study aimed to compare seasonal variations of glucose incorporation into glycogen measured by *in vitro* bioassay in *C. gigas* (Berthelin et al. 2000b) with *in vivo* levels of incorporation. This incorporation was measured after the injection of ^{14}C glucose into the adductor muscle using an experimental procedure originally used for the artificial infection of oysters with pathogens (Hervio et al. 1992).

Protein metabolism was first analyzed by measuring the incorporation of radioactive carbon into proteins. Radiolabeled proteins represented 7 to 11% of recovered radioactivity. Whereas experimental conditions were significantly different (i.e., tracer concentration, incubation time, and injection procedure), these data may be compared with the results reported by de Zwaan et al. (1975) for the mussel *Mytilus edulis* in aerobic conditions: these authors found that proteins accounted for 11% of radioactivity. In the oyster, the annual pattern of ^{14}C incorporation into proteins appeared rather stable throughout the complete gametogenetic cycle (from January 1 to October 1). Following the gametogenetic cycle (from November 1) incorporation was also stable, but slightly lower (7%).

By comparison with this relative stability, glycogen metabolism shows some significant variations: the ^{14}C incorporation into glycogen ranged from 0.6 to 1.9% of the radioactivity. Glycogen storage decreased during gonadal development from February to July and increased after the spawning event when the oysters were in the sexual resting stage. These results were then compared with *in vitro* data obtained during a previous gametogenetic cycle (Berthelin et al. 2000b). Because of possible interannual bias, additional *in vitro* measurements also were performed within the same year as the *in vivo* experiments.

Whatever the approach, ^{14}C glucose incorporation into glycogen presents the same annual pattern. Indeed, the observed variations correlated with seasonal changes in glycogen content reported in *C. gigas* (Mann 1979, Robert et al. 1993, Almeida et al. 1997) and confirm that glycogen storage occurs during early gametogenesis to support the energetic cost of the reproductive effort (Ruiz et al. 1992, Mathieu & Lubet 1993, Berthelin et al. 2000b, Heude Berthelin et al. 2001). The mobilization of glycogen also was observed *in vivo* in early spring and autumn. This matching between *in vivo* and *in vitro* data is essential to verify the previous *in vitro* approach to the study of seasonal variations in glycogen metabolism: the *in vitro* bioassay should be considered as an adjusted technical approach to investigate the cellular mechanisms involved in the regulation of these changes of metabolism.

Moreover, the correlation between the pattern of glycogen metabolism in the whole animal (i.e., the *in vivo* approach) and the gonadal vesicular cells suggests that in the oyster glycogen metabolism occurs mainly in the specialized storage tissue located in the gonadal area (Berthelin et al. 2000a), and that this metabolism is the main source of energy for reproductive effort. Further experiments should now be carried out to improve different technical aspects of the *in vivo* procedure. With the current *in vivo* procedure, glucose is supplied directly into the hemolymph sinus without taking into account digestive assimilation or possible short-term storage in the digestive gland (Berthelin et al. 2000b). Improvement may result from the use of labeled microalgae or coated beads. In addition, organ dissection should be considered to quantify the respective role of each compartment involved in glucose metabolism.

ACKNOWLEDGMENTS

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EFFECTS OF TEMPERATURE AND FEEDING REGIMES ON GAMETOGENESIS AND LARVAL PRODUCTION IN THE OYSTER *CRASSOSTREA GIGAS*

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ABSTRACT The effect of feeding regimes and temperature on the beginning of gametogenesis in the Pacific oyster *Crassostrea gigas* (Thunberg) was examined under laboratory conditions. Oysters from two different culture sites in France, Baie des Veys (Department Manche) and La Tremblade (Department Charente-Maritime), were collected in January 2000 and exposed to four treatments, involving a period of maintenance at 10°C with or without feeding followed by a conditioning period at 19°C with or with feeding. Routine conditioning procedures at 19°C (direct conditioning), with or without food, were performed at the same time and were used as controls. Oocyte size was used to describe the evolution of gametogenesis in all treatments. Contrasting responses were noticed between samples from Baie des Veys (BV-oysters) and La Tremblade (LT-oysters). BV-oysters containing more tissue reserves than specimens from the other location used carbohydrates to support gametogenesis, while LT-oysters used proteins to fuel oocyte development. During the initial period at 10°C, fed BV-oysters began gametogenesis and produced mature oocytes, while unfed BV-oysters began gametogenesis, but at a slower rate. Fed LT-oysters began gametogenesis at 10°C, whereas unfed LT-oysters remained unchanged (early gametogenesis stage) during the cold phase and only initiated gametogenesis when the temperature was increased. Oysters conditioned without food produced significantly less oocytes than specimens conditioned with food, but no differences in larval yield (D-larvae) were detected amongst the different conditions and sampling locations. Only LT-oysters kept without food throughout the experiment did not produce oocytes at the end of the conditioning period. These experiments demonstrate that oocyte production in *C. gigas* is dependent upon food supply and temperature, but that oocyte quality under controlled conditions appears to be related to stored reserves in natural settings.

KEY WORDS: conditioning, *Crassostrea gigas*, food, gametogenesis, temperature

INTRODUCTION

Accumulation of reserves in the Pacific oyster *Crassostrea gigas* (Thunberg 1793) takes place in autumn and winter, and the first signs of the beginning of gametogenesis are observed in January when temperature is still descending (Chávez-Villalba et al. 2002a). The influence of food supply and temperature on the reproduction cycle of bivalves has been noted by many authors (Dinamani 1987, Ruiz et al. 1992). However, there are few data on the influence of environmental factors on gametogenesis in *C. gigas*. The studies on this topic for the scallop *Aequipecten irradians concentricus* (Sastry 1979) and eastern oyster *Crassostrea virginica* (Thompson et al. 1996) suggest that in the early phase of gametogenesis, bivalves require an adequate food supply, as well as a suitable temperature to stimulate gonad growth. These authors proposed that, under inadequate food conditions, tissue reserves are used for maintenance metabolism rather than gametogenesis, and that food supply appears to be less critical after certain minimum reserves have accumulated in the gonad. Gonad maturation then occurs at a rate that is dependent on temperature. Gametogenesis in oysters is directly correlated with water temperature (Muranaka & Lannan 1984). However, Sastry (1968) found that low temperature can be inhibitory in well-fed scallops held at 15°C that already had started early gametogenesis, but oocytes did not enter into normal growth until exposed to higher temperatures (20°C).

Thus, normal reproductive development requires a minimum temperature and an adequate food supply.

Temperate bivalves exhibit a marked seasonal cycle in the synthesis, accumulation, and use of biochemical energy reserves. In general, reserves are stored during periods of high food availability (late summer and fall), at which time the major energy requirements for somatic and germinal growth have already been satisfied. Stored reserves are used to initiate gametogenesis and to maintain metabolism during periods of low food availability (Thompson et al. 1996). Berthelin et al. (2000) found that glycogen accumulation in the gonad of *C. gigas* occurs in fall and winter, and this compound serves as a substrate to support gametogenesis. In this way, oysters can partially uncouple temporal food availability with gamete production, allowing gametogenesis to start when food supply is at a minimum (winter).

The accumulation and use of stored reserves in bivalves depend on the state of gonad development, the influence of environmental parameters on metabolic activities, and the nutritional value of food supplied during conditioning. In French hatcheries, the beginning of broodstock conditioning of *C. gigas* starts in December to obtain viable gametes and larvae by the end of January (Chávez-Villalba et al. 2002b). Conditioning procedures consist of feeding abundantly the oysters for 7 wk at a warm temperature (19°C). The use of this technique allows animals to be conditioned from December until April, producing an increase of viable gametes and larvae with time (Chávez-Villalba 2001). Additionally, it was found that there were some groups of oysters that can produce

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viable oocytes without being fed during the conditioning procedures, and that larval hatching rates from unfed oysters were not significantly different, compared with fed animals. However, the nutritional stress produced by partial or complete deprivation of food can substantially alter the biochemical composition of bivalves. Whyte et al. (1990) demonstrated that oysters exhibit different biochemical composition if deprived of food.

Different experiments in our laboratory (Chávez-Villalba 2001, Chávez-Villalba et al. 2002a, Chávez-Villalba et al. 2002b, Chávez-Villalba et al. 2003) have shown that gametogenesis in *C. gigas* seems to be an integrated response to different environmental factors, in which temperature and food supply play significant roles. In this study, the effects of food and temperature in the period at the beginning of gametogenesis, as well as the biochemical changes in soft tissues produced by the effect of these parameters, were investigated. To achieve these objectives, we studied two oyster populations: one from Baie des Veys (BV) where oysters have low spawn rates as a result of low summer temperatures; and the other from La Tremblade (LT) where environmental conditions allow full spawning in summer.

MATERIAL AND METHODS

Experimental Conditions

At the end of January 2000, two 600-oyster samples were taken from two different culture sites in France where they had been raised in plastic mesh bags on iron tables. These animals were initially collected from the Bassin d'Arcachon (44°41.8'N, 1°8.3'W). In the Bassin, the water temperature varies from 7.5°C in January to 22 C in August, and salinity records go from 26 to 33‰ during the year. Juveniles then were grown at Le Morbihan (47°35.5'N, 3°1.3'W) until they were 18 mo old and subsequently were dispatched to one of two culture sites, where they were raised for about 1 y (Fig. 1). In Le Morbihan, water temperature fluctuates from 3 to 5°C in the winter to 20 to 22°C in the summer, and salinity values remain stable throughout the year (34.8–35.4‰). One of the culture sites is on the Baie de la Seine in the BV in the

Departments of Manche and Calvados (49°21.5'N, 1°6.9'W). In this zone, the average temperature in January is about 6°C, about 17°C in August, and during spring and summer temperature increases on the surface. Salinity records in the bay are always under 34.5‰, showing a decreasing pattern toward the coast (30‰). The other site is located on the Atlantic coast in the estuary of the Seudre River at LT (45°31.6N, 1°1.7W) in the Department of Charente-Maritime (Fig. 1). In this area, the temperature varies from 7°C in winter to 22°C during the summer, and salinity records are closer to 15‰ (www.ifremer.fr).

Oyster samples were transported from the culture sites to the Brest-IFREMER center where the samples from the two sites were divided into several groups. Fifty animals from each site were exposed to standard conditioning (19°C with ample food). Two more 50-oyster groups from each site were exposed to standard conditioning (19°C) but without food. These groups were conditioned (direct conditioning) from February 8 to March 30, 2000, and were used as controls.

Four groups of 250-oysters each (two from BV and two from LT) were placed in maintenance tanks at 10°C. One group from each site was fed continuously with the same diet as that used for conditioning, and one group from each site was maintained without food. These maintenance treatments were sustained for 60 days (8 February–9 April, 2000). At the end of this period, each group was divided into two subgroups that were conditioned under two different treatments, with and without food (Fig. 2).

For the conditioning experiments, seawater temperature in the tanks was increased 1°C per day until it reached 19°C (heating period), and the photoperiod was adjusted to 16 h of daylight and 8 h of night (spring conditions). Oysters were fed a diet commonly used in experimental hatcheries for conditioning: a mixture of two micro-algae species (10^9 cells of each species per day per animal) from monospecific cultures of *Isochrysis aff. galbana* Green (Clone T-Iso; Tahiti *Isochrysis*) and *Chaetoceros calcitrans* Takano.

Sampling

During the direct conditioning, the groups were sampled (20 oysters per site) two times; the first sample was obtained before the heating period (10°C), and the second sample was taken after 6 wk at 19°C. For the four treatments, oyster samples (20 from each group) were taken at the beginning, in the middle, and at the end of the period at 10°C. The last samples were obtained at the end of the four conditioning procedures (Fig. 2). From each sample (20 oysters), 10 specimens were used for histologic examination, and the other 10 were used for biochemical analyses.

Semi-Quantitative Histology

Procedures in this part of the study generally followed the methods of Chávez-Villalba et al. (2002a, 2002b). Oysters used for histology were opened, and a section of approximately 1 cm^3 of visceral mass was taken from above the pericardial area and fixed in Bouin's solution for at least 48 h. Samples were dehydrated with a series of ethanol treatments of increasing concentration, cleared in toluene, and embedded in paraffin following a standard procedure. Sections 5 μm wide were cut, mounted on glass slides, and colored with Groat's hematoxylin and eosin Y solution (Martoja and Martoja-Pierson 1967). The histology slides were examined under a microscope connected to a video camera to determine
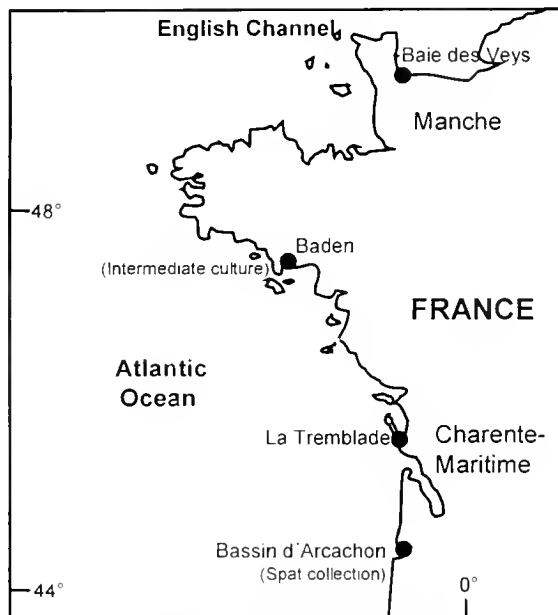
Figure 1. Location of *C. gigas* sites.
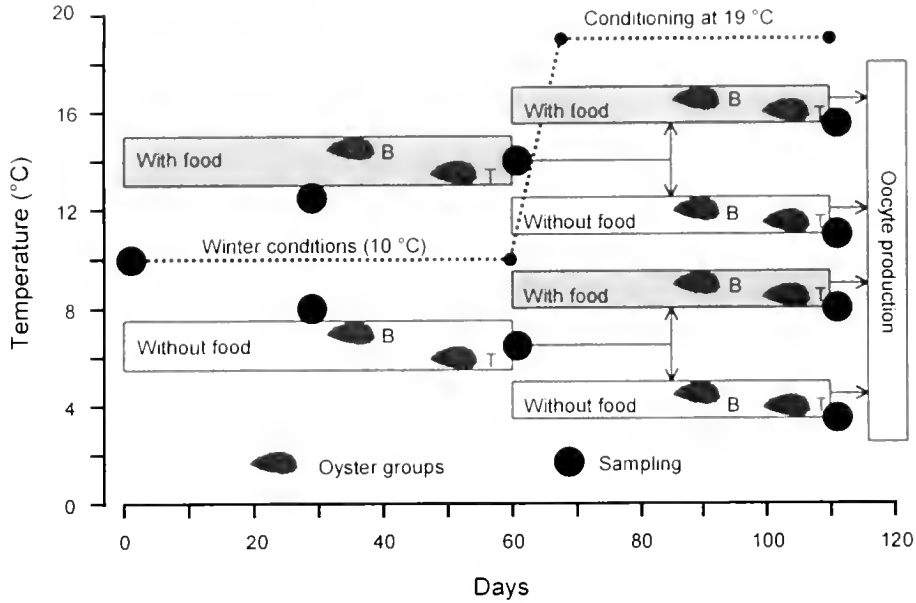
Figure 2. Experimental conditions of the four treatments (black circles = samples taken for histologic and biochemical studies). B = BV specimens; T = LT specimens. Starting date is February 8, 2000.

oocyte size and frequency, and gametogenic activity. Recorded images were processed by digital image analysis.

Oocytes were measured and histology classified following the description by Lango-Reynoso et al. (2000). These operations were conducted on 100 randomly chosen oocytes per oyster, and measurements followed a standard bias reduction procedure for selecting measurement fields. Transects of gonad preparations were oriented to maximize coverage of the larger vertical or horizontal oocyte field axis. All oocytes with a well-defined germinal vesicle in a field were measured, and every oocyte measured was assigned to a reproductive stage based on the diameter and histologic characteristics of the gonad (Table 1). In the case of male

oysters, the evolution of the spermatogenesis was described according to the histologic characteristics of the gonad. Three developmental stages were recognized (Table 1).

Biochemical Analyses

We used 10 oysters per sample for biochemical analyses. Specimens were dissected, and soft tissues were divided into two sections: gonad-digestive gland portion (called "gonad"); and the remaining tissue (called "meat"). All samples were ground by adding 1 mL of distilled water per gram of tissue at 5°C in an ice bath. A 400-μL aliquot was used for lipid determination using the

TABLE 1.

Reproductive stages in the female and male oyster *C. gigas:* Cytologic characteristics corresponding to each stage are included.

Stages	Stage Interval (μm)	Histologic Description
Females		
Early gametogenesis	3.0 ± 12.0	Follicles are elongated and often isolated in the abundant connective tissue, with walls consisting of primary oocytes of homogeneous size.
Growing	12.1–30.0	Start of oocyte growth. A large range of oocyte size at all gametogenic stages can be observed, including some free oocytes. Interfollicular connective tissue disappears.
Mature	30.1–41.0	Follicles of relatively homogeneous size completely filled with mature oocytes with distinct nucleus.
Degenerating	41.1–60.0	Follicles containing degenerating oocytes, often elongated in shape, sometimes broken. Obvious redevelopment indicated by increased number of primary oocytes.
Males		
Early gametogenesis		Abundant connective tissue containing elongated follicles with walls consisting of germinal epithelium with some spermatogonia and spermatocytes
Growing		Connective tissue is reduced, follicles become larger, and normal sequences of spermatogenesis are observable with spermatocytes I and II, spermatides, and some spermatozoids organized in the lumen
Mature		Connective tissue almost disappeared. Follicles filled with packages of spermatozoids oriented with tails toward the follicle lumen

Reproductive stages in female specimens are based on an oocyte diameter (μm) interval (Lango-Reynoso et al. 2000).

Bligh and Dyer (1959) method. Carbohydrates were analyzed in a 300-µL sample by the method of Dubois et al. (1956), and proteins were analyzed in a 300-µL aliquot by the method of Lowry et al. (1951). Tissue dry weights were calculated from the macerate of each sample; 2 mL were emptied into preweighed aluminum containers and dried in an oven at 80°C for 48 h. Finally, aluminum containers were reweighed after cooling in a desiccator.

Given that no significant differences were observed between total dry weights of oysters (from BV and LT locations) at the beginning and by the end of the experiments, the dry weight percentages of each biochemical compound were multiplied by the total dry weight of each tissue sample to express the results in milligram equivalence of each biochemical compound per total dry weight of the tissue (gonad and meat).

Oocyte Production and Larval Yield Estimation (D-Larvae)

Oocyte production and larval yield estimations followed the recommendations of Chávez-Villalba et al. (2002a). Oysters from each group were taken from the experimental tanks at the end of conditioning procedures; 20 animals per group were opened, and their sex was determined by observing a fresh smear sample from the gonad under a microscope. After this procedure, females and males were separated, and gametes from both sexes were recovered using the scarification technique described by Allen and Bushek (1992). The gonads of all oysters were scarified by a light incision of the gonadal tegument. Oocytes were collected in beakers by rinsing the gonad with filtered seawater. The oocytes were passed through a 60-µm sieve to eliminate undesirable material. Mature oocytes were retained in a 20-µm sieve. These were rinsed several times and placed in 2- or 5-L beakers. To determine oocyte

production, three 50-µL samples per group were examined and counted under a profile projector. Males underwent the same procedure, but spermatozoa suspensions were examined under a microscope for motility. Batches of spermatozoa of low motility were discarded. A minimum of three batches was mixed together and 10 to 20 mL were used for fertilization. Oocytes were fertilized in 5-L beakers and checked for normal progress about 0.5 to 1 h later (Robert and Gérard 1999).

After fertilization, an equal number of embryos from all oysters of each group were pooled together and placed, one group per tank, in 150-L tanks at a 33 embryos mL^{-1} concentration. After 48 h, the tanks were emptied, and the larvae were recovered by sieving. Three 50-µL larvae samples from each tank were taken for larval yield estimation (number of D-larvae after 48 h of culture/initial number of embryos).

Data Analysis

The oocyte proportion corresponding to each reproductive stage was calculated according to Lango-Reynoso et al. (2000), and the arcsine was transformed (Snedecor and Cochran 1972) for each oyster. The logarithms of oocyte production data were calculated. The transformed proportions and logarithms were compared using the Kruskal-Wallis test. A two-way analysis of variance (ANOVA) test was used to examine the effect of origin and feeding regimen on early, growing, and mature oocyte categories for the direct conditioning. A three-way ANOVA test was run to analyze the following: (1) the effect of time (between days 30 and 60), origin, and feeding regimen on the different oocyte categories during the period at 10°C; (2) the effect of time (beginning and end of conditioning), origin, and the four tested conditions on the oo-

TABLE 2.

Mean proportion[a] of different oocyte stages: early gametogenesis, growing, and mature in *C. gigas* under direct conditioning with and without food, and four treatments during a second phase of conditioning.

Oyster Sample	Day 0				End of Conditioning (Day 56)			Characteristics
	E	G	M		E	G	M	
Direct conditioning								
BV	69	31	0		6	22	72	Conditioning WF
					30	51	19	Conditioning WOF
				→ 7 wk at 19°C →	21	41	38	Conditioning WF
LT	63	37	0		52	46	2	Conditioning WOF

Oyster Sample	← Period at 10 C →									End of Conditioning (Day 110)			Characteristics
	Day 0			Day 30			Day 60						
	E	G	M	E	G	M	E	G	M	E	G	M	
Four treatments													
BV	69	31	0	58	42	0	33	66	1	2	22	76	Treatment 1 (WF/WF)
										1	26	73	Treatment 2 (WF/WOF)
				84	16	0	60	40	0	0	12	88	Treatment 3 (WOF/WF)
										11	76	13	Treatment 4 (WOF/WOF)
LT	63	37	0	90	10	0	38	62	0	5	28	67	Treatment 1 (WF/WF)
										1	27	72	Treatment 2 (WF/WOF)
				96	4	0	87	13	0	10	34	56	Treatment 3 (WOF/WF)
										57	43	0	Treatment 4 (WOF/WOF)

Presented as %. E, early gametogenesis; G, growing; M, mature; WF, with food; WO, without food.

[a] Mean of values found in a 10-oyster sample.

cytes (early, growing, and mature) during the conditioning phase; (3) the effect of origin, time, and feeding regimen on the lipid, protein, and carbohydrate content in the gonad and meat of oysters maintained at 10°C for 60 days; and (4) the effect of origin, time, and four tested conditions on the lipid, protein, and carbohydrate content in the gonad and meat of oysters during the conditioning procedures. Statistics were analyzed at a significance level α = 0.05.

RESULTS

Oogenesis

Direct Conditioning

The proportions of the different oocyte categories found at the beginning and the end of direct conditionings are presented in Table 2. Mature oocytes were observed in all BV and LT groups at the end of conditioning, but statistical results (Table 3) showed a significant effect of feeding regimen and place of origin. There was a higher proportion of mature oocytes in specimens conditioned with food, and BV oysters produced a higher proportion of mature oocytes than LT specimens.

Four Tested Conditions

The proportions of the oocyte categories found during the cold phase and at the end of conditioning in both BV and LT oysters for

TABLE 3.

Results of two-way and three-way ANOVA tests for early, growing, and mature oocyte categories, and for biochemical content in the gonad and meat of oysters *C. gigas*, respectively (direct conditioning).

Source of Variation	Df	MS	F	P
Oogenesis				
Early gametogenesis stage				
Factor A (feeding regime)	1	1840.1	8.53	0.0079
Factor B (origin)	1	1194.1	5.54	0.0280
Interaction (A × B)	1	11.1	0.05	0.8225
Growing stage				
Factor A (feeding regime)	1	475.2	3.45	0.0768
Factor B (origin)	1	68.5	0.5	0.4882
Interaction (A × B)	1	360.2	2.61	0.1202
Mature stage				
Factor A (feeding regime)	1	6201.8	34.8	0.0000
Factor B (origin)	1	1615.2	9.05	0.0065
Interaction (A × B)	1	78.9	0.44	0.5128
Biochemistry				
Meat				
Factor A (origin)	1	1.03E6	54.2	0.0000
Factor B (feeding regime)	1	936.7	0.05	0.8246
Factor C (time)	1	433395	22.7	0.0000
Interaction (A × B)	1	814.4	0.04	0.8366
Interaction (A × C)	1	28714	1.5	0.2217
Interaction (B × C)	1	1179.6	0.06	0.8040
Gonad				
Factor A (origin)	1	2.25E6	126	0.0000
Factor B (feeding regime)	1	324793	18.2	0.0000
Factor C (time)	1	349092	19.5	0.0000
Interaction (A × B)	1	7675.7	0.43	0.5132
Interaction (A × C)	1	1445.8	0.08	0.7765
Interaction (B × C)	1	339690	18.9	0.0000

DF, degrees of freedom; MS, mean square; F, ratio; and P, probability.

all conditions are summarized in Table 2. Statistical analyses (Table 4) for the period at 10°C showed a significant effect of feeding regimen, place of origin, and time. In the treatment with food, there was a higher proportion of growing oocytes than in the treatment without food. The production of growing oocytes was significantly higher in BV oysters, and there was a significant increase of growing oocytes over time. For the conditioning period, we found a significant effect of treatment (the proportion of mature oocytes was significantly lower in treatment 4) and time (mature oocytes increased significantly with time). Nevertheless, there was not a significant effect of place of origin for mature oocytes (Table 4).

Spermatogenesis

The number of male oysters found in this study did not allow observation of any pattern of change in spermatogenesis. The number of males detected in the four treatments is presented in Table 5.

Biochemistry

Direct conditioning. Place of origin and time had a significant effect on the biochemical content in both the gonad and meat of oysters (Table 3). Specimens from BV had a higher content of biochemical compounds in the gonad and meat compared with oysters from LT, and the biochemical content increased significantly over time in the conditioning procedures. Feeding regimen also had a significant effect but only on the biochemical content in the gonad. There was a higher content of proteins, lipids, and carbohydrates in the gonad of oysters conditioned with food (Table 6).

Four tested conditions. Three-way ANOVA during the phase at 10°C (Table 4) showed a significant effect of feeding regimen, place of origin, and time on the biochemical content of the gonad, and a significant effect of place of origin and time on the biochemical content in the meat. A significantly higher biochemical content was found in the gonads of oysters maintained with food. No significant differences were noted relating biochemical content in the meat of animals kept with or without food. Significant biochemical differences in meat and gonad tissue favored the BV oysters. The biochemical content increased significantly over time. Statistical analyses (Table 4) during the conditioning procedures showed a significant effect of treatment, place of origin, and time on the biochemical content of oyster gonads. For meat, a significant effect of place of origin favored the BV specimens. Concerning conditioning effects on the biochemical content of gonads, the highest content was found in treatment 1 (BV-1 and LT-1) compared with the other treatments, and there were no significant differences between treatments 2 and 3. The lowest concentration was observed in treatment 4. Finally, the biochemical content was significantly higher in oysters from BV, and compound concentrations increased significantly over time (Figs. 3 and 4).

Oocyte Production and Larval Yield

Direct conditioning. LT oysters conditioned without food did not produce any oocytes at the end of the direct-conditioning phase. In the other three treatments, BV oysters produced more oocytes than LT specimens. Concerning the yield of larvae (D-larvae), similar values were obtained (85% and 78%) for fed oysters coming from both sources and a yield of larvae (62%) in BV specimens conditioned without food. There were no significant

TABLE 4.

Results of three-way ANOVA tests for early, growing, and mature oocyte categories, and for biochemical content in the gonad and meat of oysters *C. gigas* in the period at 10°C and conditioning.

Source of Variation	Df	MS	F	P
Oogenesis				
Period at 10°C				
Early gametogenesis stage				
Factor A (origin)	1	1016.3	4.36	0.0491
Factor B (feeding regime)	1	1808.2	7.76	0.0111
Factor C (time)	1	2378.9	10.2	0.0043
Interaction (A × B)	1	21.03	0.09	0.7668
Interaction (A × C)	1	7.38	0.03	0.8604
Interaction (B × C)	1	131.58	0.56	0.4607
Growing stage				
Factor A (origin)	1	993.1	4.33	0.0499
Factor B (feeding regime)	1	1768.2	7.71	0.0113
Factor C (time)	1	2343.1	10.2	0.0043
Interaction (A × B)	1	25.6	0.11	0.7417
Interaction (A × C)	1	9.51	0.04	0.8406
Interaction (B × C)	1	120.9	0.53	0.4758
Mature stage				
Factor A (origin)	1	2.57	0.45	0.5079
Factor B (feeding regime)	1	4.3	0.76	0.3938
Factor C (time)	1	2.57	0.45	0.5079
Interaction (A × B)	1	4.3	0.76	0.3938
Interaction (A × C)	1	2.57	0.45	0.5079
Interaction (B × C)	1	4.3	0.76	0.3938
Conditioning				
Early gametogenesis stage				
Factor A (treatment)	3	2193.3	16.9	0.0000
Factor B (origin)	1	2152.8	16.6	0.0000
Factor C (time)	1	16340	126	0.0000
Interaction (A × B)	3	353.2	2.73	0.0523
Interaction (A × C)	3	552.8	4.27	0.0087
Interaction (B × C)	2	39.2	0.3	0.5842
Growing stage				
Factor A (treatment)	3	546.5	2.97	0.0395
Factor B (origin)	1	354	1.92	0.1711
Factor C (time)	1	561.4	3.05	0.0862
Interaction (A × B)	3	340.6	1.85	0.1486
Interaction (A × C)	3	2121.5	11.5	0.0000
Interaction (B × C)	1	560.7	3.04	0.0864
Mature stage				
Factor A (treatment)	3	2396.8	26.7	0.0000
Factor B (origin)	1	701.3	7.81	0.0071
Factor C (time)	1	29084	324	0.0000
Interaction (A × B)	3	55.9	0.02	0.6030
Interaction (A × C)	3	2337.6	26.1	0.0000
Interaction (B × C)	1	614.6	6.85	0.0114
Biochemistry				
Period at 10°C				
Meat				
Factor A (origin)	1	2.73E6	100	0.0000
Factor B (feeding regime)	1	21011	0.77	0.3816
Factor C (time)	1	224169	8.22	0.0048
Interaction (A × B)	1	8410.4	0.31	0.5796
Interaction (A × C)	1	5842.8	0.21	0.6442
Interaction (B × C)	1	2307.9	0.08	0.7716
Gonad				
Factor A (origin)	1	1.9E6	229	0.0000
Factor B (feeding regime)	1	149781	18	0.0000
Factor C (time)	1	127086	15.3	0.0008

TABLE 4.

continued

Source of Variation	Df	MS	F	P
Interaction (A × B)	1	39084	4.7	0.0318
Interaction (A × C)	1	20100	2.42	0.1222
Interaction (B × C)	1	96709	11.6	0.0008
Conditioning				
Meat				
Factor A (treatment)	3	32563	1.02	0.3829
Factor B (origin)	1	5.02E6	158	0.0000
Factor C (time)	1	147545	4.63	0.0321
Interaction (A × B)	3	65159	2.14	0.0950
Interaction (A × C)	3	10153	0.32	0.8119
Interaction (B × C)	1	236027	7.41	0.0068
Gonad				
Factor A (treatment)	3	492786	17.9	0.0000
Factor B (origin)	1	5.51E6	201	0.0000
Factor C (time)	1	308652	11.3	0.0009
Interaction (A × B)	1	32763	1.2	0.3113
Interaction (A × C)	3	307844	11.2	0.0000
Interaction (B × C)	1	324.5	0.01	0.9134

Df, degrees of freedom; MS, mean square; F, ratio; and P, probability.

differences in oocyte production among the three treatments in this part of the experiment (Fig. 5).

Four tested conditions. The highest oocyte production occurred in oysters fed during the experiment, in particular in specimens under conditions BV-1 and BV-3. Specimens raised under condition LT-4 (without food) did not produce any oocytes by the end of the experiment. The highest yield of larvae was detected in BV and LT oysters that were not fed during the cold phase and in oysters raised with food during conditioning. The lowest yield of larvae was observed in specimens from BV maintained under treatment 1, even though the mean oocyte production in these oysters was 42.5 million. The statistical analysis showed that treatments involving feeding produced significantly more oocytes than did treatments involving oysters kept without food, with the highest values favoring BV oysters. There are no significant differences in oocyte production among unfed oysters (Fig. 5).

DISCUSSION

Oocytes in early gametogenesis and growing stages in BV oyster samples were observed at the beginning of February in our laboratory in previous experiments (1999). These oocyte categories were detected later in LT oysters (Chávez-Villalba et al. 2002), which is in agreement with the results of Lango-Reynoso (1999), who found oocytes in the growing stage in oysters from Marennes-Oleron (near LT) by the end of February 1998. In this study, oocytes in early gametogenesis and growing stages in both oyster samples were detected at the beginning of the direct conditioning (February 2000), showing that the oysters in LT began gametogenesis earlier in the year. Even though similar proportions of early gametogenesis and growing oocytes were measured at the beginning of conditioning in both samples, by the end of conditioning lower proportions of mature oocytes were found in LT oysters in both treatments, indicating differences in the environmental patterns regulating the beginning of gametogenesis between these samples.

With decreasing latitude, the temperature required for the ini-

TABLE 5.

Number of male oysters (*C. gigas*) and their respective developmental stage found during the four treatments.

	10 C						End C 110 Days	
	0 Days		30 Days		60 Days			
	BV	LT	BV	LT	BV	LT	BV	LT
Treatment 1								
Early gametogenesis	1	—	2	1	—	4	—	—
Growing	1	—	—	1	2	—	—	—
Mature	—	—	—	—	—	—	4	7
Treatment 2								
Early gametogenesis	1	—	2	1	—	4	—	—
Growing	1	—	—	1	2	—	2	2
Mature	—	—	—	—	—	—	1	4
Treatment 3								
Early gametogenesis	1	—	1	—	—	—	—	—
Growing	1	—	—	—	2	—	—	1
Mature	—	—	—	—	—	—	3	5
Treatment 4								
Early gametogenesis	1	—	1	—	—	—	—	1
Growing	1	—	—	—	2	—	4	—
Mature	—	—	—	—	—	—	—	—

End C, end of conditioning.

tiation of the gametogenic cycle increases, and, as a result, reproductive cycles occur later in the year (Barber and Blake 1983). This study showed different responses between northern and southern oysters to food and temperature during the period considered, such as the beginning of gametogenesis in *C. gigas*. Oysters from BV (in the north of France), within a high productivity ecosystem (Goulletquer et al. 1996), acclimated to colder water than southern populations, developed mature oocytes after 60 days

TABLE 6.

Protein, carbohydrate, and lipid content (mg/equivalent tissue[a]) in the gonad and meat of *C. gigas* samples from two culture sites at the beginning and by the end of the direct conditioning experiment conducted under two types of conditions: with and without food.

Oyster Sample	Tissue	Compound	Conditioning		Conditioning Characteristics
			Beginning	End	
BV	Gonad	Carbohydrates	283 ± 27	211 ± 16	With food
				256 ± 37	Without food
		Proteins	264 ± 11	732 ± 58	With food
				307 ± 28	Without food
		Lipids	113 ± 7	278 ± 30	With food
				119 ± 23	Without food
	Meat	Carbohydrates	195 ± 25	144 ± 28	With food
				206 ± 32	Without food
		Proteins	253 ± 17	557 ± 34	With food
				502 ± 31	Without food
		Lipids	45 ± 2	128 ± 16	With food
				106 ± 20	Without food
LT	Gonad	Carbohydrates	5 ± 1.5	70 ± 9	With food
				16 ± 5	Without food
		Proteins	47 ± 12	341 ± 36	With food
				63 ± 9	Without food
		Lipids	8 ± 2	73 ± 11	With food
				66 ± 37	Without food
	Meat	Carbohydrates	15 ± 3.5	36 ± 6	With food
				18 ± 7	Without food
		Proteins	93 ± 11	235 ± 30	With food
				262 ± 47	Without food
		Lipids	31 ± 4.5	40 ± 5	With food
				53 ± 20	Without food

[a] Presented as mean ± SE in a sample size of 10 oysters.

Figure 3. Protein, carbohydrate, and lipid content in the meat and gonad of *C. gigas* from the BV site during the four treatments (10°C for the period of 0–60 days, and conditioning at 19 C for the period of 60–110 days).

at 10°C when maintained with food. When kept without food, oysters produced a large proportion of growing oocytes during this period. In contrast, the LT oysters (cultured in a central coastal bay), where conditions included a limited food supply (Pastoureaud et al. 1996), produced some growing oocytes when raised with food during the cold phase. LT oysters kept without food remain blocked in the early gametogenesis stage. Considering that BV oysters can initiate gametogenesis and continue oocyte development even if winter conditions are artificially extended, this eliminates temperature as the principal regulator of gametogenesis in *C. gigas*. Bivalves require sufficient energy to meet maintenance and reproductive requirements during their gametogenesis cycles. The differences found in this study are probably a result of northern oysters having a better storage reserve than animals from southern locations. Moreover, since both samples of oysters were

collected from the same more southern location (Bassin dArcachon), no genetic diversity is considered.

Lubet (1976) found that the initiation of gametogenesis in *Mytilus edulis* and *Mytilus galloprovincialis* is not dependent on thermal conditions. This author emphasizes that populations of *C. gigas* and *Ostrea edulis* in the English Channel confirmed similar results, since the reinitiation of gametogenic activity coincides with low temperatures (8–9°C). It seems that these facts are valid for the oysters in BV, but in the case of oysters in LT it appears that if enough food is available to build a reserve, these oysters can initiate gametogenesis under low-temperature conditions. Nevertheless, LT oysters kept without food initiated gamete development until the temperature increased, but the mature stage was not reached after conditioning, probably because reserves were used to assure maintenance instead of gametogenesis. Lubet (1976) hy-
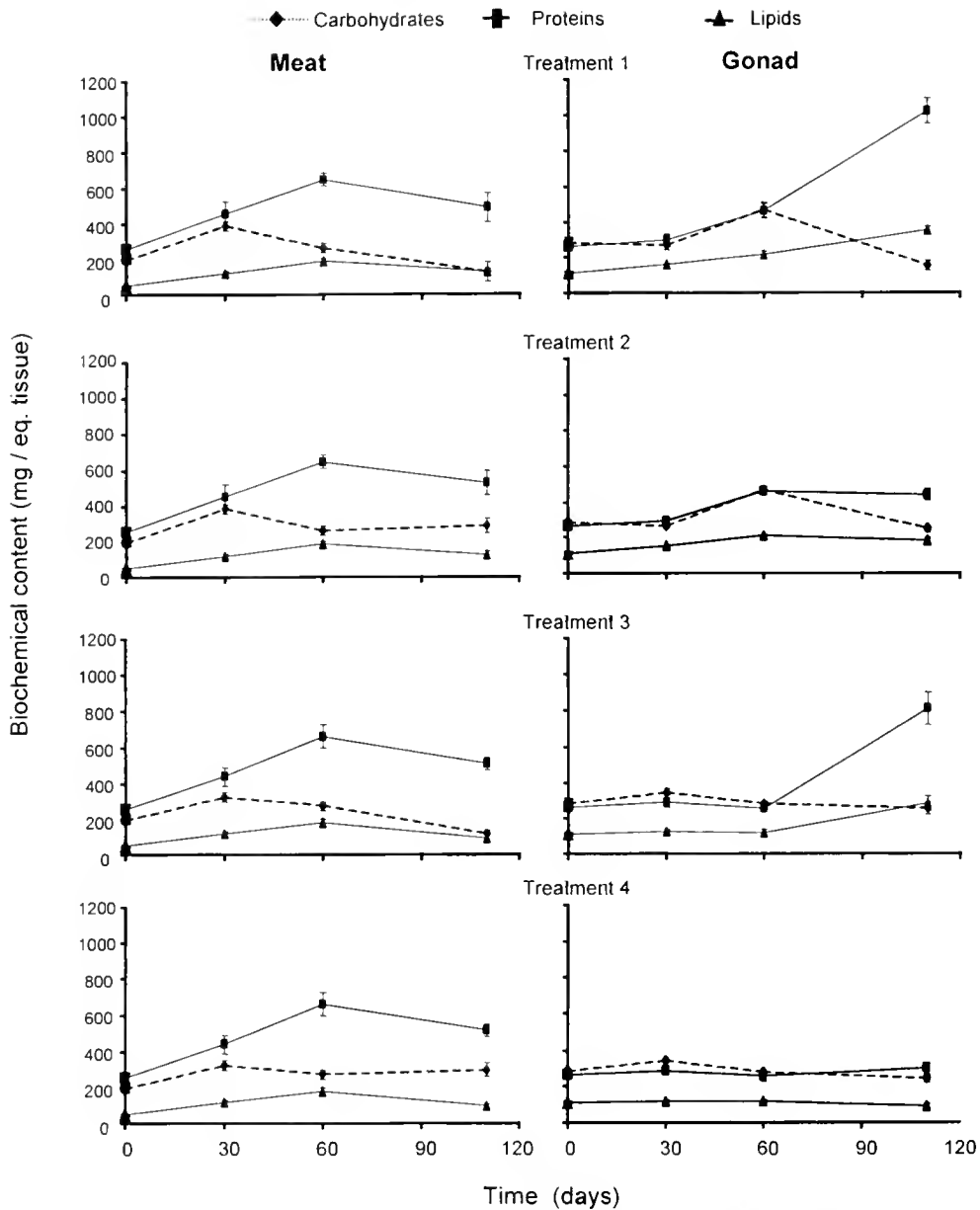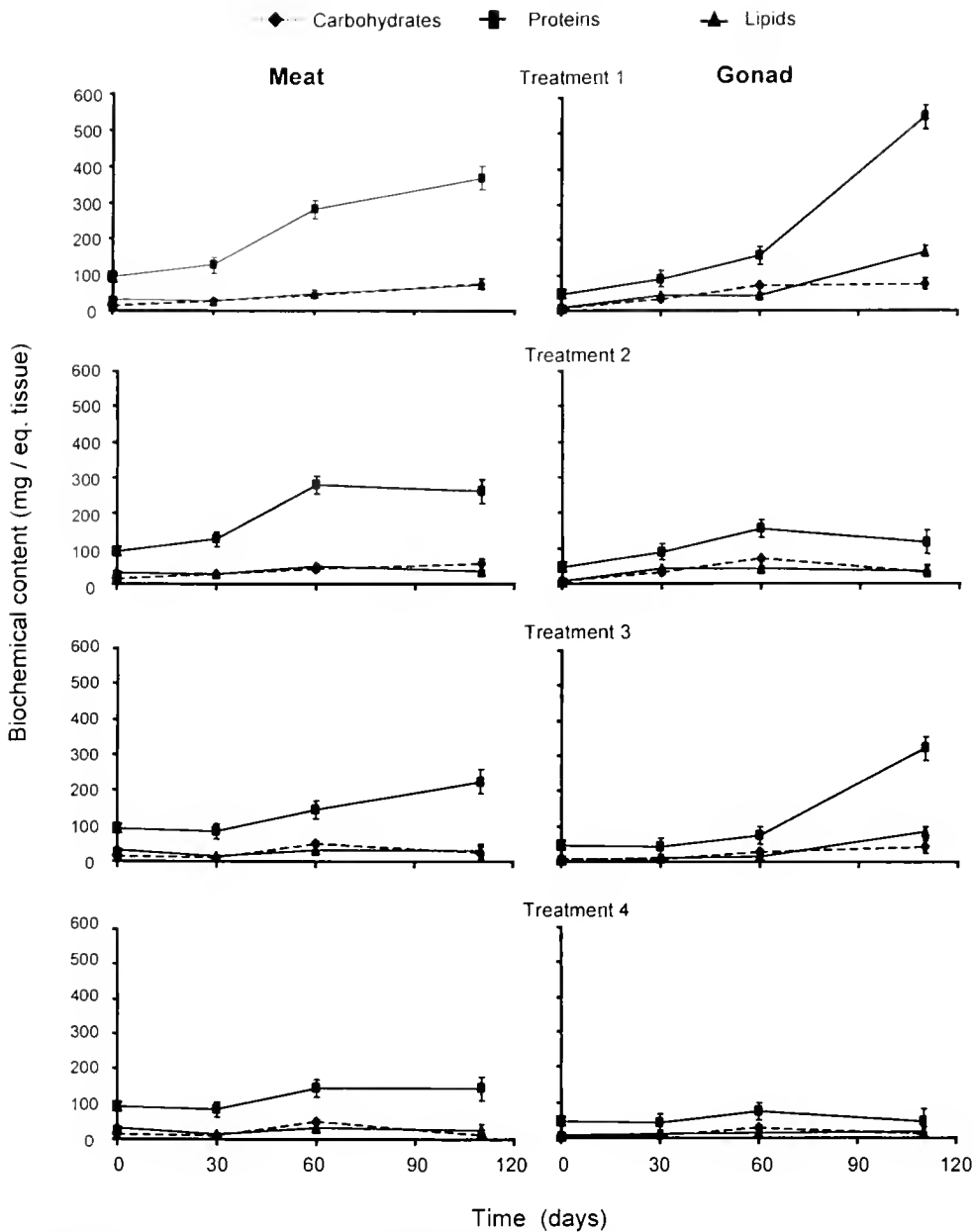
Figure 4. Protein, carbohydrate, and lipid content in the meat and gonad of *C. gigas* from the LT site during the four treatments (10°C for the period of 0–60 days, and conditioning at 19°C for the period of 60–110 days).

pothesized that the beginning of gametogenic activity is under the influence of a neurosecretion internal clock that determines the initiation and extent of the sexual cycle, and this clock may be modified by external factors, of which temperature would play an essential role. Nevertheless, the results of this study showed that, apart of temperature, the beginning of gametogenesis is also dependent on reserves accumulated during the previous year. As a consequence, we can hypothesize that, even if the beginning of gametogenesis is dependent upon temperature, oocyte development will not occur in conditions of low temperature unless a minimum reserve stock had been accumulated. Thompson et al. (1996) pointed out that food supply seems to be less critical once a minimum quantity of reserves is accumulated in the bivalve gonad. Some studies have demonstrated a site-specific variation of the gametogenesis cycle associated with phenotypic adaptations to local food supply variations (MacDonald & Thompson 1988). Therefore, we assume that oocyte growth in *C. gigas* is dependent upon food supply and a certain minimum temperature that varies with geographic location. This minimum temperature of about 10°C for BV oysters accords with the observations of Lubet (1976). The higher water temperature for LT oysters takes into consideration environmental conditions in that geographic location.

It is known that there is a relationship between the proximate biochemical composition of oysters and the gametogenic cycle (Deslous-Paoli & Héral 1988). According to Berthelin et al. (2000), glycogen concentration in *C. gigas* is minimal immediately after spawning, and increases during fall and early winter, reaching maximum values prior to gametogenesis. Protein and lipid concentrations follow a similar pattern, with a fairly uniform percent-

Figure 5. Oocyte production and larval yield (D-larvae) of *C. gigas* conditioned in February to March 2000 (direct conditioning at 19 C: W = with food; WO = without food).

age composition during autumn and winter, but with the highest amounts in the gonad from April to June. Even though BV oysters were exposed to conditions that affected physiologic activities, they have an important quantity of reserves, using carbohydrates as the principal source to support gametogenesis un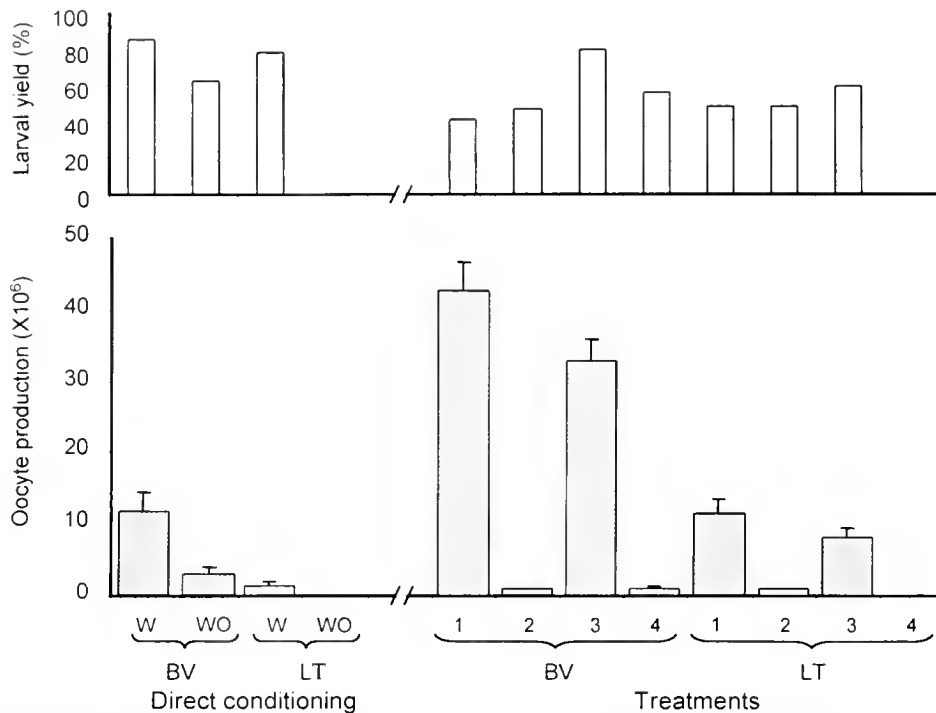der all conditions. In contrast, LT oysters used proteins to fuel gamete development. Probably, southern oysters used proteins because they have a net loss of glycogen during the winter, when it may be catabolized to meet maintenance requirements during poor food conditions (Deslous-Paoli & Héral 1988). Whyte et al. (1990) found that protein can contribute more than carbohydrates for the metabolic processes of oysters maintained in unfavorable food conditions. Moreover, Barber and Blake (1983) found that the source of reproductive energy for *Argopecten irradians* over its latitudinal range could be affected by food supply and metabolic rates. These authors suggest that, with decreasing latitude, the bay scallop has a greater metabolic rate as well as a smaller food supply, with less energy available for reproduction. This concept may be true for this study if northern oysters are in more favorable food conditions than southern populations, and that metabolic rates in this species are influenced by temperature (Bougrier et al. 1995)

BV and LT oysters conditioned with food produced more oocytes by the end of the conditioning period than those conditioned without food. Similar results in *C. gigas* were obtained by Robinson (1992). These results indicate that the oocyte quantity produced under controlled conditions is dependent on the food offered during conditioning, since the oysters fed during the phase at 10°C, but conditioned without food, have produced significantly fewer oocytes than animals conditioned with food, but maintained without food during the cold phase. Oocyte production was significantly lower in the oysters kept with food during the direct-conditioning procedure than in oysters maintained in treatments 1 and 3 (BV-1, BV-3 and LT-1, LT-3, respectively). If it is consid-

ered that oysters in treatments 1 and 3 were maintained for 60 days at the same temperature as that at the beginning of the experiments (10°C), then the difference in terms of oocyte production may indicate that animals during the cold phase continue their oogonie multiplication with or without the influence of food. It would be interesting to study changes at the cellular level and try to quantify oogonie multiplication under similar experimental conditions.

Le Pennec et al. (1990) found a significant relationship between the lipid index of oocytes in *Pecten maximus* and the parameters involved in the endotrophic phase of larval rearing. They emphasize that D-larvae and the anomaly rates of prodisoconch 1 are strongly related to the mean lipid index; the greater the lipid content in the oocytes, the greater the quality of larval rearing required in the first 2 days of culture. We observed that lipids accumulated in the gonads of oysters conditioned with food, and these animals produced more oocytes than oysters maintained without food, but the larval yield of the two groups was similar. Muranaka and Lannan (1984) observed higher fecundity rates in oysters conditioned with food when compared with oysters conditioned without food. Nevertheless, the results of this study did not show significant differences in larval yields between samples conditioned with or without food. On the contrary, the lowest larval yield was found in the oysters kept in condition 1. These observations suggest that lipid reserves in unfed oysters are maintained even in conditions of the absence of food, and, although there are few oocytes in these animals, these gametes will yield good quality D-larvae. In previous experiments, Chávez-Villalba et al. (2003) observed that unfed oysters not only yielded good-quality D-larvae but that larvae presented similar growth and survival patterns as larvae from fed animals throughout 19 days of trials. Thus, oocyte quality seems to be related not only to food quality during conditioning, but also to reserves accumulated in nature prior to experiments.

In this study, oysters having the same place of origin show flexible reproductive patterns that are responses to varying environmental factors, most notably food availability. Northern oysters, having a larger reserve stock than southern oysters, initiate gamete development in conditions of low temperature, which confirms that the beginning of gametogenesis is not dependent on thermal conditions. The amount of gametogenic material is also dependent on food supply, but oocyte quality seems to depend, to a large extent, on accumulated reserves. The differences found in this study are that the stored reserves in BV oysters are used to initiate gametogenesis and to maintain metabolism under low food availability, while LT oysters operate closer to their energetic limit at the production site, and require supplementary energy from spring planktonic blooms to continue gametogenesis and to produce viable oocytes and larvae.

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TWO SPECIES OF OYSTER LARVAE SHOW DIFFERENT DEPTH DISTRIBUTIONS IN A SHALLOW, WELL-MIXED ESTUARY

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ABSTRACT The vertical distribution of late stage, or pediveliger, larvae of several bivalve mollusks was examined in a west Florida estuary. The study site was an artificial canal, and the water was shallow (1.5 m) and well mixed, with only modest currents. Pediveligers of three bivalve taxa were collected: the eastern oyster *Crassostrea virginica*; the crested oyster *Ostrea equestris*; and unidentified shipworms (Teredinidae). Despite the shallow and well-mixed water column, larvae exhibited vertical zonation, with most larvae of all three species collected from lower in the water column. The larvae of *C. virginica* and shipworms showed no significant effect of time of day, but larvae of *O. equestris* reversed their distribution pattern at night, with most larvae being near the surface. Pediveliger larvae were not behaving as neutrally buoyant particles but appeared to regulate their depth even in this well-mixed and shallow water column. Given that the larvae of the two oyster species were probably competent to settle, their vertical distribution patterns do not fit what has been reported about their adult depth distribution.

KEY WORDS: *Crassostrea virginica*, estuary, larvae, *Ostrea equestris*, pediveliger, plankton, Teredinidae

INTRODUCTION

A variety of studies over the years have attempted to address the issue of whether larval distribution in estuaries is controlled mainly by hydrologic forces, or whether there is a significant larval behavioral component that also affects distribution. For some crustacean larvae, the case seems to be fairly well made that behavior plays a large part in planktonic distribution, usually (but not always) for late-stage larvae or post-larvae (Shanks 1986, 1995, Benfield & Aldrich 1992, Gherardi 1995).

Bivalve mollusks also have been the focus of studies on larval distribution in estuaries, but there is no consensus in the literature on whether bivalve veligers are distributed as neutrally buoyant particles or whether behavior significantly affects their distribution. Like crustacean larvae, bivalve larvae clearly exhibit oriented swimming, at least in the laboratory (Feeny 1984, Hidu & Haskin 1978). Some field studies have appeared to show nonrandom bivalve larval distribution, relative to hydrodynamic processes (Tremblay & Sinclair 1990, Shanks et al. 2002, Baker & Mann 2003). Compared with crustacean postlarvae, however, bivalve pediveligers are small and slow swimming, and Banse (1986) questioned whether the weak swimming rates observed for these larvae are sufficient to produce distribution patterns. The distribution of bivalve larvae in estuaries may be attributed to hydrodynamic processes alone in some cases, if larvae are treated as neutrally buoyant particles (Wood & Hargis 1971, Mann 1988).

This author examined the above question (i.e., does bivalve larval distribution in an estuary have a behavioral component?) under the most restrictive conditions possible for an estuarine system. The estuarine system in question was simple in shape (an artificial inlet), very shallow, and well mixed throughout the study, although it was a low-energy system. Only late-stage bivalve larvae were included in the study. If bivalve larvae behave as neutrally buoyant particles, their distribution should be fairly even throughout the water column (allowing for boundary-layer effects), and the species should have similar distributions.

MATERIALS AND METHODS

Research was conducted at the Harbor Branch Oceanographic Institute, near Fort Pierce, FL, in May 1993. The study site was about halfway along a 1-km artificial canal that opened into the Indian River Lagoon. The sides of the canal were concrete and steel seawalls, heavily fouled by eastern oysters, *Crassostrea virginica*, and the mean water depth at the wall were about 1 m, gradually increasing toward the center of the canal. The observed currents were mostly tidal, with velocities near the seawalls of 1 to 3 cm s^{-1}, and the tidal range was up to 0.5 m.

Plankton was sampled with two modified 12-V bilge pumps, each rated at 1800 L h^{-1}. Power came from a standard 110-V outlet with a transformer to regulate voltage. Pumps were suspended about 2 m out from the canal wall, where the mean water depth was about 1.5 m. One pump was maintained at a depth of about 20 cm above the bottom, which was determined by preliminary samples to be the maximum depth achievable without entraining significant quantities of sediment. The other pump was adjusted for each sampling episode to a depth of about 20 cm below the surface. Mann (1986) and Mohlenberg (1987) found no avoidance of a plankton pump intake by bivalve mollusk larvae, which swim slowly compared with many zooplankton.

Water from each pump was delivered by a garden hose to a separate sieve on the banks of the canal. Each sieve consisted of a 400-μm coarse filter and a 150-μm final filter on which the sample was retained. Plankton was sampled twice daily, at mid-morning (full daylight) and mid-evening (after nightfall), for about 2 h at a time. The volume sampled at each depth was calculated from the time, to the nearest minute, multiplied by the mean pumping rate. The pumping rate was estimated before and after each sample, for each pump, by the time required to fill a 20-L container. (If sampling episodes included high or low water, the pumping rate measurements also were taken then and factored into volume calculations.) Samples were taken into the laboratory, and bivalve larvae were counted and identified to the lowest possible taxonomic level.

The identification of oyster pediveligers (*C. virginica* and *Ostrea equestris*) was verified by collecting newly settled juveniles on shell-strings (Haven & Fritz 1985) that had been immersed at the study site for <24 h, marking individuals, and letting them grow in the canal for several weeks. By the end of this time, *O.*

equestris shells had developed the diagnostic dorsal-marginal dentition, or chomata (Galtsoff & Merrill 1962).

Only samples that had six or more pediveligers of a given taxa from the two pumps combined were used in the analysis. Data for each pump were converted to proportions of total larvae of a given species collected in a sampling episode and were arcsine-square root-transformed prior to statistical analysis (Zar 1996). Analysis of variance tests were used to test null hypotheses of equal proportions of larvae collected by either pump (top vs. bottom) at either time of day (morning vs. evening), with no interaction (Zar 1996).

RESULTS

Two species of oyster larvae were collected in plankton samples on the majority of days sampled: the eastern oyster, *C. virginica*; and the crested oyster, *O. equestris*. Pediveligers, or late-stage larvae, of these species could be distinguished on the basis of shape (*O. equestris* pediveligers were nearly identical to those of *C. virginica* in size but were more rounded, with a broader, less pronounced umbo). Living pediveliger larvae were clearly distinguishable on the basis of color. *C. virginica* pediveligers at this site were tan to brown and opaque, while *O. equestris* pediveligers were transparent except for their visceral masses, which were green to brown. The only other common bivalve larvae were shipworms (Teredinidae) of unknown species, which were treated in this study as if they were a single taxon. Unidentified pediveligers of other bivalve taxa were occasionally collected.

The abundance of all three species was highly variable, but fairly low. *C. virginica* and *O. equestris* reached peak densities of just over 12 per m^3, but teredinids peaked at less than half of that. All three taxa showed peak densities near the beginning of the study. Density data for all three taxa from the lower intake are shown in Fig. 1.

The plankton pumps at the two sample depths did not collect equal densities of larvae, for any species. About 85% of *C. virginica* pediveligers and 75% of teredinid pediveligers were collected from the bottom pump, and time of day had no significant effect. During the day, the distribution patterns for *O. equestris* pediveliger larvae appeared to be similar to the above taxa, but at night 61% of *O. equestris* pediveligers were collected by the near-surface pump. Thus, for *O. equestris*, abundance differed significantly for neither time of day nor depth, but the interaction of depth and time of day was significant at $\alpha = 0.05$. The proportions for each species collected for each time and daylight treatment are presented in Table 1, and the results of the analysis of variance are presented in Table 2.

DISCUSSION

The above study rose serendipitously from an attempt to locate an estuarine environment in which oyster pediveliger larvae (*C. virginica*) were randomly distributed throughout the water column, for a separate study (Baker 1993). Clearly, nonrandom distribution complicates the effort to quantify the larval supply. Yet, even in this highly simplified estuarine environment, in <2 m of water, all three bivalve taxa exhibited strong vertical distribution patterns. The vertical distribution patterns from this study were similar to those observed for *C. virginica* and teredinid larvae in a more complex estuarine environment in Virginia (Baker 1993). The major difference noted from that prior study was the effect of time of day on the distribution of *O. equestris* larvae; no effects of time of
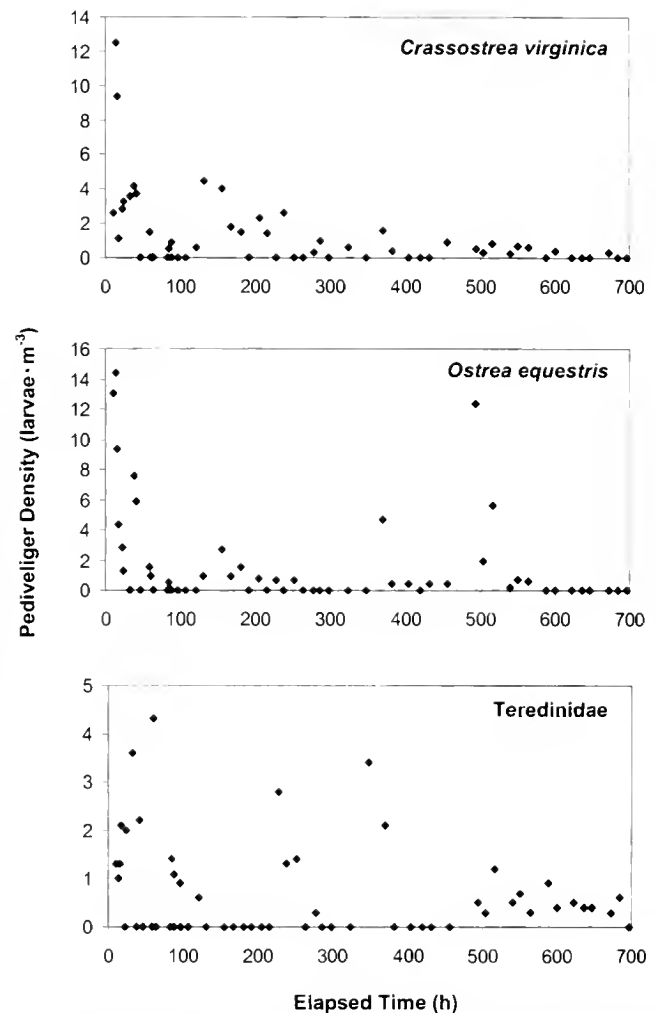
Figure 1. Abundance (density per cubic meter) of three taxa of bivalve pediveligers at the Harbor Branch Oceanographic Institute canal during May 1993, from the near-bottom plankton pump.

day were reported for any species in the Virginia study. The sparseness of pediveliger larvae also was noted by Carriker (1951), who collected only 56 pediveligers from >14,500 *C. virginica* larvae across six samples.

TABLE 1.

Mean proportional (%) abundances of three taxa of bivalve pediveligers at two times (morning vs. evening) and two depths (top vs. bottom) in the Harbor Branch Oceanographic Institute canal during May 1993.

Taxon	Depth	Morning	Evening	All Times
C. virginica	Top	14.6 (18.8)	16.8 (30.3)	15.5 (24.5)
	Bottom	85.4 (18.8)	83.2 (30.3)	84.5 (24.5)
		(*n* = 19)	(*n* = 13)	(*n* = 32)
O. equestris	Top	18.6 (29.4)	61.0 (41.9)	33.2 (38.6)
	Bottom	81.4 (29.4)	39.0 (41.9)	66.8 (38.6)
		(*n* = 19)	(*n* = 10)	(*n* = 29)
Unidentified teredinids	Top	23.2 (39.1)	26.8 (33.4)	24.2 (38.3)
	Bottom	76.8 (39.1)	73.2 (33.4)	75.8 (38.3)
		(*n* = 20)	(*n* = 8)	(*n* = 28)

SDs are given in parentheses.

TABLE 2.

Summary of analyses of variance for the effects of time of day (morning vs. evening) and depth (top vs. bottom) on proportional abundance of three taxa of bivalve pediveligers in the Harbor Branch Oceanographic Institute canal during May 1993.

Source	DF	Seq SS	Adj SS	Adj MS	F Value	P Value
Analysis of variance for *C. virginica*						
Time of day	1	7.779	7.779	7.779	1.89	0.174
Depth	1	46.685	36.225	36.225	8.81	0.004
Time × depth	1	7.967	7.967	7.967	1.94	0.169
Error	58	238.417	238.417	4.111		
Total	61	300.848				
Analysis of variance for *O. equestris*						
Time of day	1	10.701	10.701	10.701	1.39	0.244
Depth	1	41.905	15.269	15.269	1.98	0.165
Time × depth	1	52.386	52.386	52.386	6.80	0.012
Error	54	416.069	416.069	7.705		
Total	57	521.062				
Analysis of variance for teredinids						
Time of day	1	0.0161	0.0161	0.0161	0.02	0.898
Depth	1	16.6334	10.9201	10.9201	11.26	0.001
Time × depth	1	0.7875	0.7875	0.7875	0.81	0.372
Error	52	50.4180	50.4180	9.9696		
Total	55	67.8550				

Seq SS = sequential sum of squares; Adj SS = adjusted sum of squares; Adj MS = adjusted mean square.

Several authors have reported the vertical stratification of bivalve larvae in estuaries (Nelson 1927, Perkins 1932, Wood & Hargis 1971, Sekiguchi et al. 1991), although they did not attempt to demonstrate that this was due to larval behavior. Vertical stratification or the migration of bivalve larvae also has been observed in the absence of estuarine stratification (Tremblay & Sinclair 1990, Raby et al. 1994), but those studies were in systems significantly deeper than 1.5 m.

Deksheineks et al. (1996) modeled *C. virginica* larval distribution in the water column of a well-mixed estuary, and predicted, as observed here, that the majority of late-stage larvae would be within a meter of the benthos. As larvae grow, they sink faster (due to an increased shell/cilia ratio), and the swim-sink behavioral pattern observed for this species by Hidu and Haskins (1978) would result in a net sinking rate for older larvae, according to the model (Deksheineks et al. 1996). The above model, however, does not include bottom avoidance; larvae must either increase swimming rates in response to the benthos or spend a certain amount of time resting on the benthos. The latter behavior (except for benthic explorations by competent-to-settle larvae; Prytherch 1934, Cranfield 1973) has not been reported, and increased contact with the benthos also exposes the larva to a new guild of predators (Breese & Phibbs 1972, Steinberg & Kennedy 1979, Cowden et al. 1984, Osman et al. 1989, André et al. 1993). It is likely, therefore, that size-related sinking/swimming ratios provide only a partial explanation for pediveliger distribution in *C. virginica*. *O. equestris* pediveligers, which in this study were about the same size as *C.*

virginica pediveligers, were not constrained to the lower reaches of the water column by the weight of their shell, at least not during the night.

If pediveliger larvae were no more than negatively buoyant particles, they could not remain in the water column in a low-energy environment. If they were neutrally buoyant particles, they would be distributed evenly in a well-mixed water column. None of the species observed in this study were evenly distributed, and one species (*O. equestris*) differed from the others, altering its depth distribution on a diurnal cycle. Thus, while neutral buoyant models may be sufficient to describe broad distribution patterns (Wood & Hargis 1971, Mann 1988), ciliated larvae are clearly not inert particles, and species-specific larval behavior must be invoked to describe at least some scales of distribution.

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DIOXIN/FURAN AND POLYCHLORINATED BIPHENYL CONCENTRATIONS IN EASTERN OYSTER (*CRASSOSTREA VIRGINICA*, GMELIN) TISSUES AND THE EFFECTS ON EGG FERTILIZATION AND DEVELOPMENT

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ABSTRACT A 10-mo field study was conducted to evaluate the bioaccumulation of dioxins/furans and polychlorinated biphenyls (PCBs) in transplanted adult eastern oysters (*Crassostrea virginica, Gemlin*) to Newark Bay and the Raritan Complex, New Jersey. Adult oysters (mean size 86.4 ± 14.2 mm) were deployed from September 2000 until June 2001. Oysters transplanted to Newark Bay, Arthur Kill, and Sandy Hook, NJ, accumulated 3.2/2.1, 1.3/1.7, and 0.15/2.3 parts per trillion (pptr) of 2,3,7,8-Tetrachlorodibenzo-*p*-dioxin (TCDD)/2,3,7,8- Tetrachlorodibenzo-*p*-furan, respectively. In addition, oysters transplanted to Newark Bay, Arthur Kill, and Sandy Hook, NJ, had bioaccumulation levels of 68.6, 64.5, and 35.3 parts per billion total PCBs, respectively. The number of fertilized eggs (±SD) from strip spawned transplanted oysters from Newark Bay, Arthur Kill, and Sandy Hook, NJ, was 107 (±6.00), 54 (±36.11), and 113 (±13.61), respectively, and the number of unfertilized eggs was 164 (±25.6), 178 (±15.9), and 97 (±39.9), respectively. The number of veliger larvae that resulted from fertilized eggs (*n* = 100) was 3 (±1.7), 4 (±2.31), and 82 (±12.2), respectively, for Newark Bay, Arthur Kill, and Sandy Hook, NJ. Survival data from a laboratory study using an acute static 48-h *in vivo* and *ex vivo* exposure regiment to 2,3,7,8-TCDD showed that exposure to 2 pptr dioxin caused adverse effects on egg fertilization and development. Exposure to dioxin-like compounds at the low parts per trillion ranges can result in altered gonadal development and altered embryonic development.

KEY WORDS: *Crassostrea virginica*, dioxins/furans, egg fertilization, polychlorinated biphenyls, transplant study

INTRODUCTION

Since the early 1970s there has been concern about the impacts of 2,3,7,8-Tetrachlorodibenzo-*p*-dioxin (TCDD) and related compounds because of their potential hazard to humans and animals. TCDD is a byproduct of anthropogenic processes such as paper and chemical manufacturing, incineration, the manufacturing of pesticides and herbicides, the production of iron and steel, and enzymatic reactions in sewage sludge (Rappe 1992, Alonso et al. 1996, Poland et al. 1982). The most important source of TCDD for humans is food, especially diary products, meat, and fish (Pohjavirta et al. 1994, EPA 2000).

Concern about TCDD stimulated numerous studies to assess its behavior in the environment and its effects on living organisms. Studies conducted in contaminated areas have shown a positive correlation between dioxin levels in animals and their soil contact (Pohjavirta et al. 1994). Studies in aquatic model ecosystems also have shown that TCDD and other organochlorine pollutants bioaccumulate in organisms in concentrations approximately equal to those in the sediment (Isensee et al. 1975, Chen et al. 2002). The effects of TCDD on feeding, growth, and development are most pronounced in young, growing organisms compared with adults (ASTM 1994, Davis & Herber 1969, Calabrese et al. 1973, Capuzzo, 1989, Capuzzo 1996). Because of the lipophilicity of these compounds, they are associated with lipid stores and high lipid-containing tissues (Cooper 1989, EPA 2000). Prior to spawning, bivalves have a high lipid and glycogen content in gonadal tissue. Therefore, the spawning status of the bivalve would affect the amount of dioxin present over the spawning season in a similar fashion to that observed in fish (Capuzzo 1989, Vashchenko et al. 1993, Bayne et al. 1972, Bayne et al. 1978).

Oysters release their gametes into the water column; therefore, planktonic larvae will have limited exposure to TCDD via water due to the low water solubility of dioxin (EPA 2000.). Newly settled bivalve spat and adult bivalve molluscs may be exposed to TCDD through their sediment contact and feeding on resuspended materials, while the developing eggs would receive the majority of exposure from the adult female (Cooper 1989). Bivalve embryos begin to accumulate TCDD at the two-cell embryonic stage (ASTM 1994). This may explain the sensitivity of young, growing organisms to low-level concentrations of dioxins.

There has been limited work on the bioaccumulation of dioxin in the eggs of aquatic organisms. Isensee and Jones (1975) reported no effect of 2,3,7,8-TCDD exposures on snail egg survival, but there was a reduction in the number of viable eggs. There have been several studies on both resident and migratory species of fish and crustaceans in New Jersey. Aquatic organisms in the tidal Passaic River were found to contain elevated levels of TCDD in the edible tissue, ranging from 38 parts per trillion (pptr) in the American eel (*Anguilla rostrata*) to 476 pptr in the blue crab (*Callinectes sapidus*) hepatopancreas (Tucker and Prince 1993). Cooper et al. (1993) found that the TCDD levels in the Arthur Kill organisms accumulated within higher trophic levels. For example, the soft-shell clam (*Mya arenaria*) contained 6.9 pptr TCDD, and the killifish (*Fundulus heteroclitus*) contained 100 pptr TCDD, total body burden.

Changes in the gonadal tissue of bivalves after exposure to a wide variety of pollutants such as oil, heavy metals, and lipophilic organic compounds have been reported (Vashchenko et al. 1993, Capuzzo 1996, Moore et al. 1980, Gardner et al. 1991, Lowe & Pipe 1985, 1986, 1987; Capuzzo & Leavitt 1988; Lowe, 1988; Moore 1988, Widdows & Johnson 1988). For instance, oocyte mass resorption observed in the sea urchin as well as other invertebrates at prespawning is considered to be a reaction to pollution (Vashchenko et al. 1993, Lowe & Pipe 1985, 1986, 1987, Capuzzo 1996). The abnormal development of oocytes, and altered egg shape and size have been correlated with polluted sites (Wintermyer 1998, Lowe & Pipe 1985). The accumulation of pollutants in

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bivalves can cause stress. Capuzzo (1996) reported that pollution-induced sites can lower biochemical reserve, and contribute to poor egg quality and fertilization rates in bivalves. Bayne et al. (1972, 1978) similarity reported that under stressful conditions the mussel (*Mytilus edulis*) produced fewer and smaller eggs, and that larvae that developed from the gametes of stressed adults had a lower growth rate. In a study comparing egg size and larval survival of the hard-shell clam (*Mercenaria mercenaria*) and the bay scallop (*Argopecten irradians*), Kraeuter et al. (1982) reported that for both species, smaller eggs (20–25 μm) had a significantly less than expected survival rate, while larger eggs (35–44 μm) had a significantly greater than expected survival rate. Intermediate size eggs (25–35 μm) showed no difference between the expected and observed survival rates.

The objectives of this study were to transplant adult oysters into sites contaminated with different levels of dioxin and dioxin-like compounds to measure the effects on egg development and fertilization, and to evaluate the potential for restoring oyster populations into the New Jersey bay area.

METHOD AND MATERIALS

Deployment

Adult eastern oysters (*n* = 180) were purchased from Prince Edward Island, Canada, and were transplanted in September 2000 at three study sites (*n* = 60 per site): Newark Bay, NJ; Arthur Kill, NJ; and Sandy Hook Bay, NJ (reference site). The oysters were determined to be disease free by histologic examination prior to deployment. Oyster bags (*n* = 2) were suspended in the water column in Sandy Hook Bay located north of the bridge connecting the Highlands entrance to Sandy Hook State Park. For the Arthur Kill site, oyster bags (*n* = 2) were suspended in the water column from General Anline Works building dock (longitude 74°12.312W, latitude 40°36.647N) in Elizabeth, NJ. For the Newark Bay site, oyster bags (*n* = 2) were suspended in the water column from

an abandoned dock on Shooter's Island (longitude 74°09.788W, latitude 40°38.482N) in Newark, NJ (Fig. 1).

Each oyster was filed, numbered (1–60), and weighed (in grams), and the dimensions were measured [i.e., length, width, and height (in millimeters)] prior to being placed into marked, mesh polyethylene bags (0.5 × 0.5 inch mesh). Each site was equipped with two bags containing 30 oysters each suspended into the water column 1.8 to 2.4 m (6–8 feet) below the water surface. The depth was selected to avoid low-tide exposure and icing during the winter. Oyster bags were collected in June 2001, terminating the 10-mo field study. Oysters were wet weighed immediately upon collection, and were prepared for tissue chemical analysis, histologic evaluation, and fertilization assays.

Chemical Analysis

Samples of shucked oysters (50 g, *n* = 7) from each site were sent to Triangle Laboratories (Research Triangle Park, NC) for dioxin, furan, and polychlorinated biphenyl (PCB) tissue analysis. Samples were analyzed by high-resolution chromatography and high-resolution mass spectrometry [method 1613B (9/97) and modified method 680 (11/85), Triangle Laboratories]. Tissues were sent in labeled amber-colored jars and were frozen during shipment.

Histologic Evaluation

Oysters from each site (*n* = 15) were selected randomly for histologic evaluation. Shucked oyster samples were preserved in a 10% phosphate formalin buffer for several days followed by 70% ethanol. Transverse cuts were made with a scalpel through the mid-visceral region of the oyster to obtain a segment approximately 5 mm thick. Segments were embedded in paraffin after processing (i.e., dehydration and clearance through an alcohol:xylene series). Sections (6 μm) were cut and stained with Harris' hematoxylin and eosin. Histologic grading was based on a scale
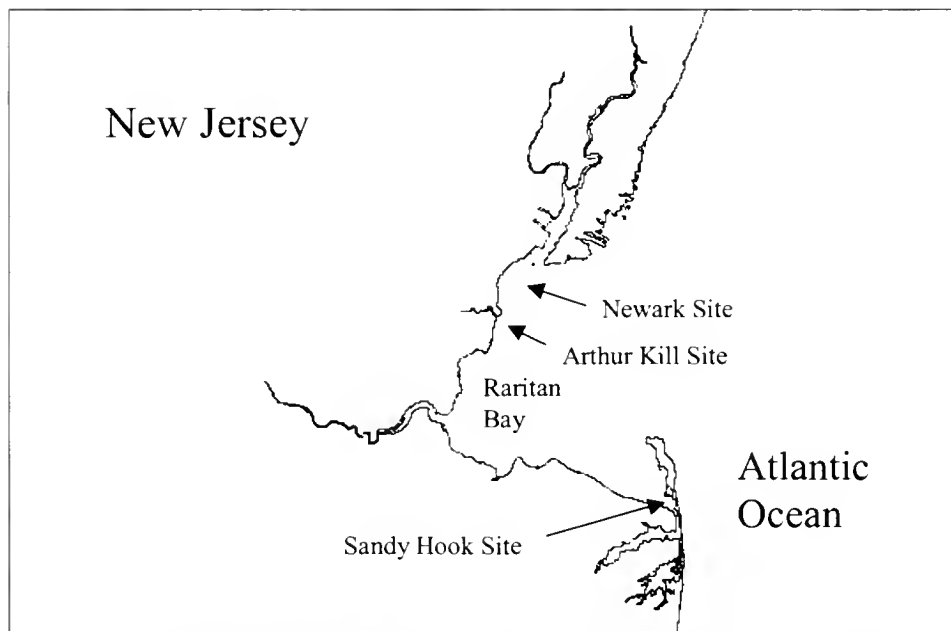

Figure 1. Locations of New Jersey field study sites in the Newark/Raritan Bay Complex.

from mild (↑) to severe (↑↑↑) for lesions, inflammation-like responses, and infectious diseases.

Gonad condition was graded according to Kennedy (1977):

Stage 0 = resting stage
Stage I = early development
Stage II = later development
Stage III = sexual maturity
IIIa = maturity
IIIb = spawning
IIIc = redevelopment
IIId = recently spent

Tissues evaluated were gills, mantle, adductor muscle, kidney/heart, digestive gland, and gonadal condition.

Fertilization Assay: Strip Spawning

Field Study

A total of six ripe oysters from each site were strip spawned (male = 3, female = 3). Eggs and sperm were extracted from the gonadal region using a scalpel and lightly lacerating the gonad (Allen et al. 1989). Collected eggs were sieved on a 25-μm screen and were washed with seawater collected from the respective site. Eggs were viewed under a microscope for maturation before being fertilized with the collected oyster sperm (sperm was diluted to 50 mL). Once sperm (1 mL) was added to the egg suspension (200 eggs per mL), the eggs were set aside for 1 h before being assayed to allow for fertilization. The total number of fertilized and unfertilized eggs, in three 1-mL replicate samples, was ascertained before eggs were dispensed into petri dishes. To each 10-mL glass petri dish (n = 3 per site), 10 mL of the site-collected water and fertilized eggs (n = 100) from each site were dispensed into the appropriate petri dish. Fertilized eggs were allowed to develop for 48 h at room temperature without aeration or food. After 48 h, the larvae were sieved on a 53-μm screen, and the number of larvae that had developed to the straight hinge stage was counted.

Laboratory Study

In vivo. Adult eastern oysters (*Crassostrea virginica*) were purchased from Haskin Shellfish Research Laboratory (Rutgers University, Piscataway, NJ). Oysters (n = 32) were exposed to two treatments of tritium-labeled 2,3,7,8-TCDD via adductor muscle injections. The study was conducted for 28 days to allow the circulation and distribution of dioxin throughout the oyster. This time period was selected based on results obtained from a distribution study using 2,3,7,8-TCDD (Wintermyer 1998). Oysters (n = 48) were weighed (mean weight 50 g), numbered and notched, and their dimensions were measured (i.e., height, length, and width). Oysters were notched on the left side of the valves for access to the adductor muscle. Control oysters (n = 16) were injected (via adductor muscle) with 100 μL (0.1 mL) of 20 parts per thousand filtered seawater. The nominal 2.0 pptr treatment group (n = 16) was injected with 100 μL (0.1 mL) of 0.996 pg/g ^{3}H-TCDD. The nominal 20.0 pptr treatment group (n = 16) was injected with 100 μL (0.1 mL) of 27.7 pg/g ^{3}H-TCDD. ^{3}H-TCDD equivalents were based on radioactivity in 0.1-mL injection volumes in a 50-g oyster (pg/g) (n = 3). All oysters were placed on absorbent paper for 1 h before being put into 76-L aquarium tanks for 24 h. This procedure was performed to allow the discharging and recirculation of dioxin by the oysters. Oysters were not fed 24 h before or 24 h after the injections. Treatment groups

(i.e., control, 2.0 pptr, and 20.0 pptr groups) were placed into separate recirculating seawater systems 24 h after the injections. All oysters were reinjected on day 14 of the study according to the procedure described above. This procedure was performed to maintain dioxin concentrations in the oysters over 28 days (Wintermyer 1998). Treatment groups were strip spawned on day 28 according to the procedure described above (field study). Eggs (10 eggs per mL) from each treatment group were fertilized with sperm (1 mL; sperm was diluted to 100 mL) collected from the corresponding treatment group.

Ex vivo. The 48-h static *ex vivo* assay consisted of control eggs (9 eggs per mL) fertilized with control sperm (1 mL; sperm was diluted to 100 mL). Glass exposure beakers (150 mL) (n = 3) consisted of 0.1 mL of nominal 2.0 pptr TCDD, and 0.1 mL of nominal 20.0 pptr TCDD and 0.0 pptr TCDD, respectively. To each treatment beaker, a 10-mL egg suspension and a 2-mL sperm suspension were added, and allowed to set for 2 h for fertilization.

Both 48-h *in vivo* and *ex vivo* assays were conducted in 20-mL glass petri dishes. Fertilized eggs (10 mL) from each treatment group was pipetted into individual petri dishes (n = 20 per group) and were incubated at 22°C for 48 h. After 48 h, each petri dish in both the *in vivo* and *ex vivo* assays was examined for the number of fertilized and unfertilized eggs, as well as for the number of living and dead larvae and their development stages.

Radiolabeled Compounds

[^{3}H] 2,3,7,8-TCDD (34.7 Ci/mM, 98% pure by high-performance liquid chromatography, with carbons 1 and 6 radiolabeled) was purchased from Chemsyn Science Laboratories (Lenexa, KA). Oysters were exposed to 0.996 pg/g (2 pptr) or 27.7 pg/g (20.0 pptr) of [^{3}H]-TCDD via adductor muscle injection. All [^{3}H]-TCDD values were based on equivalents.

RESULTS

Deployment and Retrieval

In this study, a total of six bags containing eastern oysters was transplanted to the Newark Bay and the Raritan Bay Complex from September 2000 until June 2001. Oysters transplanted to Newark Bay for 10 mo had the second highest increase in total weight gain (+6 g). Oysters transplanted to Arthur Kill had a decrease in total weight gain (−10.9 g), and oysters transplanted to Sandy Hook Bay had the highest increase in weight gain (+10.3 g). There was not a significant difference in shell growth among the Newark Bay, Arthur Kill, or Sandy Hook transplanted oysters over the 10-mo field study (Table 1).

Tissue Analysis

Oyster tissues were analyzed for dioxin, furan, and PCB analytes. Newark Bay oysters had the highest tissue levels of 2,3,7,8-TCDD (3.2 pptr), total TCDD (16.5 pptr), total TCDF (93.8 pptr), and total PCBs [1.7 parts per billion (ppb)]. Arthur Kill transplanted oysters had the second highest tissue levels of 2,3,7,8-TCDD (1.3 pptr), total TCDD (13.3 pptr), total TCDF (56.7 pptr), and total PCB (64.5 ppb). Sandy Hook oysters had the lowest levels of 2,3,7,8-TCDD (0.15 pptr), total dioxin (2.5 pptr), total furan (47.6 pptr), and total PCBs (35.3 ppb) (Tables 2 and 3).

Deployment and retrieval data from *C. virginica* transplanted to Newark Bay, NJ, Arthur Kill, NJ, and Sandy Hook Bay, NJ, field sites.[a]

Sites	Date	No. of Oysters[b]	Temp. (°C)	Salinity (ppt)	Weight (g)	H (mm)	L (mm)	W (mm)
Deployment								
Newark Bay	9/12/00	60	18.5	20	57.5 ± 15.3	81.4 ± 13.6	45.8 ± 5.0	19.8 ± 2.8
Arthur Kill	9/12/00	60	19.5	20	66.8 ± 19.9	88.7 ± 14.0	46.9 ± 7.4	20.5 ± 2.9
Sandy Hook	9/12/00	60	18	23	68.1 ± 25	89.7 ± 15.0	46.6 ± 5.0	20.8 ± 4.0
Retrieval								
Newark Bay	6/1/01	47/13[c] (2 bags recovered)	14.3	16	63.5 ± 18.0	81.7 ± 13.2	45.4 ± 4.8	19.5 ± 2.6
Arthur Kill	6/1/01	45/15[c] (2 bags recovered)	17.3	16	55.9 ± 13	88.2 ± 13.8	46.2 ± 7.7	20.8 ± 3.0
Sandy Hook	6/1/01	25/5[c] (1 bag recovered)	14.6	20	78.4 ± 26	89.4 ± 14.7	46.1 ± 5.5	20.5 ± 4.2

Presented as mean ± Sd, unless otherwise indicated.

[a] H, height; L, length; W, width; ppt, parts per thousand.

[b] Number of oysters per site; two bags per site.

[c] Number of live oysters/number of dead oysters.

Histologic Evaluation

Oysters transplanted to Newark Bay showed moderate signs of epithelial-severe hyperplasia, while oysters transplanted to Arthur Kill showed signs of severe epithelial-severe hyperplasia with some cells (>4) showing mitotic division, and connective tissue displaying areas of focal fibrosis. Oysters transplanted to Sandy Hook showed signs of slight epithelial-severe hyperplasia. Only the transplanted oysters to Arthur Kill were observed to have a *haplosporidium nelsoni* (MSX) infection in the digestive gland and mantle tissues (Table 4). All transplanted oysters showed slight-to-moderate gill hyperplasia ("clubbing"). Oysters transplanted to Newark Bay and Arthur Kill showed an alteration in gill cilia shape, size, and orientation. The cilia had a thickened appearance and an alteration in cilia length resulting in a distinct whip-like appearance (approximately six times the length of normal gill cilia).

Gross Body Evaluation

Oysters transplanted to Newark Bay had semi-developed gonadal tissue. The gonadal area had a slightly cream-colored appearance, and the oysters appeared to be of moderate health and were plump. The shell interior had a white, iridescent color and had no obvious scarring or discoloration. Oysters transplanted to

Oyster tissue analysis for dioxins/furans at Newark Bay, NJ, Arthur Kill, NJ, and Sandy Hook Bay, NJ, during a 10-mo water suspension field study.

Analytes	Newark Bay Concentration (pptr)[a]	Arthur Kill Concentration (pptr)[b]	Sandy Hook Concentration (pptr)[c]
2,3,7,8-TCDD	3.2	1.3	0.15[d]
1,2,3,7,8-PeCDD	<DL (0.3)[e]	<DL (0.3)	<DL (0.2)
1,2,3,4,7,8-HxCDD	<DL (0.3)	<DL (0.3)	<DL (0.1)
1,2,3,6,7,8-HxCDD	<DL (0.3)	<DL (0.3)	<DL (0.1)
1,2,3,7,8,9-HxCDD	<DL (0.3)	<DL (0.3)	<DL (0.1)
1,2,3,4,6,7,8-HpCDD	0.59	1.0	0.43
1,2,3,4,6,7,8,9-OCDD	1.8	4.8	2.3
2,3,7,8-TCDF	6.5	4.3	<DL (2.5)
1,2,3,7,8-PeCDF	<DL (0.2)	<DL (0.2)	<DL (0.1)
2,3,4,7,8-PeCDF	0.93	0.73	<DL (0.1)
1,2,3,4,7,8-HxCDF	<DL (0.2)	<DL (0.2)	<DL (0.08)
1,2,3,6,7,8-HxCDF	<DL (0.2)	<DL (0.2)	<DL (0.07)
2,3,4,6,7,8-HxCDF	<DL (0.2)	<DL (0.2)	<DL (0.09)
1,2,3,7,8,9-HxCDF	<DL (0.2)	<DL (0.2)	<DL (0.1)
1,2,3,4,6,7,8-HpCDF	<DL (0.2)	<DL (0.2)	<DL (0.1)
1,2,3,4,7,8,9-HpCDF	<DL (0.4)	<DL (0.3)	<DL (0.2)
1,2,3,4,6,7,8,9-OCDF	<DL (0.5)	<DL (0.4)	**0.47
Total TEFs[f]	4.3	2.2	0.6

DL, detection limit; TEF, total equivalent factor.

[a] Sample size, 25.15 g; 0.3% lipids.

[b] Sample size, 25.17 g; 0.2% lipids.

[c] Sample size; 25.1 g; 0.6% lipids (shown in parentheses).

[d] Concentration is below the calibration curve. Value is an estimate only.

[e] Values are less than the detection limit.

[f] Environmental Protection Agency (1989a).

TABLE 3.

Oyster tissue analysis for total PCBs at Newark Bay, NJ, Arthur Kill, NJ, and Sandy Hook Bay, NJ, during a 10-mo water suspension field study.

Analytes	Newark Bay Concentration (ppb)[a]	Arthur Kill Concentration (ppb)[b]	Sandy Hook Concentration (ppb)[c]
Total MonoCB	<DL (0.09)[d]	<DL (0.1)	<DL (0.08)
Total DiCB	<DL (0.1)	<DL (0.1)	0.54
Total TriCB	5.6	4.5	2.1
Total TetraCB	24.2	23.5	12.2
Total PentaCB	22.8	21.6	14.9
Total HexaCB	14.2	13.0	4.9
Total HeptaCB	1.7	1.9	0.68
Total OctaCB	<DL (0.8)	<DL (0.7)	<DL (0.6)
Total NonaCB	<DL (1.2)	<DL (1.0)	<DL (0.9)
DecaCB (#209)	<DL (2.2)	<DL (1.9)	<DL (1.6)
Total PCB[e]	68.6	64.5	35.3
Total PCB + EMPC[f]	71.6	69.4	35.3

DL, detection limit; EMPC, estimated maximum possible concentration.

[a] Sample size, 30.0 g; 0.3% lipids

[b] Sample size, 20.0 g; 0.2% lipids.

[c] Sample size, 24.0 g; 0.6% lipids.

[d] Values are below the DL.

[e] Newark Bay and Arthur Kill total PCB was approximately two times that of Sandy Hook.

[f] If matched sets of peaks in the time window do not have the appropriate ion mass ratios for a true PCB, the EMPC is calculated using the sum of the observed peaks (Triangle Laboratories, Inc., modified method 680 (11/85).

Arthur Kill had underdeveloped gonads, and were easily shucked and watery. The gonadal area had a vein-like appearance and a gray coloration. The shell interior had a white, iridescent color and had no obvious scarring or discoloration. Oysters transplanted to Sandy Hook were plump, had a whitish-cream coloration and well-developed gonads, and were in a prespawning state (Table 4).

Field Study Strip Spawning Assay

Results from the strip-spawning assay using oysters transplanted to Newark Bay, Arthur Kill, and Sandy Hook, NJ, showed that the majority of eggs collected from female oysters at the Newark Bay and Arthur Kill sites were not viable. There was not a difference in fertilized egg size (64 μm) among the transplanted oysters, however, oysters transplanted to Arthur Kill had a smaller unfertilized egg size (48 μm) compared with oysters transplanted to Newark Bay or Sandy Hook. This study shows that 60.5% and 76.7%, respectively, of eggs collected from oysters transplanted to Newark Bay and Arthur Kill were not fertilized, and of the eggs that were fertilized (39.5% and 23.3%) only 0.03% and 0.04%, respectively, of the eggs developed to the straight-hinge stage.

TABLE 4.

C. virginica histological evaluation for 10-mo field study (September, 2000–June, 2001).

Sites	Gill	Mantle	Adductor Muscle	Kidney/Heart	Digestive Gland (Midgut)	Gonad[a] Condition
Newark Bay (n = 15)	↑ Hyperplasia (80%) ↑ Inflam. (100%)‡ ↑↑–↑↑↑ Dysplasia (100%)	↑↑ Epithelial severe hyperplasia (70%) ↑ Inflam. (100%) ↑↑–↑↑↑ Dysplasia (100%)	↑ Brown cell (10%)‡ ↑ Inflam. (10%) ↑↑–↑↑↑ Dysplasia (100%)	↑↑–↑↑↑ Brown cell (70%) ↑ Inflam. (70%) ↑↑–↑↑↑ Dysplasia (100%)	↑↑ Epithelial severe hyperplasia (80%) ↑ Inflam. (100%) ↑↑–↑↑↑ Dysplasia (100%)	Stage 2 and 3 (80%) Stage 1 (20%)
Arthur Kill (n = 15)	↑↑–↑↑↑ Inflam. (100%) ↑ Hyperplasia (100%) ↑ Brown cell (100%)	↑↑↑ Epithelial severe hyperplasia (100%) ↑↑–↑↑↑ Inflam. (100%) ↑↑–↑↑↑ Dysplasia (100%) ↑↑–↑↑↑ MSX (70%)	↑ Inflam. (30%) ↑↑–↑↑↑ Dysplasia (100%)	↑ Inflam. (60%) ↑↑–↑↑↑ dysplasia (100%) ↑↑–↑↑↑ Brown cell (60%)	↑↑↑ Epithelial severe hyperplasia (100%) ↑↑–↑↑↑ Inflam. (100%) ↑↑–↑↑↑ Dysplasia (100%) ↑ Brown cell (100%) ↑↑–↑↑↑ MSX (70%)	Stage 1 and 2 (40%) Stage 3 (60%)
Sandy Hook (n = 15)	↑ Dysplasia and filament fusion (100%) ↑ Hyperplasia (100%)	↑ Epithelial severe hyperplasia (40%) ↑ Brown cell (100%) ↑ Dysplasia (100%)	↑ Dysplasia (100%)	↑ Dysplasia (100%)	↑ Epithelial severe hyperplasia (40%) ↑ Brown cell (100%) ↑ Dysplasia (100%)	Stage 3a and 3b (80%) Stage 0 (0.01%)

Numbers in parentheses are % of oysters. Lesion grading definitions: (–), absent; (↑), slight; (↑↑), moderate; (↑↑↑), severe; inflam., inflammatory like response; brown cell, brown cell accumulation.

[a] Gonad grading: stage 0, resting stage; stage 1, early development; stage 2, later development; stage 3, sexual maturity; stage 3a, maturity; stage 3b, spawning; stage 3c, redevelopment, stage d, recently spent (Kennedy 1977).

Most fertilized eggs did not develop beyond the zygote stage. The strip-spawning assay from oysters transplanted to Sandy Hook showed that 53.7% of the eggs were fertilized, and of those eggs 84% developed to the straight-hinge stage (Table 5, Fig. 2).

Acute Static 48-h In Vivo and Ex Vivo Assays

In this study using *C. virginica*, there was an observable decrease in the number of fertilized eggs in the 2 and 20 pptr TCDD groups. In Table 6, controls for the *in vivo* and *ex vivo* assays had high rates of egg fertilization and larvae development to the straight hinge stage (80.3%). The 2.0 pptr *in vivo* assay had 48% egg fertilization, but 100% mortality at the zygote development stage. In the 20.0 pptr *in vivo* assay, to which viable control eggs were fertilized with 20.0 pptr sperm, there was very little fertilization (0.9%), which resulted in a high egg mortality rate (99.%). The 2.0 pptr *ex vivo* and 20.0 pptr *ex vivo* assays also had low fertilization rates (3% and 2%, respectively), which resulted in high egg mortality rates (97% and 98%, respectively). In both the 48-h acute *in vivo* and *ex vivo* studies, there were large decreases in the number of veliger larvae compared with the controls. Within treatment groups (nominal 2.0 pptr TCDD and 20.0 pptr TCDD), there were 52 to 99% unfertilized eggs. Eggs that were fertilized had a 98 to 100% mortality rate and did not develop beyond the zygote stage. In contrast, the control eggs had an 80% survival rate to the straight-hinge stage (Table 6, Fig. 3).

DISCUSSION

PCBs were first commercially produced in 1929 (NJDEP 1993). PCBs were commonly used in transformer oils and electrical products. In 1977, the U.S. Environmental Protection Agency banned the production of PCBs. However, many PCB-laden transformers, capacitors, and other electrical equipment remain in service (NJDEP 1993). PCBs have been and continue to be dispersed throughout the environment through spills, effluent discharges, and incineration.

In the 1970s and 1980s, the levels of TCDD in Newark, NJ, and Arthur Kill, NJ, shellfish approached the no-consumption advisory level suggested by the U.S. Food and Drug Administration of 25 pptr (Belton et al. 1985). The levels of other isomers such as PCBs, polychlorinate dibenzo-p-dioxin (PCDDs), and polychlorinated dibenzo-p-furan (PCDFs) found in aquatic organisms (striped bass and blue crab) in Newark Bay and Arthur Kill resulted in the closing of the waterways to fishing beginning in 1984 (NJDEP 1990). Extensive soil contamination with dioxin, specifically 2,3,7,8-TCDD, discovered at a site adjacent to the Passaic River in Newark, NJ, prompted an intensive study of dioxin levels in sediments and biota in 1983 and 1984 (NJDEP 1990).

In this study, a total of six bags were deployed in the field in September 2000. The field sites were selected based on historical data about the bay system and accessibility via boat. Sandy Hook, NJ, was selected as the reference site, and Arthur Kill and Newark Bay, NJ, were selected as the exposure sites due to the high level of industrialization along the waterways. The approximate distance between the Newark site and the Arthur Kill site is 5 miles. The distance between the Sandy Hook site and the Newark-Arthur Kill site is approximately 32 miles. Oysters were put in the field at the completion of the 2000 spawning season and were collected prior to the 2001 spawning season to ensure bioaccumulation levels prior to and during gametogenesis. Oyster tissues were analyzed for dioxin, furan, and PCB analytes. Newark Bay oysters had the highest tissue levels of 2,3,7,8-TCDD (3.2 pptr), total TCDD (16.5 pptr), total TCDF (93.8 pptr), and total PCBs (68.6 ppb). Oysters transplanted to Arthur Kill had slightly lower tissue levels of 2,3,7,8-TCDD (1.3 pptr), total TCDD levels (13.3 pptr), total TCDF levels (56.7 pptr), and a slightly lower total PCB level (64.5 ppb) than those of the Newark Bay oysters (Tables 3 and 4). Sandy Hook oysters had the lowest levels of 2,3,7,8-TCDD (0.15 pptr), total dioxin (2.5 pptr), total furan (47.6 pptr), and total PCBs (35.3 ppb) (Tables 2 and 3).

Oysters transplanted to Newark Bay showed moderate signs of epithelial-severe hyperplasia, and oysters transplanted to Arthur Kill showed signs of severe epithelial-severe hyperplasia, with some cells (>4) showing mitotic division and connective tissue displaying areas of focal fibrosis. Oysters transplanted to Sandy Hook showed signs of slight epithelial-severe hyperplasia. The epithelial-severe hyperplasia could be interpreted as preneoplastic in nature, however, further research is needed to verify that these lesions can progress to a neoplastic condition. Only the Arthur Kill oysters were observed to have a moderate-to-severe MSX infection in the digestive gland and mantle tissues (Table 4). Sandy

TABLE 5.

Summary of the strip-spawning assay from Newark Bay, NJ, Arthur Kill, NJ, and Sandy Hook Bay, NJ, 10-mo field study (September, 2000–June, 2001).

	Newark Bay, NJ	Arthur Kill, NJ	Sandy Hook, NJ
Weight of oysters at time of deployment (g) (9/00)	57.5 ± 15.3 (n = 60)	66.8 ± 19.9 (n = 60)	68.1 ± 24.9 (n = 60)
Weight of oysters at termination of study (g) (6/01)	63.5 ± 18.4 (n = 45)	55.9 ± 13.1 (n = 47)	78.4 ± 25.6 (n = 25)
% lipid (6/01)	0.3	0.2	0.6
Egg size fertilized vs. unfertilized (μ at 40×) (n = 5)	64 μm fertilized 56 μm unfertilized	64 μm fertilized 48 μm unfertilized	64 μm fertilized 56 μm unfertilized
Total number of fertilized eggs[a]	107 ± 6.00	54 ± 30.11	113 ± 13.61
Total number of unfertilized eggs[a]	164 ± 25.6	178 ± 15.9	97 ± 39.9
Number of veligar larvae after 48 h[b]	3 ± 1.7	4 ± 2.31	82 ± 12.2

Presented as mean ± SD, unless otherwise indicated.

[a] Numbers represent the average of 1-mL replicate samples (n = 3).

[b] Number of veligar larvae resulting from approximately 100 fertilized eggs (n = 3 replicates).
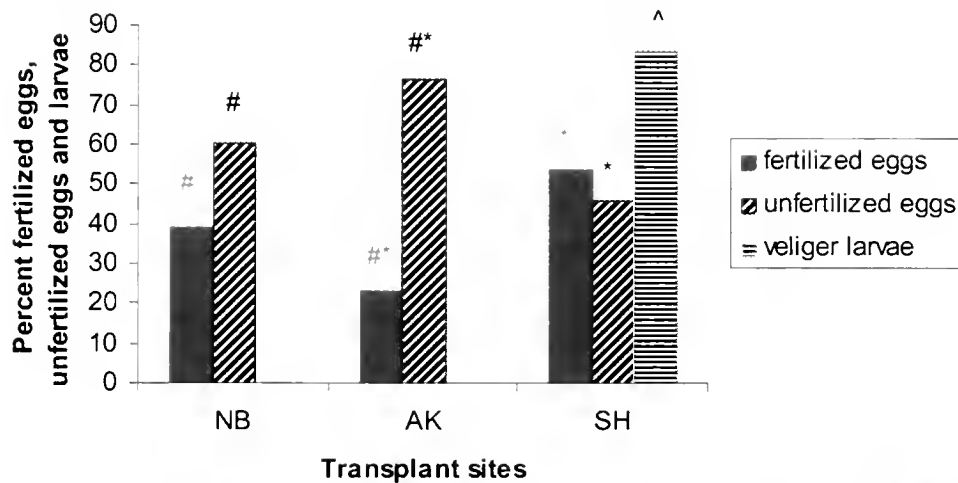
Figure 2. The percentage of fertilized eggs, unfertilized eggs, and veliger larvae resulting from the strip-spawning assay using transplanted oysters from Newark Bay, NJ, Arthur Kill, NJ, and Sandy Hook Bay, NJ (10-mo field study, September 2000–June 2001). Number of fertilized and unfertilized eggs are averages of 1-mL replicates (three per site). Numbers of veliger larvae are those resulting from 100 fertilized eggs after 48 h (three sites). #* (light), fertilized egg groups that are significantly different ($P < 0.05$; ANOVA); #* (dark), unfertilized egg groups that are significantly different ($P < 0.05$; ANOVA); ∧, veliger larvae groups that are significantly different ($P < 0.05$; ANOVA); NB, Newark Bay; AK, Arthur Kill; SH, Sandy Hook.

Hook oysters had fully developed gonads and were in a prespawning state. Newark Bay and Arthur Kill oysters were slightly moderately underdeveloped due to a lack of gonadal development compared with Sandy Hook oysters at the time of collection (Table 4). All transplanted oysters showed slight-to-moderate gill hyperplasia (clubbing). Oysters transplanted to Newark Bay and Arthur Kill showed an alteration in gill cilia shape, size, and orientation. The cilia had a thickened appearance and an alteration in cilia length resulting in a distinct whip-like appearance (approximately six times the length of normal gill cilia). This alteration in gill cilia could be a result of chronic exposure over time. The lesions observed in the transplanted oysters would be consistent with those resulting from chronic exposure to chemicals. The lesions are not pathoneumonic but are consistent with a wide variety of chemical and physical irritants.

Oysters transplanted to the Newark Bay site had the second highest increase in weight gain (+ 6 g), percentage of lipids (0.3%), egg fertilization (39.5%), and larval development (0.03%). Oysters

transplanted to the Arthur Kill site had a decrease in weight over the 10-mo study (−10.9 g), the lowest percentage of lipid content (0.2%), the lowest percentage of egg fertilization (23.3%), and a decrease in larval development (0.04%). Oysters transplanted to the Sandy Hook site had the greatest increase in weight gain (+10.3 g), the highest percentage of lipids (0.6%), the highest percentage of egg fertilization (53.7%), and the highest percentage of larval development (84%) (Table 5, Fig. 2). Weight gain and the percentage of lipid content of the oyster contribute greatly to egg development and production, egg fertilization success, and larval development (Capuzzo 1996, Capuzzo & Leavitt 1988, Lowe 1988, Moore 1988). Oysters transplanted to Sandy Hook had the highest level of fitness followed by oysters transplanted to Newark Bay and Arthur Kill, based on lesion grading, inflammatory-like responses, infectious disease states, weight gain/loss, and the degree of gonadal development.

Results from the strip-spawning assay using oysters transplanted to Newark Bay, Arthur Kill, and Sandy Hook, NJ, showed

TABLE 6.

Summary of an acute static 48-h *in vivo* and *ex vivo* strip-spawning bioassay for *C. virginica* exposed to 2 and 20 pptr 2,3,7,8-TCDD.

	Initial (Egg)		After 48 h (Veliger Larvae)			
	Number of Fertilized Eggs	Number of Unfertilized Eggs	Number Dead after 48 h	Stage of Development	Number Alive after 48 h	Stage of Development[a]
Control *in vivo*	196 ± 143	2 ± 0.63	39 ± 1.45	Trochophore, egg, and D-stage	159 ± 1.66	D-stage
2 pptr *in vivo*	152 ± 3.12	166 ± 3.80	318 ± 3.45	Egg	0	NA
20 pptr *in vivo*[b]	6 ± 0.801	660 ± 16.2	663 ± 17.94	Egg	3 ± 0.52	D-stage
Control *ex vivo*	194 ± 2.17	4 ± 0.84	48 ± 2.10	Trochophore, egg, and D-stage	150 ± 2.36	D-stage
2 pptr *ex vivo*	13 ± 0.489	420 ± 10.8	423 ± 12.0	Egg and D-stage	10 ± 0.513	Trochophore and D-stage
20 pptr *ex vivo*	16 ± 0.410	803 ± 27.3	810 ± 27.6	Egg and D-stage	9 ± 0.510	D-stage

Presented as mean ± SD, unless otherwise indicated. NA, not applicable.
In vivo represents eggs exposed to TCDD during gametogenesis and *ex vivo* represent eggs exposed to TCDD in petri dishes during fertilization (n = 20 for each group). Table taken from Wintermyer (1998).
[a] Stage of fertilized egg (Loosanoff and Davis 1963).
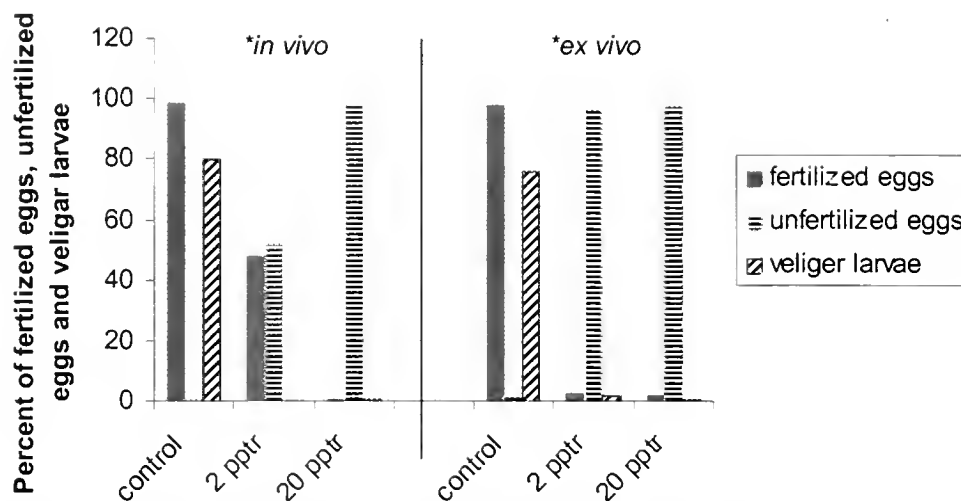[b] Viable control eggs were fertilized with 20 pptr sperm.

Figure 3. The percentage of fertilized eggs, unfertilized eggs, and veliger larvae resulting from an acute static 48-h *in vivo* and *ex vivo* strip-spawning assay using *C. virginica* exposed to 2 and 20 pptr 2,3,7,8-TCDD. *in vivo*, eggs exposed to TCDD during gametogenesis; *ex vivo*, eggs exposed to TCDD in petri dishes during fertilization (*n* = 20 for each group). Table from Wintermyer (1998).

that the majority of eggs collected from female oysters at the Newark Bay and Arthur Kill sites were not viable (Fig. 2). This study shows that 60.5% and 76.7%, respectively, of eggs collected from Newark Bay and Arthur Kill transplanted oysters were not fertilized, and of the eggs that were fertilized (39.5% and 23.3%, respectively) only 0.03% and 0.04%, respectively, of the eggs developed to the straight-hinge stage. Most fertilized eggs did not develop beyond the zygote stage. The strip-spawning assay for oysters transplanted to Sandy Hook showed that 53.7% of the eggs were fertilized, and of those eggs 84% developed to the straight-hinge stage (Fig. 2). This study was performed to evaluate the potential for restoring oysters in to the bay area. Based on the field study and strip-spawning assay, transplanting oysters into the Newark bay and Arthur Kill sites at this time would not result in successful recruitment of the bay area. However, the Sandy Hook site would be an ideal area for oyster restoration.

In the laboratory studies, the 2.0 pptr and 20.0 pptr treatment concentrations of 2,3,7,8-TCDD used in the 48-h acute *in vivo* and *ex vivo* studies were based on tissue concentrations that were reported from the soft-shelled clam (*Mya arenaria*) living in Newark, NJ, (11–20 pptr TCDD) and Tuckerton, NJ, (0.1–0.6 pptr) (Brown et al. 1993) and on sediment samples from Newark Bay (20 pptr), Arthur Kill (10 pptr), and Tuckerton (0.5 to 1.0 pptr) (Brown et al. 1993). In this study using *C. virginica*, there was an observable decrease in the number of fertilized eggs within the 2 and 20 pptr TCDD treatment groups. In Fig. 3, controls for the *in vivo* and *ex vivo* assays had high rates of egg fertilization and larvae development to the straight-hinge stage (80.3%). The 2.0 pptr *in vivo* assay had a 47.8% egg fertilization rate, but a 100% mortality rate at the zygote development stage. In the 20.0 pptr *in vivo* assay to which viable control eggs were fertilized with 20.0 pptr sperm, there was very little fertilization (0.901%), which resulted in a high egg mortality rate (99.6%). The 20-pptr treatment group did not have any female oysters remaining due to toxicant-induced stress and mortality by the end of the 28-day period. The 2.0 pptr *ex vivo* and 20.0 pptr *ex vivo* assays also had low fertilization, which resulted in high egg mortality (Fig. 3). In both the 48-h acute *in vivo* and *ex vivo* studies, there was a large decrease in the number of fertilized eggs respective to treatment group compared with the controls. Within treatment groups (nominal 2.0

pptr TCDD and 20.0 pptr TCDD), there were 52 to 99% unfertilized eggs. Eggs that were fertilized had a 98 to 100% mortality rate and did not develop beyond the zygote stage. In contrast, the control eggs had an 80% survival rate to the straight-hinge stage (Table 6, Fig. 3). This laboratory study is important in understanding the effects of 2,3,7,8-TCDD independent of other lipophilic compounds on oyster gametogenesis and egg fertilization. We cannot state that the field study results were solely due to 2,3,7,8-TCDD, but laboratory studies demonstrate that TCDD can result in a significant decrease in gametogenesis and egg viability.

CONCLUSION

In conclusion, this study was designed to investigate two points of interest: (1) the dioxin/furan and PCB concentrations in the eastern oyster during gametogenesis and the effects on egg fertilization and development; and (2) to evaluate the potential for restoring oysters back into the New Jersey bay area. Oysters transplanted to Sandy Hook, NJ, had the greatest weight gain, percentage of lipid content, percentage of egg fertilization, and percentage of larval development to the straight-hinge stage, followed by oysters transplanted to Newark Bay and Arthur Kill, NJ. The laboratory *in vivo* and *ex vivo* strip-spawning assays showed that exposure to compounds such as dioxin can accumulate in animal tissues and can interfere with normal metabolic processes that affect gonadal development and egg fertilization. While we cannot separate the effects of different gonadal development on strip-spawning fertilization and larval development, the laboratory studies support the effect of 2,3,7,8-TCDD on gonadal development at levels observed in the field.

This study demonstrated that dioxins, furans, and PCBs are still bioavailable in the Newark Bay estuary. The levels approach concentrations that in the laboratory result in impacts on gonadal development and egg viability. This study clearly demonstrates that 2,3,7,8-TCDD effects gonadal development and egg viability in the eastern oyster in a similar fashion to fish species.

ACKNOWLEDGMENTS

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AN IMPROVEMENT TO THE DETERMINATION OF MEAT CONDITION INDEX FOR THE EASTERN OYSTER *CRASSOSTREA VIRGINICA* (GMELIN 1791)

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ABSTRACT The meat condition index (MCI) of a bivalve is a numerical representation of the quality of its soft tissue. Based on the percentage of the internal shell volume occupied by a bivalve's soft body tissue, the enumeration of a quantitative index is possible. Early methods sought to measure the shell cavity volumetrically; however, this technique is both slow and difficult to perform accurately. In 1982, Lawrence and Scott developed a method to determine the MCI for oysters gravimetrically, in which shell cavity capacity was determined by the difference between whole oyster weight and empty shell weight after drying for 24 h. This technique was not only as accurate as the volumetric method, but it was faster and easier. However, since any water contained within the shells themselves (not between them) was included in the initial whole oyster weight, it seemed logical that this should be included in the weight of empty shells as well. Drying shells for 24 h could make the calculated shell cavity appear larger, resulting in reduced meat condition. To determine the significance of weighing shells after 0 h versus 24 h of drying, the MCIs were determined by shell cavity casts and were compared with MCIs determined by the two gravimetric methods. Weighing shells immediately after processing (0 h) was determined to more accurately estimate cavity volume whenever shells lost >3% of their weight due to drying. Of 1749 oysters examined from the Patuxent River, Maryland, over 3 y, 74% lost >3% of their shell weight. Several other sites in the Chesapeake Bay were also examined, yielding similar results. Weighing shells at 0 h not only increased accuracy for most of the oysters examined, but also saved time, as shells did not need to be held for an additional 24 h. Differences in shell morphology and fouling community structure may influence shell porosity, favoring one technique over the other.

KEY WORDS: oyster, *Crassostrea virginica*, meat condition, Chesapeake Bay

INTRODUCTION

The meat condition index (MCI) of a bivalve is a numerical representation of the quality (i.e., nutritive status or "fatness") of its soft tissue. Quantitative methods of determining the meat condition of bivalves have been conducted by various researchers as far back as the early 1900s. Crosby and Gale (1990) presented a brief history of the development of condition indices, which mentioned the works of Moore (1908), Milroy (1909), Grave (1912), Higgins (1938), Haven (1962), Walne (1970), Lawrence and Scott (1982), and Hawkins et al. (1987). Most of these indices were calculated using a formula that relates the weight of the soft tissue to the shell cavity volume. The Hopkins formula (Higgins 1938) used dry tissue weight (g) × 100/internal cavity volume (cm³). In many of the earlier methods, shell cavity volume was determined by displacement, until Lawrence and Scott (1982) showed that the weight of the whole oyster in air (g), less the weight of the empty valves in air (g), gave a very close approximation of cavity volume (cm³). Their formula for determining MCI became:

$$MCI = \frac{dry \ soft \ tissue \ wt \ (g) \ \times \ 100}{internal \ shell \ cavity \ capacity \ (g)}$$

Although some authors prefer to use dry soft tissue weight × 1000 (Hawkins et al. 1987, Crosby & Gale 1990), this formula simply results in a condition index value an order of magnitude larger than that of Lawrence and Scott (1982). Internal shell cavity volume (cm³) and shell cavity capacity (g) are the same when the cavity contents are assumed to have a density of 1 g cm⁻³.

This index represents meat quality or nutritive status while not necessarily reflecting the health of the individual. A fat oyster or one with a high MCI generally has a rich creamy color due to stored glycogen reserves and shows little of the internal organs beneath the mantle (Fig. 1A). An oyster of lesser quality will show

some internal structure because the mantle is thinner and more transparent (Fig. 1B). A very poor quality oyster will be watery and almost entirely clear.

Condition indices of oysters (*Crassostrea virginica*) in the Chesapeake Bay normally display a cyclical pattern, with the highest levels occurring in late fall and winter, and the lowest levels occurring in late summer after spawning is completed, but low conditions may occur at any time of year, possibly indicating a disease problem or unfavorable environmental conditions (Haven 1962, Abbe & Sanders 1988). In fact, Scott and Middaugh (1978), Scott and Vernberg (1979), and Lawrence and Scott (1982) all suggested the use of an MCI to monitor the effects of waterborne pollutants. Low salinity may have the opposite effect on condition since gametogenesis may be depressed or halted at salinities between 5 and 7.5‰ (Butler 1949, Loosanoff 1953). Oysters that do not ripen their gonads, and thus fail to spawn, may attain higher condition indices than would otherwise be observed if gametes are resorbed or if the energy intended for gamete production is funneled into glycogen production. A visual assessment of oyster meats, at least in some primitive form, has probably been conducted almost as long as humans have consumed oysters. As an index based on opacity due to glycogen content, however, visual condition indices are overly subjective and somewhat impractical.

Lawrence and Scott (1982) determined shell cavity capacity by weighing the whole oyster after air drying for 45 to 60 min, and then subtracting the weight of the valves after air drying for an additional 24 to 30 h. After using this method for more than 15 y to determine the MCIs of the eastern oyster *C. virginica* (Gmelin, 1791), we questioned its accuracy when shells were dried for 24 h. We suspected that the elapsed time between when the oyster is shucked and when the valves are weighed might have a major effect on the calculated value of shell cavity volume. When the whole oyster is weighed, there is a certain amount of water within the shells (not between them) in the spaces created by shell-boring animals. Since this weight is included in the initial weight of the

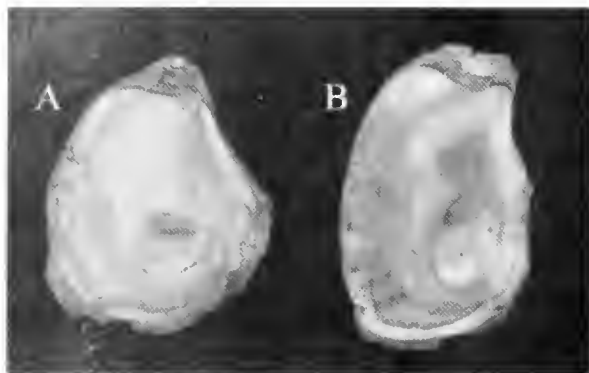*Corresponding author. E-mail: abbe@acnatsci.org

Figure 1. A fat oyster (A) with high MCI and one of lower meat condition (B). Note that the mantle along the left edge of B is transparent allowing shell structure to be observed beneath it.

Figure 2. Locations of 12 natural oyster bars in the Patuxent River where oysters were collected during 1997-2000: Teague Point (TP); Holland Point (HP); Macks Hallow (MH); Broad Neck (BN); Jacks Marsh (JM); Gatton (GAT); Peterson (PT); Hellen (HEL); Hawks Nest (HN); Town Creek (TC); Southeast Middleground (SM); and Little Cove Point (LCP).

whole oyster, it should also be included in the weight of the empty valves because it does not represent any part of the internal shell cavity. Drying shells for 24 h could make the calculated shell cavity volume appear larger, resulting in a reduced meat condition. We expected that valves might continue to lose weight as they dried over time for up to several days, although most of the weight loss would probably occur during the first 24 h. We suspected, therefore, that it might be necessary to weigh the empty valves immediately after the oyster is opened and the soft tissue removed to obtain a more accurate determination of shell cavity capacity or volume.

The amount of water weight within the valves also depends on the size (age) of the oyster as well as the number and size of the organisms living in (not on) the shells, which may include boring sponge (*Cliona* sp.) and mud worms (*Polydora* sp.) Young oysters have smaller shells to be inhabited by boring organisms and less time for their shells to be colonized. Older and larger oysters have more shell surface area to hold boring organisms and more time for their shells to be colonized. Regardless of size, however, if shells were weighed immediately after the meat was removed, the water weight in the shells should have a minimal effect on the calculated cavity volume, and thus on the condition index. We conducted several experiments (one that examined 1749 oysters over 36 mo) to investigate and quantify the effect of water-weight loss and drying time on oyster MCI.

MATERIALS AND METHODS

Drying Time

Thirty oysters were collected from the Holland Point oyster bar in the Patuxent River (Fig. 2) near Benedict, Maryland, in March 1997. They were cleaned of external fouling organisms and scrubbed with a nylon brush in the field. They were held in water until they were returned to the laboratory, where they were held in a raceway of running filtered river water at ambient salinity (10–14 parts per thousand). They were kept in water until processed, since it is critical that they release no cavity fluid when weights are used to determine cavity volume. Oysters were removed from water, rinsed, blotted dry, numbered, measured for right valve length (height), and weighed before they could gape and lose fluid. Once weights were determined, the loss of some cavity fluid by animals that gaped had no effect on subsequent measurements. Oysters were then shucked into preweighed beakers and dried to constant
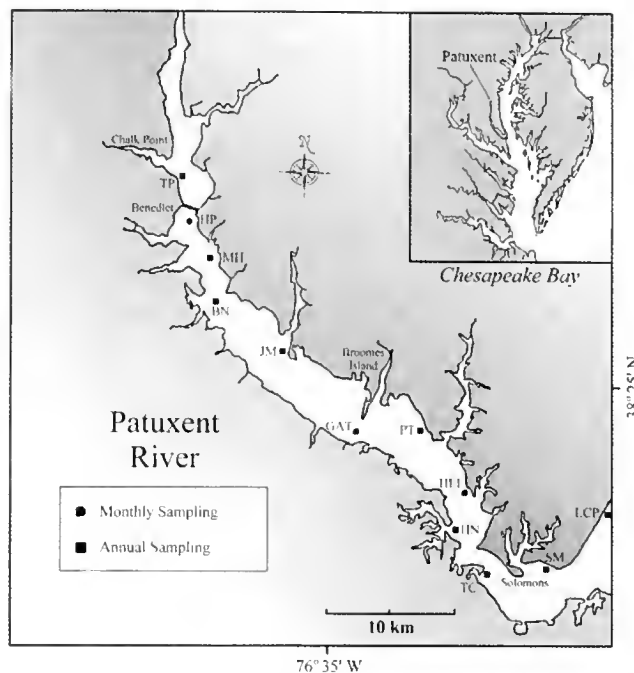
weight at 60 to 70°C (5–7 days). Dry meat weight was measured to the nearest 0.001 g. After shucking, the internal surfaces of the valves of each oyster were wiped dry, and the valves were weighed immediately to the nearest 0.001 g. They were weighed again after 6, 24, 48, and 72 h

Weight Loss

To determine whether valves weighed immediately after oysters were shucked (0 h) or after drying for 24 h resulted in the closest estimate to true cavity volume, it was necessary to first determine the true volume as accurately as possible. Several methods were tried, all of which were discussed by Crosby and Gale (1990), but most gave highly variable results and were time-consuming. The best technique that we found used a liquid casting medium of known density that was poured into each valve until slightly overfilled. As the liquid began to harden, the two valves were realigned and pressed tightly together so that excess casting material was squeezed out. Valves were banded to keep them tightly together until the cast was solid, which took only a few minutes. When the cast was hard, it was removed from the shells, the flashing was trimmed from the edges, and the volume of the cast was then determined volumetrically by displacement or gravimetrically by weighing and dividing by the density of the casting medium. MCIs were determined for 169 oysters using 0 and 24 h valve weights and cavity volumes using casts.

From March 1997 to February 2000, monthly sampling of oysters from four beds (Holland Point, Gatton, Hellen, and Southeast Middleground) and annual sampling from eight additional beds (Teague Point, Macks Hollow, Broad Neck, Jacks Marsh, Peterson, Hawks Nest, Town Creek, and Little Cove Point) in the Patux-

ent River (Fig. 2) was conducted with MCIs determined for 1749 oysters using shell weights at both 0 h and after drying for 24 h. Following the analysis of Patuxent River oysters, oysters were examined from two sites in the upper Chesapeake Bay (Eastern Bay and near Shady Side) and from two tributaries of the Potomac River (Wicomico and St. Mary's Rivers) (Fig. 3) to determine whether oysters from these areas were similar in weight loss to those from the Patuxent River. Approximately 50 oysters were collected and analyzed from each of these four areas.

RESULTS

Drying Time

Oysters used to determine drying time ranged from 72 to 89 mm shell length (SL), with a mean (±SD) SL of 80.4 ± 4.1 mm. Whole weights were 89.6 to 163.0 g, with a mean weight of 128.2 ± 23.2 g. The mean shell weight at 0 h was 104.4 g, and 101.5 g after drying for 6 h (a loss of 2.8%). Shells continued to lose weight out to 72 h when they averaged 100.0 g (Fig. 4A), although the rate of decrease declined over time. As shell weight decreased over 72 h, calculated cavity volume increased from 23.8 to 28.2 cm^3, resulting in a decrease in mean condition from 9.5 at 0 h to 8.1 after 72 h (Fig. 4B). However, since the mean condition had already decreased to 8.2 after just 24 h, the decrease over the next 48 h was minimal.

Weight Loss

When the percentage shell weight losses were averaged by 2-mm SL increments, the means ranged from 2.8% for oysters of
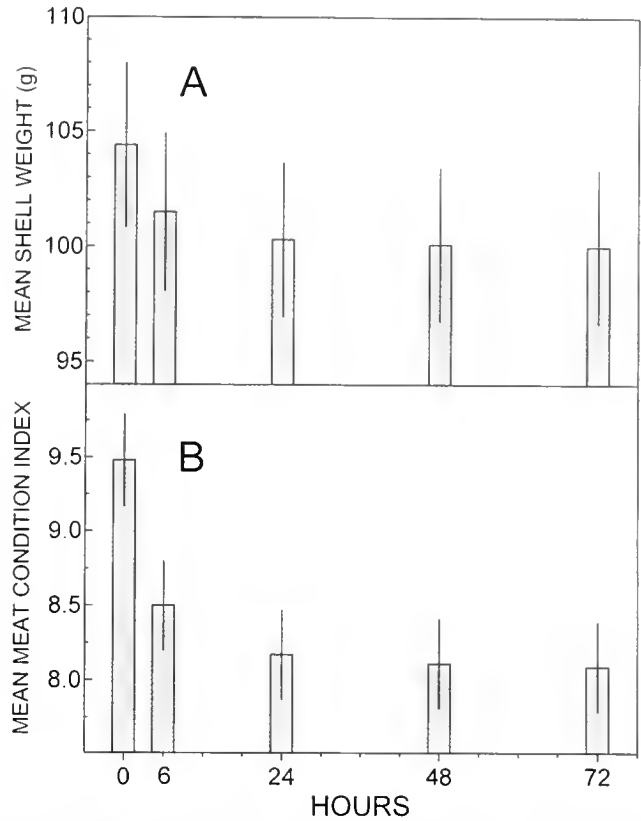
Figure 4. Mean shell weight over time as shells dried (A) and resulting loss in condition index (B). Drying was nearly complete after 24 h, but continued to 72 h.

62 mm SL to 5.0% for those of 110 mm SL, and they exhibited a highly significant relationship ($P < 0.001$) between SL and weight loss (Fig. 5). Figure 5 also shows that the three smallest groups were well below the regression line. The individual percentage weight loss for all 1749 oysters, however, ranged from as little as 1.2% to as much as 13.0%, with distribution skewed to the right, although most were in the 2 to 8% range (Fig. 6).

Shells were weighed at 0 and 24 h, and calculated cavity vol-

Figure 3. Other sites in Maryland from which oysters were collected during 2000 including Eastern Bay (EB), near Shady Side (SS), the Wicomico River (WR), and the St. Mary's River (SMR).
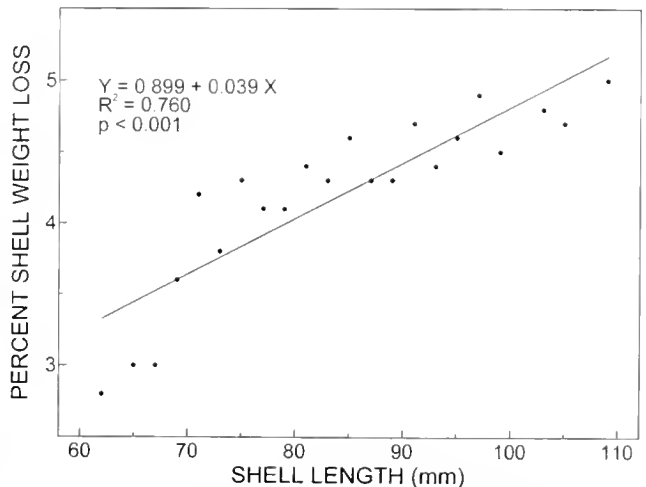
Figure 5. Linear regression of SL (by 2-mm increments) and shell weight loss after drying for 24 h.
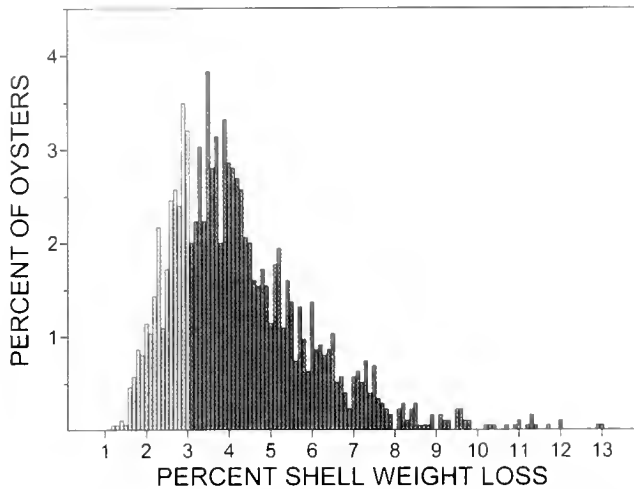
Figure 6. Percent weight loss frequency for shells dried for 24 h. More than 74% of the 1749 oysters examined lost at least 3% of their initial shell weight (darker bars).

umes were compared, with "true" volumes determined by casts for 169 oysters (Fig. 7). Shells weighed at 0 h underestimated true volume by about 5.8% and remained nearly unchanged as shell weight loss increased from 1 to 12% (Fig. 7A). Shells weighed at 24 h, however, estimated a cavity volume that was fairly close to true volume when shell weight loss was 1 to 2%, but as weight loss increased to 12%, cavity volume was overestimated by as much as 20% (Fig. 7B). The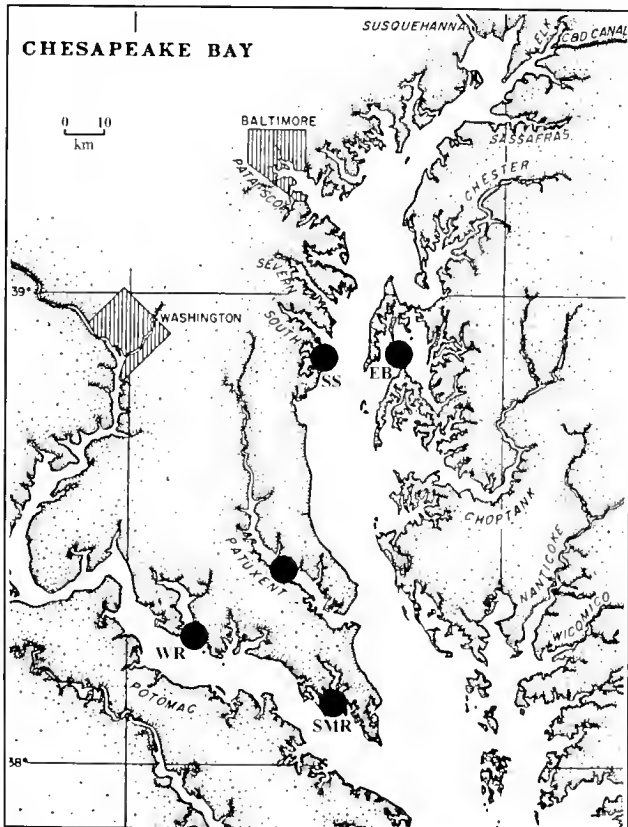 point at which the underestimation of cavity volume by 0-h shell weight equals the overestimate of cavity volume by the 24-h weight was at a shell weight loss of slightly <3%. Thus, 3% allowed a fairly conservative breakeven point, below which the 24-h weight gave better estimates of cavity volume and above which the 0-h weight gave better estimates.

Since cavity volume is the denominator in the MCI equation,

overestimated cavity volume results in underestimated meat condition by an equal amount. Although 0-h weights overestimated meat condition by about 5.8% (range −2 to 15%), they remained relatively constant over the range of weight loss from 1 to 12%. However, the 24-h shell weights underestimated meat condition by about 7.5% (range 10 to −20%), and the estimates became worse as the percentage of shell weight loss increased.

Meat condition determined gravimetrically was correlated with meat condition determined by casts for 169 oysters after 0 h (Fig. 8A) and after 24 h drying (Fig. 8B), and both gave significant correlations. The 24-h shell weights had a highly significant r^2 of 0.952 ($P < 0.001$), but the 0-h shell weights were slightly better with an r^2 of 0.979 ($P < 0.001$).

Because the oysters used in this analysis were all native to the Patuxent River, the possibility existed that oysters from elsewhere in the Chesapeake Bay might show different weight loss properties after drying for 24 h and might reduce the validity of using 0-h shell cavity volumes. In order for this technique to be valid elsewhere, the average shell weight loss after drying for 24 h should exceed 3%. For those areas examined, 24-h weight losses were >3% in all cases. Eastern Bay oysters lost 4.6%, Shady Side oysters lost 5.3%, the Wicomico River oysters lost 6.1%, and the St. Mary's River oysters lost 8.3%. All of these were greater than the 4.3% for Patuxent oysters, indicating that this technique would be valid in at least those areas, but probably in many other areas of the state, and perhaps other areas of the east coast, as well.

DISCUSSION

The value of gravimetric meat condition measurements has been demonstrated by Lawrence and Scott (1982) and Crosby and Gale (1990). Dry weight measured at 24 h provides an excellent estimate of cavity volume with comparisons between cavity capacity (in mL) by water displacement and cavity volume (in cm^3)
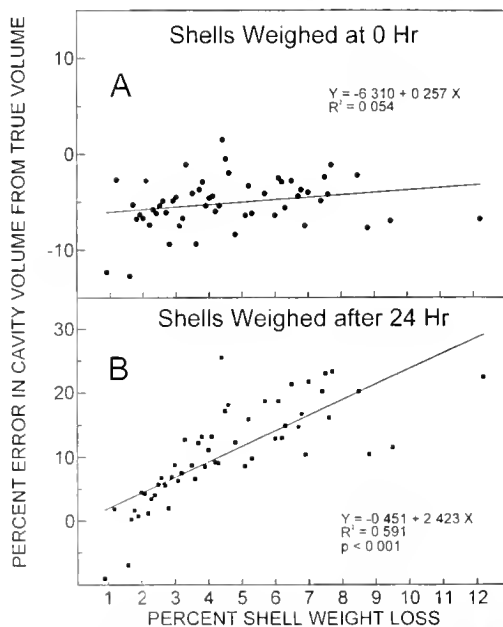

Figure 7. Linear regressions of the percentage error in cavity volume and the percentage shell weight loss for shells weighed at 0 h (A) and after 24 h (B).
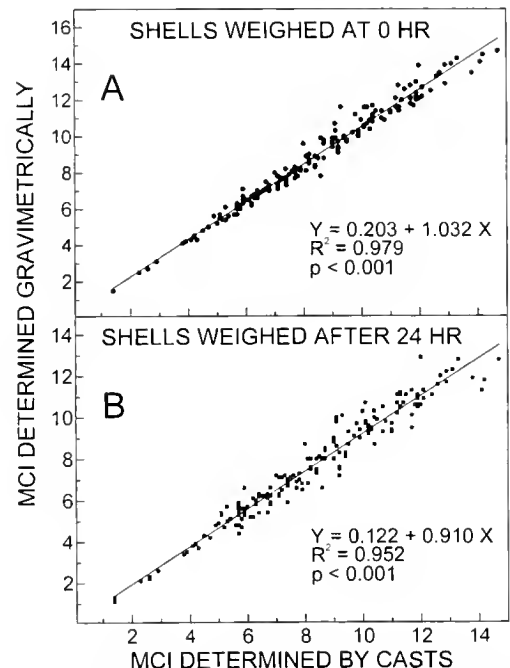

Figure 8. Linear regressions of MCI determined gravimetrically and by cast. For shells weighed at 0 h (A) the points fit the line slightly better than for shells weighed at 24 h (B).

Figure 9. An oyster with a smooth shell typical of one that will lose ≤3% of its shell weight after drying for 24 h. Insets show enlarged detail.

Figure 10. An oyster with a rough shell typical of one that will lose >3% of its shell weight after drying for 24 h. Insets show enlarged detail.

yielding correlation coefficients of 0.93 to 0.98 for oysters from three sites in South Carolina (Lawrence & Scott 1982). Our 24-h dry weights also yielded a correlation coefficient of 0.98, but 0-h dry weight coefficients reached 0.99 (Fig. 8A and B). Either method appears to yield a good estimate of meat condition, but since the water in the shell pores was weighed initially, there is no reason to exclude it in the second weight. The arbitrary removal of a variable amount of weight (water) artificially increases the volume (capacity) of the shell cavity, which in turn artificially and unnecessarily decreases the meat condition.

In addition to slightly increased accuracy, the use of 0-h weights means that all the shell weighing is completed within minutes. There is no need to allow shells to dry overnight and to return the next day to weigh them again.

While this technique appears to be an improved method for estimating the meat condition of *C. virginica* in much of the Maryland Chesapeake Bay, in terms of time and accuracy, it has not been tested elsewhere. The amount of water in the shell depends on porosity, which can be a function of oyster size, rate of growth, boring organisms, and shell structure. Small oysters generally lost less weight after 24 h, as a percentage of 0-h weight, than larger oysters because their shells had less time for shell-boring organisms to inhabit them. Oysters with smooth shells (Fig. 9) will often lose ≤3% because there are few places for water to enter the shell. Oysters with rough shells (Fig. 10) will generally lose >3% and

sometimes a great deal more (up to 13%). If the shells lose an average of just ≤3% of their weight from shell water, then a 24-h shell weight is the best estimator of cavity volume and thus of MCI. However, if shells lose >3%, then the 0-h weight proves to be the best estimator. It should be relatively easy to determine whether oysters from any particular area lose (on average) >3% or <3% of their shell weight upon drying for 24 h, and thus determine which method is more accurate for that site. Investigations of condition index have been conducted with *Crassostrea gigas* on the west coast (Schumacker et al. 1998, Brett Dumbauld, Washington Department of Fish and Wildlife, pers. comm.), and since the shell structure and porosity of *C. gigas* may differ from that of *C. virginica*, we await the results of these investigations.

ACKNOWLEDGMENTS

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EFFECTS OF OYSTER REEFS ON WATER QUALITY IN A TIDAL CREEK ESTUARY

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ABSTRACT The importance of oyster filtering in moderating aspects of water quality has received increased attention over the past several years. This study examined the influence of intertidal oyster reefs on chlorophyll *a*, fecal coliform bacteria, and total suspended solid concentrations under field conditions in a tidal creek estuary. Oyster reefs of varying live oyster density were sampled during summer 2002, winter 2003, and spring 2003. Water samples were taken upstream and downstream of each reef as well as over a mud flat control area on an ebb tide and analyzed for concentrations of these water column constituents. Summer data showed consistent, significant decreases in chlorophyll *a* concentrations as water moved over the reefs, usually by 10–25%. Fecal coliform counts were frequently lower downstream, by up to 45%, but were much more variable and not statistically different in most cases. Data taken in winter, when temperatures and oyster feeding rates were lower, showed less consistency in upstream versus downstream patterns. In spring, chlorophyll *a* decreases were less frequent than in summer, but significant fecal coliform decreases were more frequent. Total suspended solid concentrations were not changed by the presence of oyster reefs during any season. Data from this study indicate that feeding by oysters and changes in water flow caused by the presence of reefs may both play a role in reducing chlorophyll *a* and bacterial concentrations in the water column.

KEY WORDS: *Crassostrea virginica*, fecal coliform bacteria, chlorophyll *a*, tidal creek

INTRODUCTION

Increasing coastal populations and watershed development have led to concerns over water quality for both shellfishing and human contact waters. Among the water quality concerns in coastal areas are water-borne pathogens, eutrophication, increased turbidity, and sediment loads. Nutrients, sediments, and pathogens enter natural water bodies through runoff and can have both human health and ecosystem-level impacts.

Microbial pathogens, particularly those from human and animal feces, can pose concerns for human health (Grimes 1991). Fecal coliform bacteria, indicators of pathogens associated with human and animal wastes, have been shown to be positively correlated with impervious surface cover in a watershed as well as with nitrate and orthophosphate concentrations (Mallin et al. 2000) and turbidity (Pommepuy et al. 1992, Mallin et al. 2000), and inversely correlated with salinity (Goyal et al. 1977, Mallin et al. 1999, Mallin et al. 2000). Suspended solids and turbidity can contribute to survival and even growth of fecal coliform bacteria by providing protection from light, an organic substrate, and a mechanism for transport downstream (Gerba & McLeod 1976, Pommepuy et al. 1992, Sayler et al. 1975). Rainfall events have also been correlated with increases in fecal coliform concentrations (Goyal et al. 1977, Struck 1988, Howell et al. 1995) due to runoff inputs.

Increasing sedimentation and turbidity are concerns not only for their role in the survival of fecal coliforms, but also because of their effects on water column irradiance. Suspended solids and turbidity can prevent light from penetrating the water column and thus can negatively impact the growth of primary producers such as rooted aquatic macrophytes, benthic microalgae, and phytoplankton (Cordone & Kelley 1961). Benthic community structure, including the occurrence of shellfish beds, can be affected through burial by sediments and interference with filter feeding (Loosanoff & Tommers 1948, Posey 1990, Shumway 1996).

Eutrophication, caused mainly by nutrient loading, can also have detrimental effects on ecosystems (Nixon 1995, Bricker et al. 1999). Direct effects of eutrophication include initial increases in chlorophyll and primary production, changes in phytoplankton and macroalgal communities, and loss of seagrass (Burkholder 2001, Cloern 2001). Indirect effects include changes in water transparency, nutrient cycling, benthic communities, and food web structure (Cloern 2001, Posey et al. 2002). These effects are moderated by system attributes, with some areas being more sensitive to nutrient loading than others (Cloern 2001, Posey et al. 2002).

In response to the potential deterioration of water quality associated with watershed development, natural measures are being examined as possible remediation techniques. Several recent studies have concentrated on the role of bivalves in regulating suspended particulate loads in estuarine systems. Models based on laboratory studies of bivalve filtration rates predict that bivalves, when sufficiently abundant in shallow waters, can control phytoplankton biomass (Cloern 1982, Officer et al. 1982, Gerritsen et al. 1994). These models, however, are often based on high estimates of feeding rates from laboratory trials and fail to take into account variability in bivalve feeding rates under field conditions or bivalves' release of nutrients, which could actually stimulate phytoplankton growth. Oyster feeding rates can be affected by temperature, salinity, suspended solid concentrations, and other factors (Shumway 1996). While filter feeding is hypothesized to remove substantial amounts of particulate matter, removal may also be caused by physical effects of oyster reefs on water flow (Dame 1987). The presence of reefs can cause eddies and turbulence, which lead to the settling of fine particles.

Field studies regarding removal of particulate matter by oyster reefs are somewhat limited. Dame et al. (1984, 1985, 1989) and Dame & Dankers (1988) found significant decreases in total organic carbon, particulate organic carbon, total suspended solids, nitrite+nitrate, and chlorophyll *a*. Ammonium concentrations increased downstream of oyster reefs, suggesting a role for oyster reefs in nutrient cycling (Dame et al. 1984, 1985, 1989; Dame & Dankers 1988; Nelson et al. 2003). In one study, tidal creeks with oysters did not show significantly lower chlorophyll *a* levels than

creeks without oysters (Dame & Libes 1993); however, another study found significantly lower chlorophyll *a* (especially phototrophic flagellates) in creeks with oysters (Wetz et al. 2002).

The eastern oyster, *Crassostrea virginica* (Gmelin), is a filter feeder that is widely believed to reduce the amount of particulate matter in the water column. Field evidence to support this idea is limited, however, and no field tests of fecal coliform reductions over oyster reefs have been published. The research described here assessed the impacts of intertidal oyster reefs on suspended solids, chlorophyll *a*, and fecal coliform bacteria in a human-impacted tidal creek and also examined whether live oyster density over natural ranges influenced rates of seston removal.

MATERIALS AND METHODS

Study Site

Six natural, intertidal oyster reefs were examined in Hewletts Creek, southeastern North Carolina. Hewletts Creek is an anthropogenically impacted tidal creek with a watershed that is approximately 70% developed, with 18% impervious surface coverage (Mallin et al. 2000). The reefs used in this study were bar reefs approximately 10 m wide and were selected to provide a gradient of ambient live oyster density from "low" (79 live oysters m^{-2}) to "high" (167 live oysters m^{-2}: Table 1) based on live densities available in the study area. Because the amount of shell hash covering oyster reefs may contribute to physical effects on water flow, reefs with different amounts of shell cover were used. Two of the reefs had low dead shell cover (approximately 60–80% of the reef consisted of live oysters, and the rest of the substrate was exposed sediment); the others were completely covered by live and dead shell. All reefs were located near a channel in the creek to ensure sufficient flow and were at least 5 m distant from other reefs. Reefs were not located immediately adjacent to marsh, thus reducing potential effects of sedimentation associated with marshes. A mud flat area immediately upstream of the selected reefs was used as a no-oyster control. The mud flat area lacked any shell cover, was more than 20 m distant from oyster reefs, and was dominated by sediment of similar grain size (fine sands) as that adjacent to the studied oyster reefs. The vertical height and vertical complexity of each reef were measured, as they may impact physical effects such as flow velocity (Lenihan 1999, Posey & Alphin, unpubl. Table 1). Reef height was measured while water covered the crest of the reef by recording the depth of water over the crest and subtracting this from the depth of water covering the edges of the reef. Vertical complexity was calculated by allowing a 1 m long chain to conform to the vertical contours of the reef and measuring the actual horizontal distance covered by the chain. Complexity was quantified as the ratio of straight distance after conforming to the contours divided by 1 m. Values for complexity range from 0 to 1, with smaller values indicative of higher complexity.

Because flow speed can affect bivalve growth and filtration (Lenihan et al. 1996) as well as sediment deposition, it was important to characterize the flow regimen of each reef in this study. Flow measurements were taken with a Marsh McBirney, Inc., (Frederick, MD) Flo-mate Model 2000 handheld current meter once in the summer and during sample collection in winter and spring. Further, because oyster reefs may cause settling of fine particles, it was desirable to determine whether sediment composition was different upstream versus downstream of the reefs in this study. Sediment samples were taken at approximate upstream and downstream water column sampling locations during a low tide in June 2003, and grain size fractions were determined using a Beckman LS Coulter Counter (Miami, FL).

Sampling

Fecal coliform and chlorophyll *a* concentrations in tidal creeks have been shown to be highest at approximately mid-to-low tide (Mallin et al. 1999). Additionally, significant decreases in chlorophyll *a* concentrations downstream of a created oyster reef near the study area were observed 3 h after high tide (Nelson et al. 2003). To increase the likelihood of detecting effects, water samples were taken as close as possible to mid-ebb tide (generally about 2 h after high tide). Samples were taken from a canoe to avoid disturbing sediment. All sampling was conducted on ebb tides with a predicted range of 0.9–1.1 m after a high tide of approximately 1 m. Water depth was less than 35 cm on the upstream and downstream sides of the reef at the time of sampling and only a few cm of water were present over the crest, thereby maximizing the amount of water that came into contact with the oysters.

Samples were taken at two locations upstream and two locations downstream of each reef. The two upstream samples were approximately 1 m apart from each other, as were the downstream samples. Upstream samples were taken at mid-depth in the water column. Because dye studies conducted prior to sampling showed that water from mid-depth flowed up over the crest of the reef and stayed near the surface, downstream samples were taken just under the surface of the water. Downstream samples were taken before upstream samples to avoid the collection of sediments that had been stirred up by prior sampling. For the same reason, the first reef sampled in a day was downstream of the second reef.

Sampling of the six reefs, as well as a mud-bottom control area,

TABLE 1.

Physical characteristics of oyster reefs used in the study. Live oyster densities (m^{-2}) were measured in Summer 2002 and Spring 2003. Also indicated is % shell cover, which is indicative of the amount of dead shell covering the reef. Width is the distance water traveled over the reef between upstream and downstream sampling locations; height is the vertical difference between the crest and base of the reef.

Reef	Summer Density (per m^{-2})	Spring Density (per m^{-2})	% Shell Cover	Length (m)	Width (m)	Height (m)	Vertical Complexity
1	79	132	100	14.5	13.5	0.29	0.68
2	113	129	100	10.0	15.0	0.15	0.64
3	114	150	60	13.0	8.0	0.40	0.68
4	116	163	80	13.0	9.5	0.50	0.75
5	129	176	100	13.0	8.0	0.30	0.70
6	167	183	100	17.7	5.5	0.65	0.73

was accomplished over a period of three days during each sampling period, with two reefs sampled per day. Sampling was conducted twice per season during summer 2002 (once in July and once in August) and spring 2003 (twice in May, approximately two weeks apart). Due to low concentrations of water column constituents as well as weather limitations, only one sampling set was conducted in winter 2003 (February). Sampling within 24 h of rain was avoided due to potential effects of storm water runoff on water column constituents. In winter, however, there were such low concentrations of the water column constituents of interest that it was necessary to sample after a rain event, in addition to the scheduled sampling period, to have sufficiently high chlorophyll a and fecal coliform concentrations to allow detection of potential effects. The two reefs with the highest live-oyster density and the mud flat control area were all sampled the day after a rainfall of approximately 3 cm in February 2003.

Chlorophyll a samples were taken in triplicate into 125-mL opaque plastic bottles. A fourth bottle was used to ensure collection of enough water for total suspended solids (TSS) analysis. Fecal coliform samples were collected using autoclaved 500-mL glass bottles. All samples were kept on ice until they were filtered. Water remaining after filtration of fecal coliforms and chlorophyll a was combined and stored at 4°C until it could be used in analysis of TSS. Originally, this project was intended to focus on changes in turbidity rather than TSS. However, initial attempts to measure turbidity met with methodologic difficulties, and TSS analysis was added to the study in the second summer sampling period.

Sample Processing

Fecal coliform and chlorophyll a samples were filtered upon return to the laboratory and within 6 h of collection. Fecal coliform bacteria concentrations were determined according to the membrane filter procedure, using mFC medium (Sparks, MD) (APHA 1995). Chlorophyll a samples were filtered through Gelman (Clifton, NJ) A/E glass fiber filters with 1.0 μm pore size. The filters were wrapped individually in aluminum foil and frozen in a sealed container with desiccant. Concentrations were determined fluorometrically (Welschmeyer 1994) within three weeks. TSS were analyzed gravimetrically (APHA 1995) using 500 mL of water from each sampling location. TSS were filtered through predried Gelman A/E 47 mm diameter glass fiber filters with 1.0 μm pore size.

Statistical Analysis

The parameters of chlorophyll a and fecal coliform concentrations were tested for normality and nonheterogeneity of variances. Variances upstream and downstream of reefs were nonheterogeneous for both parameters. However, neither showed a normal distribution, even after standard transformations, leading to the use of nonparametric tests. Kruskal–Wallis tests were used (Sokal & Rohlf 1995) to test upstream versus downstream concentrations of the sampled variables and to determine whether they were significantly different across each individual reef for each sampling period. In all other analyses, which involved concentration changes of variables and not the non-normally distributed concentrations themselves, parametric methods were used. Multiple regression was used to determine whether the concentration changes of the studied variables were related to live oyster density, mean upstream flow speed, tidal range, and the time elapsed between high tide and actual sampling. An ANOVA was used to test for differences between the high-shell and low-shell reefs of the same live

oyster density. A t-test was used to test for overall reef effects within a season (i.e., did the reefs show consistently decreased concentrations downstream?). All analyses used SAS (SAS Institute, Inc. 1989).

RESULTS

Summer

Mean chlorophyll a concentrations ranged from 2.3–10.6 μg L^{-1} over the reefs and mud flat during the summer sampling periods. Mean fecal coliform concentrations ranged from 1.3–54.8 colony forming units (CFU) 100 mL^{-1}. Total suspended solid concentrations ranged from 10–27 mg L^{-1}. Temperature was approximately 25–27 C and salinity ranged from 30–36 ppt at the study site during these sampling periods.

Chlorophyll a was significantly lower downstream of reefs than upstream in summer for 9 of 12 comparisons (two comparisons for each of the six reefs; Table 2). This overall reef effect was significant for all reefs combined ($P = 0.002$), for high-shell-cover reefs ($P = 0.023$) and for low-shell-cover reefs ($P = 0.053$). Each reef demonstrated a significant decrease in chlorophyll a at least one of the two times it was sampled over the summer. There was no significant difference in percent removal of chlorophyll a between the high-shell-cover and low-shell-cover reefs of the same live oyster density ($P = 0.52$). The control was sampled only once during summer, and at that time chlorophyll a was significantly lower downstream than upstream ($P = 0.010$). Changes in chlorophyll a concentrations were not significantly related to live oyster density (Fig. 1A) or tidal range.

Fecal coliform concentrations were often lower downstream of reefs than upstream (8 out of 12 comparisons), although only two differences were statistically significant and there was not a significant overall reef effect ($P = 0.22$). Fecal coliform concentrations were higher downstream on the mud flat than upstream, but this difference was not significant. Changes in fecal coliform concentrations were not significantly related to live oyster density (Fig. 1B) or tidal range. There was no significant difference in percent fecal coliform removal between the high-shell and low-shell reefs of the same live oyster density ($P = 0.86$).

Because of difficulties encountered when measuring turbidity, TSS concentrations were added to sampling during the second summer sampling period. There were three instances of lower (24–38%) TSS concentrations downstream of reefs, two instances of higher (25–43%) concentrations downstream, and one instance with very little change. The mud flat showed no change in TSS concentration. Due to a lack of replication (only two samples upstream and two downstream), no statistical test could be run on the differences across each reef or the mud flat. There was no significant overall reef effect on TSS concentrations ($P = 0.44$). Changes in TSS concentrations were not significantly related to live oyster density (Fig. 1C) or tidal range, and percent change was not significantly different between the high-shell and low-shell reefs of the same live oyster density ($P = 0.80$).

Winter

Mean chlorophyll a concentrations ranged from 0.3–1.5 μg L^{-1} over the reefs and control during the winter sampling period. Mean fecal coliform concentrations ranged from 0.2–8.0 CFU 100 mL^{-1} over the reefs and 22.5–36.7 CFU 100 mL^{-1} over the nonreef mud flat area (control). Temperature was approximately 4°C and salin-

TABLE 2.

Results of Kruskal–Wallis tests on upstream vs. downstream concentrations of chlorophyll a (chl) and fecal coliform bacteria (fc) concentrations. Significant differences are in bold. All significant changes were reductions (lower downstream) except for one, designated with a. Each reef was sampled twice in summer 2002 and spring 2003 and once in winter 2003. The mudflat was sampled only once in summer, and reef 6 was sampled twice in winter.

Reef	Season	Parameter	df	Chi-square	K-W P-Value
1	Summer 1	chl	10	5.810	**0.016**
	Summer 2	chl	10	0.819	0.366
	Winter	chl	5	3.667	0.056
	Spring 1	chl	10	0.850	0.357
	Spring 2	chl	10	9.000	**0.003**
	Summer 1	fc	9	3.427	0.064
	Summer 2	fc	10	0.315	0.575
	Winter	fc	10	4.046	**0.044**
	Spring 1	fc	8	0.099	0.753
	Spring 2	fc	10	0.660	0.417
2	Summer 1	chl	9	0.138	0.711
	Summer 2	chl	10	8.768	**0.003**
	Winter	chl	10	1.000	0.317
	Spring 1	chl	10	1.169	0.280
	Spring 2	chl	10	4.373	**0.037**
	Summer 1	fc	10	6.322	**0.012**
	Summer 2	fc	10	1.664	0.197
	Winter	fc	9	0.222	0.637
	Spring 1	fc	10	0.523	0.470
	Spring 2	fc	10	0.241	0.624
3	Summer 1	chl	10	8.366	**0.004**
	Summer 2	chl	7	5.492	**0.019**
	Winter	chl	10	4.083	**0.043**
	Spring 1	chl	10	8.366	**0.004**
	Spring 2	chl	9	5.307	**0.021**
	Summer 1	fc	10	0.007	0.934
	Summer 2	fc	8	0.702	0.402
	Winter	fc	10	2.898	0.089
	Spring 1	fc	10	6.657	**0.010**
	Spring 2	fc	10	3.718	0.054
4	Summer 1	chl	10	8.426	**0.004**
	Summer 2	chl	10	4.790	**0.029**
	Winter	chl	10	5.978	**0.015**
	Spring 1	chl	10	4.333	**0.037**
	Spring 2	chl	10	4.889	**0.027**ᵃ
	Summer 1	fc	10	0.058	0.810
	Summer 2	fc	9	0.533	0.465
	Winter	fc	10	0.946	0.331
	Spring 1	fc	10	0.410	0.522
	Spring 2	fc	10	0.235	0.628

TABLE 2.

(Continued).

Reef	Season	Parameter	df	Chi-square	K-W P-Value
5	Summer 1	chl	10	8.396	**0.004**
	Summer 2	chl	10	8.640	**0.003**
	Winter	chl	10	0.000	1.000
	Spring 1	chl	10	1.331	0.249
	Spring 2	chl	10	5.843	**0.016**
	Summer 1	fc	10	2.857	0.091
	Summer 2	fc	10	0.103	0.749
	Winter	fc	10	0.244	0.622
	Spring 1	fc	8	2.455	0.117
	Spring 2	fc	10	0.026	0.871
6	Summer 1	chl	10	2.929	0.087
	Summer 2	chl	10	5.810	**0.016**
	Winter 1	chl	10	3.008	0.083
	Winter 2	chl	10	1.637	0.201
	Spring 1	chl	10	0.232	0.630
	Spring 2	chl	10	0.164	0.686
	Summer 1	fc	9	1.656	0.198
	Summer 2	fc	9	7.569	**0.006**
	Winter 1	fc	10	5.507	**0.019**
	Winter 2	fc	10	0.007	0.933
	Spring 1	fc	10	6.564	**0.010**
	Spring 2	fc	10	1.713	0.191
Mudflat	Summer	chl	10	6.610	**0.010**
	Winter	chl	10	3.209	0.073
	Spring 1	chl	10	0.058	0.810
	Spring 2	chl	10	3.274	0.070
	Summer 2	fc	9	0.307	0.580
	Winter	fc	10	6.587	**0.010**
	Spring 1	fc	10	5.043	**0.025**
	Spring 2	fc	10	0.000	1.000

ity ranged from 17–35 ppt at the study site during this sampling period. Turbidity was very low, ranging from 1.5–5.0 NTU, and TSS concentrations ranged from 1.8–7.5 mg L⁻¹.

Because concentrations of the studied water column constituents were so low, the two highest live-oyster-density reefs (both with high dead shell cover) and the mud flat were also sampled after approximately 3 cm of rain, when the creek water level was higher than normal. After this rain event, mean chlorophyll a concentrations ranged from 1.8–2.6 µg L⁻¹ and mean fecal coliform concentrations were approximately 146–516 CFU 100 mL⁻¹. Temperature was 4°C and salinity ranged from 15–29 ppt among sites

on the same day. Water flow speed was higher than normal after the rain event. This was due partly to a larger tidal range than was normally sampled (1.5 m versus a usual range of 0.9–1.1 m) as well as flow effects from storm water runoff. Turbidity was comparable to spring and summer turbidity, ranging from 7.8–12.5 NTU. TSS concentrations were 9.0–15.4 mg L⁻¹.

During the regular winter sampling period, there were 2 significant decreases ($P < 0.05$) in chlorophyll a concentrations over the reefs (Table 2). These differences were observed over low-shell-cover reefs, but there was not a significant difference between these reefs and the high-shell-cover-reef of the same density ($P = 0.564$). A t-test did not show a significant overall reef effect on this variable for all reefs combined ($P = 0.691$), for high-shell-cover reefs ($P = 0.582$), or for low-shell-cover reefs ($P = 0.323$). Over the mud flat, there was no significant change in chlorophyll a. Changes in chlorophyll a in winter were not significantly related to live oyster density (Fig. 2A), mean flow speed upstream of the reefs, or change in flow speed. After the rain event, both reefs and the mud flat showed slight, nonsignificant increases in chlorophyll a.

In the regular winter sampling period, fecal coliforms were lower downstream than upstream five times (out of seven comparisons: the highest-density reef was sampled twice in winter), but this overall reef effect was not significant for all reefs combined ($P = 0.26$), for high-shell-cover reefs ($P = 0.22$), or for low-shell-cover reefs ($P = 0.86$). Two of the fecal coliform decreases were significant, and these occurred over the highest-
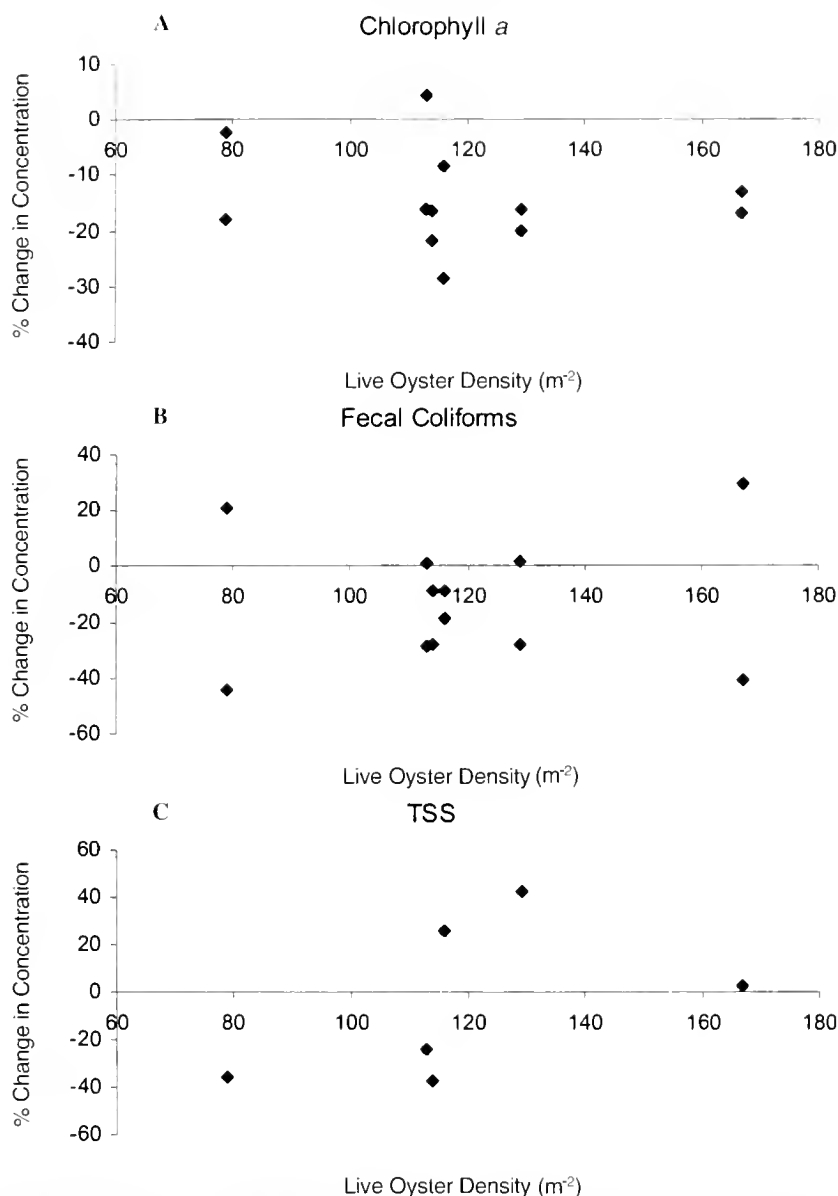
Figure 1. Water column constituents as related to live oyster density, summer 2002: Percent changes in chlorophyll *a*, fecal coliforms, and TSS. Negative numbers represent a lower concentration downstream of the reef than upstream.

density reef ($P = 0.019$) and the lowest-density reef ($P = 0.044$; Table 2). Fecal coliform concentrations significantly decreased over the mud flat ($P = 0.010$) during this sampling period. Changes in fecal coliform concentrations were not correlated with live oyster density (Fig. 2B), upstream flow speeds, or changes in flow. There was no significant difference between percent change in fecal coliform concentrations between the high-shell and low-shell reefs of the same live oyster density ($P = 0.67$).

After the rain event, fecal coliform concentrations were elevated above nonrain conditions. Due to crowding of the petri dishes on which the bacteria were grown, the counts were not considered reliable enough for statistical analysis. However, it was apparent that fecal coliform concentrations were highest over the mud flat (approximately 400 CFU 100 mL^{-1}), lower over the highest-density reef, which was slightly downstream of and adjacent to the mud flat (approximately 360 CFU 100 mL^{-1}), and

lowest over the most downstream reef (approximately 180 CFU 100 mL^{-1}).

During the regular winter sampling period, TSS concentrations were higher (25–36%) downstream of reefs as compared with upstream on three occasions. TSS concentrations were moderately lower (10%) once, and twice were only slightly (<5%) lower downstream. Given the low TSS concentrations during this sampling period, however, an increase of <1 mg L^{-1} could translate to a 30% change. There was no significant overall reef effect on concentration changes ($P = 0.252$). Upstream to downstream changes in TSS concentrations were not significantly related to live oyster density (Fig. 2C), flow speed of water upstream of the reefs, or changes in flow speed during the winter sampling period. There was no significant difference in TSS change between high-shell and low-shell reefs of the same live oyster density ($P = 0.744$). TSS concentrations were 0.7% higher downstream than
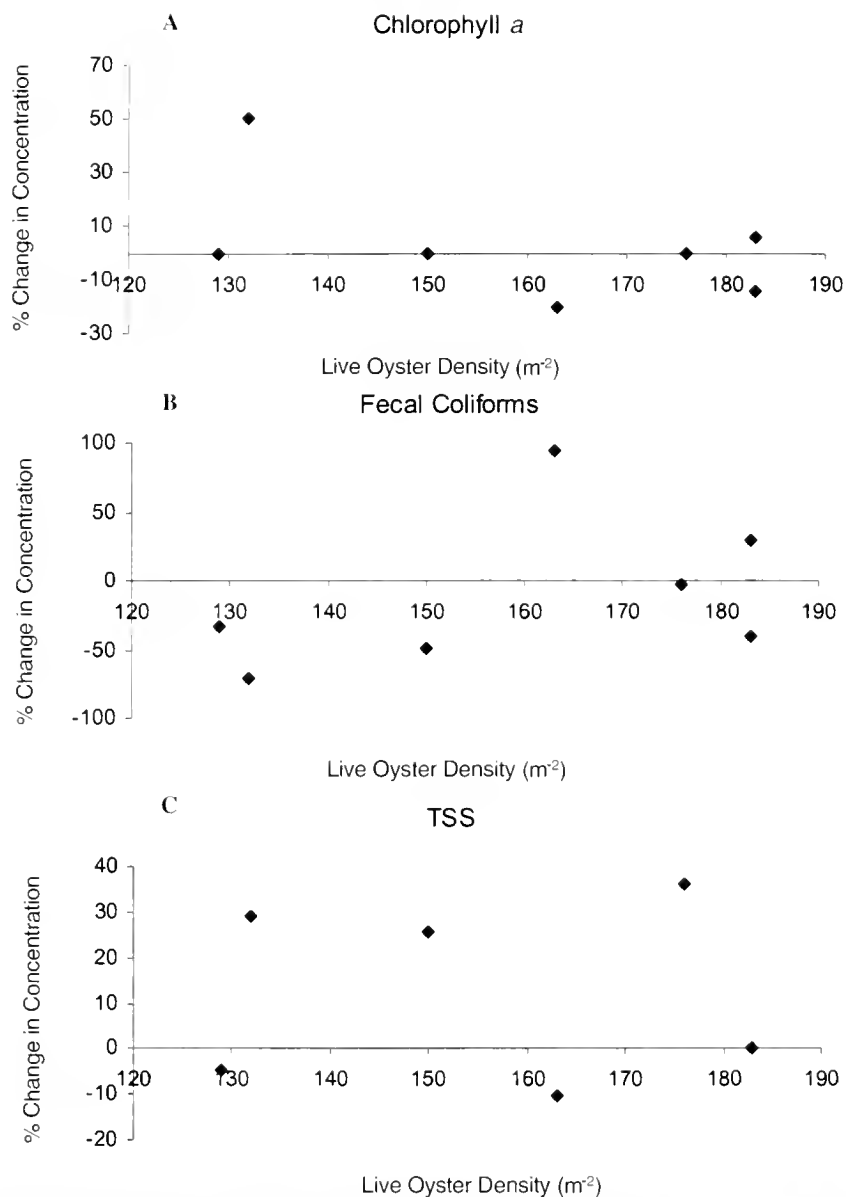
Figure 2. Water column constituents as related to live oyster density, winter 2003: Percent changes in chlorophyll *a*, fecal coliforms, and TSS. Negative numbers represent a lower concentration downstream of the reef than upstream.

upstream over the highest-density reef after the rain event, but were 30% higher over the second-highest-density reef. On the mud flat, TSS concentrations were approximately 11% lower downstream.

Spring

Mean chlorophyll *a* concentrations ranged from 1.3–7.1 μg L^{-1} over the reefs and 2.0–12.2 μg L^{-1} over the mud flat during the spring sampling period. Mean fecal coliform concentrations ranged from 8–330 CFU 100 mL^{-1} over the reefs and mud flat. Fecal coliform counts were higher during the first spring sampling period than the second due to a long rainy period preceding sampling. Samples were not taken within 24 h of rain, but the earlier rain did affect the water column. Temperature was approximately 24 C, and salinity ranged from 19–25 ppt during the first spring sampling and 30–34 ppt during the second spring sampling period. Turbidity ranged from 5.8–9.8 NTU over both spring sampling periods.

In spring, there were six significant decreases and one significant increase in chlorophyll *a* concentrations across the reefs (Table 2). There was not an overall reef effect on chlorophyll *a* concentrations for all reefs combined ($P = 0.18$), for high-shell-cover reefs ($P = 0.19$), or for low-shell-cover reefs ($P = 0.28$). Chlorophyll *a* changes also were not significantly related to live oyster density (Fig. 3A), flow speed upstream of the reefs, change in flow speed, or how long after the high tide samples were taken. There was no significant difference in percent removal of chlorophyll *a* between high- and low-shell-cover reefs of similar live oyster density ($P = 0.45$).

Ten of 12 comparisons showed fecal coliform concentrations that were lower downstream than upstream in spring. Three of these decreases were significant (Table 2), as was the overall reef effect ($P = 0.009$). The mud flat showed a significant ($P = 0.025$) downstream decrease in fecal coliforms during one of the two spring sampling periods. Changes in fecal coliform concentrations were not significantly related to live oyster density (Fig. 3B), flow
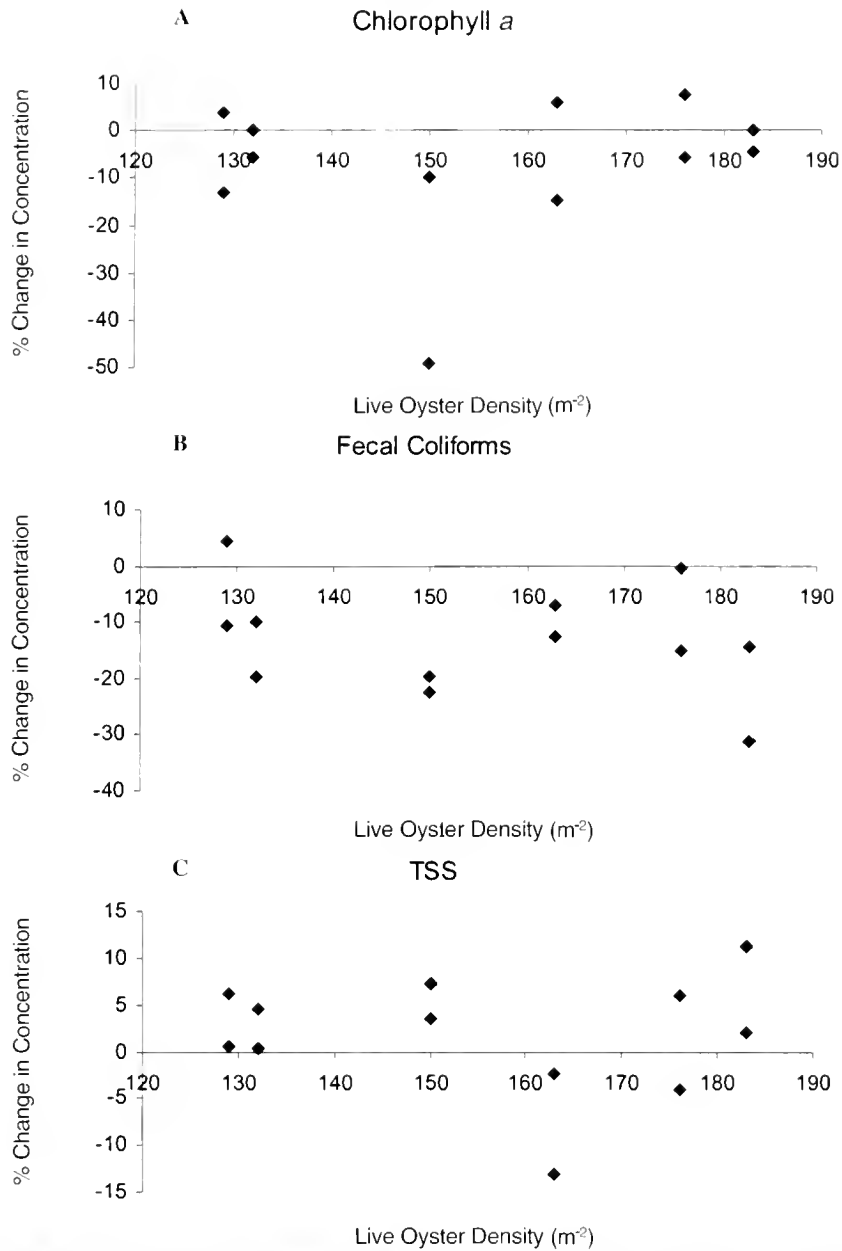
Figure 3. Water column constituents as related to live oyster density, spring 2003: Percent changes in chlorophyll *a*, fecal coliforms, and TSS. Negative numbers represent a lower concentration downstream of the reef than upstream.

speed upstream of reefs, or changes in flow. A *t*-test did show significantly decreased fecal coliform concentrations downstream of oyster reefs in spring for all reefs combined ($P = 0.009$). High-shell-cover reefs did not show this overall effect ($P = 0.10$); the pattern was driven by the low-shell-cover reefs ($P = 0.012$). However, high- and low-shell-cover reefs of similar live oyster density did not show significantly different patterns of fecal coliform removal in spring ($P = 0.16$).

TSS did not exhibit a significant pattern with respect to the variables examined in spring. Out of 12 comparisons, downstream TSS concentrations were higher seven times, lower three times, and unchanged twice. There was not a significant overall reef effect on TSS concentration changes ($P = 0.29$). TSS concentrations were higher downstream once over the mud flat and remained unchanged during the other spring sampling period. The observed changes in TSS concentrations were not significantly

related to live oyster density (Fig. 3C), water flow speed upstream of the reefs, or changes in flow. Percent removal of TSS was not significantly different between high- and low-shell-cover reefs of the same live oyster density ($P = 0.54$).

Overall

During the warm seasons of summer and spring, chlorophyll *a* was significantly lower downstream of reefs than upstream a total of 13 times (out of 24 observations). Only once was it significantly higher. In summer, chlorophyll *a* concentrations were significantly lower downstream of oyster reefs than upstream ($P = 0.002$) overall. In spring, however, there was no significant overall reef effect. Fecal coliforms were reduced the majority of the time during the warm seasons (18 of 24 comparisons), but only 4 of these decreases were statistically significant. In summer, this overall reef

effect was not statistically significant, but it was significant in spring ($P = 0.009$).

Water flow varied somewhat from reef to reef. The lowest observed flow over the parts of the reef from which samples were taken was 6 cm s^{-1}. Flow velocity reached 22 cm s^{-1} over the other reefs. The three-dimensional current study showed increases in flow speed over the crest of three of the reefs and decreases over the crests of the other three reefs. However, differences in flow speeds between reefs were not significantly related to changes in the water column constituents. Vertical complexity did not differ among the reefs (Table 1). Over five of the six reefs, downstream sediments contained a greater amount of coarse sediment than upstream (by 8–12%; Table 3). The mud flat did not exhibit the same distribution of sediment texture.

DISCUSSION

The presence of oyster reefs caused significant reductions in chlorophyll a and fecal coliform bacteria concentrations in this study. Effects on chlorophyll were greatest in summer, whereas effects on fecal coliforms were strongest in spring when bacterial counts were highest. The decreases in chlorophyll concentrations were consistent with previous studies showing that bivalve beds can have significant effects on the overlying water column (Dame et al. 1984, 1985, 1989; Asmus & Asmus 1991), and there has not been any previous investigation regarding effects of oyster reefs on fecal coliform concentrations. In this study, oyster reefs did not have any clear, consistent effects on TSS concentrations.

Haven and Morales-Alamo (1970) found that a doubling of the number of oysters in an experimental tank led to an approximate doubling of removal rates of particulate matter. Changes in suspended particulate concentrations, then, should be significantly related to live oyster density if oyster feeding is the sole or over-riding factor in particulate removal. In this study, such a relationship was not observed. One possible explanation for this observation is a threshold effect, some critical density of live oysters at which a measurable effect can be detected. Alternatively, the relationship between changes in seston and live oyster densities may be detected only over a greater density range and spatial scale. The oyster reefs used in this study provided only a small range of live oyster densities, especially after a large spatfall in summer 2002 (Posey & Alphin, unpubl.). Thus, the examined range of live oyster densities may have been too narrow for a density relationship to be detected. Because the changes in concentrations of the studied water column constituents were not significantly related to flow speeds or changes in flow speed across the reefs, it is

TABLE 3.

Sediment composition, as % fine sediment (defined as less than 63.41 μm diameter), upstream and downstream (ebb tide) of the oyster reefs.

Reef	% Fine Upstream	% Fine Downstream
1	40	32
2	38	21
3	72	70
4	85	78
5	41	30
6	64	51
Mudflat	40	41

unlikely that the observed changes were due solely to flow speed.

Live oyster lengths near the study site averaged 65 mm (Harwell, Posey & Alphin, unpubl.). Using the methods of Dame (1972), the mean dry weight for these oysters was calculated to be 1.33 g. Newell's (1988) estimate of oyster clearance rates of 5 L h^{-1} g^{-1} were used to calculate the potential volume of water that could be cleared by each oyster reef in this study. In summer, flow velocities upstream of the oyster reefs ranged from 6–21 cm s^{-1}, and the oysters on the reefs could potentially clear only 5–15% of the water moving over them. Many of the observed chlorophyll a differences in summer were greater than the potential filtration capacity of the oysters on the reefs based on these estimates (up to 30% removal), suggesting that either oyster feeding rates are higher than Newell's (1988) estimate or that factors other than oyster feeding (i.e., other filter feeders or physical effects) are important in particulate removal.

Additional calculations of approximate clearance rates, assuming 100% efficiency of particle removal, were made using the observed summer decreases in chlorophyll a concentrations. These rates ranged from 3–18 L h^{-1} g^{-1} across the reefs. The mean was 10 L h^{-1} g^{-1}, which is consistent with Jordan's (1987) laboratory estimate. Oysters do not remove all particles from water with 100% efficiency, however, so this estimate may be conservative.

Other filter feeders, such as mussels, were not abundant on these oyster reefs and therefore cannot account for the larger than expected effects. Even though flow velocities did not decrease downstream of the reefs, particle trapping within the reef crest may have occurred in flow shadow zones between oyster clumps. This explanation is consistent with chlorophyll a and fecal coliform data in that the reefs that consistently showed significant decreases in chlorophyll a and fecal coliform concentrations were the reefs with low shell cover (i.e., low areas floored by mud). These were also the reefs with the lowest flow velocities (approximately 8 cm s^{-1}). Dame et al. (1985) and Dame (1987) found that most material uptake over an oyster reef in North Inlet occurred when flow was less than 15 cm s^{-1} and attributed this to a combination of biofiltration and sedimentation. Lower flow speeds could contribute to removal of particles by increasing the time water is in contact with the oysters and thus increasing their ability to filter particulates; it could also be that particles settled out of the water at these lower speeds. Preferential ingestion of chlorophyll (microalgae) by oysters (Ward et al. 2000, Wetz et al. 2002) may interact with low flow velocities to produce the strongest effects on this parameter, consistent with the significant reduction in chlorophyll concentrations over these reefs but low influence on TSS concentrations.

Oyster reefs have been shown to play a role in nutrient cycling in tidal creeks by releasing NH$_4^+$ (Dame et al. 1984, 1985, 1989; Dame & Dankers 1988; Nelson et al. 2003). As such, it could be argued that chlorophyll a concentrations should actually be higher downstream of reefs than upstream. Ammonium released by bivalves can be taken up by phytoplankton and lead to increased phytoplankton biomass. Asmus and Asmus (1991) made this argument for systems impacted by a mussel bed, though their field study showed significant decreases in phytoplankton biomass across the bed. Increased phytoplankton production due to nutrient release is also a possibility for oyster reefs. However, there is a lag time of a few hours before the ammonium shows up as primary production in the water column, and any increased production may be appearing further downstream of the reefs than the location of sample collection for this study. In terms of the parameters examined in this study, the only change that would be immediate

enough to detect as water flows over the oyster reefs is particle removal.

Fecal coliform concentrations were often lower downstream of reefs than upstream, but the differences were rarely significant. The overall reef effect of decreased fecal coliform concentrations was significant in spring but not summer, the opposite of the effect for chlorophyll *a*. Fecal coliform counts are extremely variable, necessitating large changes before a significant effect can be detected. Because fecal coliform concentrations were higher in spring than in summer and winter, differences were slightly more detectable.

C. virginica filters unattached bacteria with an efficiency of only 5% (Langdon & Newell 1990). However, fecal coliforms have been associated with turbidity and suspended sediments in the water column (Sayler et al. 1975, Pommepuy et al. 1992, Mallin et al. 2000) and may be removed with suspended particulate matter through either filtration or settling. In this study, fecal coliform counts did not have consistent relationships with either turbidity or TSS. Changes in fecal coliform concentrations were not significantly related to live oyster density, flow speeds, or changes in flow speed across the reefs. None of these factors is readily apparent as the most influential one, and changes in fecal coliform concentrations are likely due to a combination of factors.

Changes in TSS concentrations did not exhibit any significant patterns relative to the variables examined in this study. Due to a lack of replication, statistical tests could not be used to determine whether changes across a single reef were significant. However, tests could be run to detect overall reef effects within a season, and none of these were significant for TSS. Changes in TSS were not consistently positive or negative in any season.

Water temperature in winter was 4 C, lower than the minimum temperature (5 C) at which oysters typically feed (Galtsoff 1928, in Shumway 1996). Chlorophyll *a* and fecal coliforms were consistently decreased in the warm seasons of summer and spring, but neither showed a consistent effect in winter. Feeding effects are suggested by a lack of consistent change in water column constituents during winter, even when concentrations were high enough to detect a difference (after the rain event).

The fact that the presence of oyster reefs frequently led to significant decreases in chlorophyll *a* and fecal coliform concentrations, but rarely reduced total suspended solids, leads to speculation that selective feeding by oysters occurred. In laboratory experiments, oysters have been shown to feed selectively on high quality food particles (Loosanoff 1949, Newell & Jordan 1983). In South Carolina tidal creeks, Wetz et al. (2002) found preferential feeding on phototrophic flagellates, but not heterotrophic flagellates, bacterioplankton, or cyanobacteria. Although the current study was not designed to investigate selectivity, these results do suggest that it occurs to a degree in these systems.

Flow conditions may also have contributed to changes in water column constituents; particles may have settled over the crest of the reefs (also suggested by Dame 1987). Differences in bottom sediment composition, however, may be due to larger-scale flow patterns. Sediments were finer on the sides of the reefs that were upstream during ebb tide (downstream during flood tide). Faster flow during ebb tide than flood tide would lead to greater deposition of fine particles during flood tide than ebb (as suggested by Dame 1987), which could explain the observed differences in sediment texture. In Bradley Creek, a tidal creek in southeastern North Carolina, current velocities were 14–55% higher on flood than ebb tides (Angelidaki 1997). However, high velocities lasted longer on the ebb tide than flood tide (Angelidaki 1997), possibly causing more sediment to settle on the flood tides. This study did not examine effects of oyster reefs during flood tides because chlorophyll *a* and fecal coliform concentrations are highest during ebb tides (Mallin et al. 1999), reflecting upland drainage influences.

While there was never a significant difference for changes of chlorophyll *a*, fecal coliform, or TSS concentrations between high-shell-cover and low-shell-cover reefs, the reefs themselves showed different patterns of effect. The reefs with low shell cover were also the reefs with lowest flow velocities and showed consistent removal of fecal coliforms in spring, whereas the other reefs did not. Vertical complexity was approximately equal between all reefs, and complexity may be a more important component in flow effects than the presence of shell itself. Multiple factors could be responsible for the observed effects on chlorophyll *a*, fecal coliform, and TSS concentrations. Both filtration by oysters and flow patterns over oyster reefs could contribute to particle removal in tidal creek ecosystems.

CONCLUSIONS

Significant changes in concentrations of chlorophyll *a* and fecal coliform bacteria were detected during warm seasons, even when effects on TSS concentrations were not observed. None of the examined variables were significantly related to live oyster density, flow speed, or change in flow speed across reefs, suggesting possible threshold effects. Oyster reefs do have detectable effects on chlorophyll *a* and fecal coliform concentrations under field conditions, though effects vary temporally. The degree of removal suggests physical mechanisms for removal in addition to filtration effects.

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EXPRESSION OF HSP 70 IN EXPERIMENTALLY METAL-EXPOSED EUROPEAN FLAT OYSTERS *OSTREA EDULIS*

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ABSTRACT The heat shock protein 70 family is composed of both environmentally inducible (Hsp) and constitutively expressed (Hsc) members. The expression of Hsp70 was investigated in the European flat oyster *Ostrea edulis* exposed to different metal concentrations. By using a polyclonal antibody developed in our laboratory for a recombinant Hsp70 of the oyster *Crassostrea gigas*, the soluble Hsp70 level in *O. edulis* was found metal dose dependent. An exposure to copper did not induce Hsp70 synthesis in either gills or digestive gland. A decrease of Hsp70 was observed in gill from cadmium-exposed animals, whereas digestive gland tissue showed an increase.

KEY WORDS: heat shock protein 70, *Ostrea edulis*, ELISA, expression, quantification, metal accumulation.

INTRODUCTION

The cellular stress response is involved in protecting organisms from damage caused by exposure to a great variety of stressors, including temperature, heavy metals, and other xenobiotics. The stress response entails the rapid synthesis of heat shock proteins (HSPs) to protect the proteins against denaturation (Lindquist & Craig 1988, Sanders 1993). HSPs were first described in *Drosophila busckii* (Ritossa 1962) and the genes encoding the *Drosophila* Hsp were among the first eucaryotic gene to be cloned (Craig et al. 1979). The major and the most highly conserved and studied of the HSPs in all organisms is the 70-kDa protein family (HSP70) because of its implication in protein chaperoning (Gething & Sambrook 1992) and acquired tolerance processes (Lindquist & Craig 1988, Clegg et al. 1998). The genes encoding Hsp70 are highly conserved in evolution and contain both heat-inducible (Hsp) and constitutive genes (Hsc), both of which encode stress proteins under normal conditions (Hightower 1993, Wood et al. 1998).

The types of studies conducted on stress proteins in aquatic organisms are highly variable (Sanders 1993, Gourdon et al. 1998). The synthesis of Hsp70 and induction of thermo-tolerance has been demonstrated in the Pacific oyster, *Crassostrea gigas* (Shamseldin et al. 1997, Clegg et al. 1998, Gourdon et al. 2000) and in the mussels *Mytilus edulis* and *Mytilus galloprovincialis* (Sanders 1988, Snyder et al. 2001). Piano et al. (2002) showed a rapid and significant synthesis of the inducible Hsp69 in thermal stressed flat oyster *Ostrea edulis*, but no significant variations in the constitutive isoforms level (Hsp72 and Hsp77). Recently, we characterized two HSP70 genes and quantified soluble HSP70 by enzyme-linked immunosorbent assay (ELISA) in *C. gigas* exposed to metals in the laboratory (Boutet et al. 2003). In this previous study, we showed that soluble HSP70 level decreased in tissues of experimentally metals-exposed oysters.

In the present work, the expression of Hsp70 and Hsc70 proteins in different organs of the European flat oyster, *Ostrea edulis*, exposed to a concentration gradient of metals under experimental conditions was quantified by ELISA, using a polyclonal antibody

for a recombinant Hsc72 of *C. gigas* developed in the laboratory (Boutet et al. 2003).

MATERIALS AND METHODS

Oyster Collection and Maintenance

Adult European flat oysters, *O. edulis* (3 years old; 7–8 cm), were purchased from an oyster farm of Mont Saint-Michel Bay (France) and maintained for one week in aerated 0.22-μm filtered seawater before experimentation. All the experiments were conducted in a temperature-controlled room (15°C) at a salinity of 34‰. Groups of 25 oysters were exposed to two metals, one essential (Cu^{2+}) and the other toxic (Cd^{2+}). Each metal was applied from a stock solution (100 mM) at each of two final concentrations (0.4 μM and 4 μM) and also in a mixture (0.2 μM each) for 15 days. The metal doses were chosen according to those found in most contaminated French estuaries. A group of 25 oysters was maintained in seawater, without metals, as a control. Seawater was renewed every day and oysters were fed with microalgae (*Isochrysis galbana*) every two days. The metals were reapplied to the appropriate concentrations after every water change.

Protein Extraction from Oyster Tissues

On days 0, 1, 2, 3, 5, 7, and 15 of the experiment, gills and digestive gland from exposed and control oysters (*n* = 3 for all samples) was harvested after oyster killing and homogenized in protein extraction buffer (150 mM NaCl, 10 mM NaH_2PO_4, 1 mM phenylmethanesulfonyl fluoride, pH 7.2) according to the protocol described by Tedengren et al. (1999). Samples were then centrifuged at 12,000 *g* for 10 min at 4°C and supernatant fractions containing soluble proteins were collected in fresh tubes. Total soluble proteins were quantified using the D_c Protein Assay kit (Bio-Rad) with dilutions of Bovine Serum Albumin (Sigma) as the standard. Optical density was measured at 620 nm using a microplate reader.

Metal Analysis

Pools of soft body excised from three oysters per sample day were mineralized with suprapure nitric acid. Concentrations of

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cadmium and copper were measured in each tissue sample using the potentiometric stripping method (Riso et al. 1997, Boutet et al. 2002).

Western Blot Analysis

The cross-reactivity of the anti-CgHsc72 IgG antibody developed in our laboratory (Boutet et al. 2003) was tested by Western blot as follows. Samples of *O. edulis* (control and cadmium-exposed) proteins were electrophoresed on 12% SDS-polyacrylamide gel and electrotransferred to PVDF-membrane (Bio-Rad). The membrane was blocked for 1h with blocking buffer (0.1 M Tris, 5% nonfat dry milk) and then incubated with Tris buffer containing anti-CgHsc72 antibody (1/125 diluted) for 1 h with gentle agitation at room temperature. The membrane was washed twice for 10 min with washing buffer (0.1 M Tris, 0.02% Tween 20) and incubated with Tris buffer containing 1/1,000 diluted polyclonal anti-rabbit IgG horseradish peroxidase-conjugated (Sigma) for 1 h with gentle agitation at room temperature. Again the membrane was washed twice with washing buffer, the reactive band was visualized by staining with 2.4 mM of 3-amino-9-ethyl-carbazole (Sigma) dissolved in 50 mM acetate buffer (0.2 M acetic acid, 0.2 M sodium acetate, pH 5) containing 5% of *N,N*-dimethyl Formamide (Sigma) and 12% of H_2O_2.

ELISA

Microtiter plates were coated with 20 μg per well of total proteins extracted from the digestive gland and gills of control and experimentally exposed oysters. HSP70 concentrations were quantified by ELISA developed in *C. gigas* using rabbit anti-CgHsc72 IgG and recombinant CgHsc72 as a standard (Boutet et al. 2003).

Statistical Analysis

The variations in metal and Hsp level during the experiment were analyzed by analysis of covariance ($\alpha = 0.05$) using CSS Statistica (Statsoft).

RESULTS

Metal Quantification in Oyster Tissues

Copper and cadmium concentrations in tissues of oysters experimentally exposed to Cu^{2+} and Cd^{2+} showed a significant time-dependent increase (compared with controls) during the 15 days of the experiment. Copper concentrations in the tissues of oysters exposed to 4 μM or 0.4 μM of Cu^{2+} increased from 0.17 to $0.73.10^{-6}$ M/g wet weight tissue (M/gwwt) and 0.17 to $0.37.10^{-6}$ M/gwwt, respectively. Dosing with 4 μM or 0.4 μM of Cd^{2+} resulted in an increase of Cd concentration in the gills from less than 0.01 to $0.31.10^{-6}$ M/gwwt and 0.01 to $0.075.10^{-6}$ M/gwwt. The concentration of metals in tissues of oysters exposed to a mixture of the two metals increased from 0.01 to $0.025.10^{-6}$ M/gwwt for Cd, while copper concentration did not vary.

Cross-Reactivity of Anti-CgHsc72 Antibody With O. edulis Proteins

The Western blot revealed a high cross-reactivity of our anti-CgHsc72 antibody with *O. edulis* HSP70 (Fig. 1). Two bands appeared on the membrane at a molecular weight of 68 and 70 kDa, confirming the specificity of the antibody with Heat Shock Protein 70 of this oyster species.
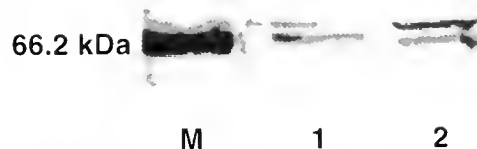

Figure 1. Western Blot of digestive gland protein sample from control (lane 1) and cadmium-exposed oyster (lane 2) electrophoresed and probed with anti-*Cghsc72* antibody. Marker (M) is SDS-PAGE Standard broad range (Bio-Rad Laboratories, Hercules, CA).

Quantification of Heat Shock Proteins 70 by ELISA

Application of the ELISA to protein samples extracted from gill and digestive glands of control oysters showed significant differences between these tissues in basal level of Hsp70. Quantities of 46.5 ± 2.6 and 59.3 ± 3.4 mg Hsp/g^{-1} protein, corresponding to approximately 4.7 and 5.9% of total proteins, were measured respectively in the gills and the digestive gland of control oysters. Hsp levels decreased significantly (compared with the control, $\alpha = 0.05$) in the gill of oysters exposed to a mixture of the two metal and 4μM of Cd (Fig. 2, A and B). A decrease (not significant) of Hsp70 levels in the gill of oysters exposed to copper was also observed. In contrast, a significant increase of Hsp concentration occurred in the digestive gland of animals exposed to 0.4 μM of Cd (Fig. 2E). No differences were observed in gills of individuals exposed to 0.4 μM of Cd (Fig. 2B) or to Cu (Fig. 2C) and in digestive gland of oysters exposed to a mixture of metals (Fig. 2D), to 4μM Cd (Fig. 2E) or to Cu (Fig. 2F). A stronger dosage-effect of cadmium was observed as either a decrease or increase of Hsp levels in the two organs. No dosage-effect of copper could be demonstrated in either organ.

DISCUSSION

In this study, we quantified soluble HSP70 by ELISA in experimentally metal-exposed *O. edulis*. The cross-reactivity of the purified rabbit anti-CgHsc72 IgG demonstrated here with *OeHsp70* supported the suitability of using these reagents to quantify HSP70. An increase in intensity of the 70 kDa bands was also observed in digestive gland of a cadmium-exposed oyster, in agreement with measurement of Hsp70 by ELISA. Now, our results showed that HSP70 level is different in gill and digestive gland (4.7 vs. 5.9% of total protein). We previously reported a concentration of about 6% in the oyster *C. gigas* (Boutet et al. 2003), and Feige and Polla (1994) observed a general HSP level of about 5% under normal conditions (without stress) in other organisms. In comparison to these basal levels, the quantification of soluble HSP70 in experimentally exposed *O. edulis* showed a metal-dosage response. A decrease of soluble HSP70 was observed in gills of oysters exposed to the highest concentration of cadmium or to a mixture of the two metals, in spite of a significant increase of metal concentration in the tissues. In contrast, an exposure to the lowest cadmium concentration induced an increase of HSP70 in digestive gland. Furthermore copper did not modify HSP70 levels in oyster tissues. We previously showed that metal exposure induced a significant decrease of HSP70 in tissues of *C. gigas* with the same treatments (Boutet et al. 2003). Veldhuizen-Tsoerkan et al. (1991) found no variation in HSP70 in *M. edulis* caged in seawater with various concentrations of cadmium, like the response in copper-exposed *O. edulis*. In contrast, Lewis et al. (2001) showed an inhibitory effect of metals, particularly copper.
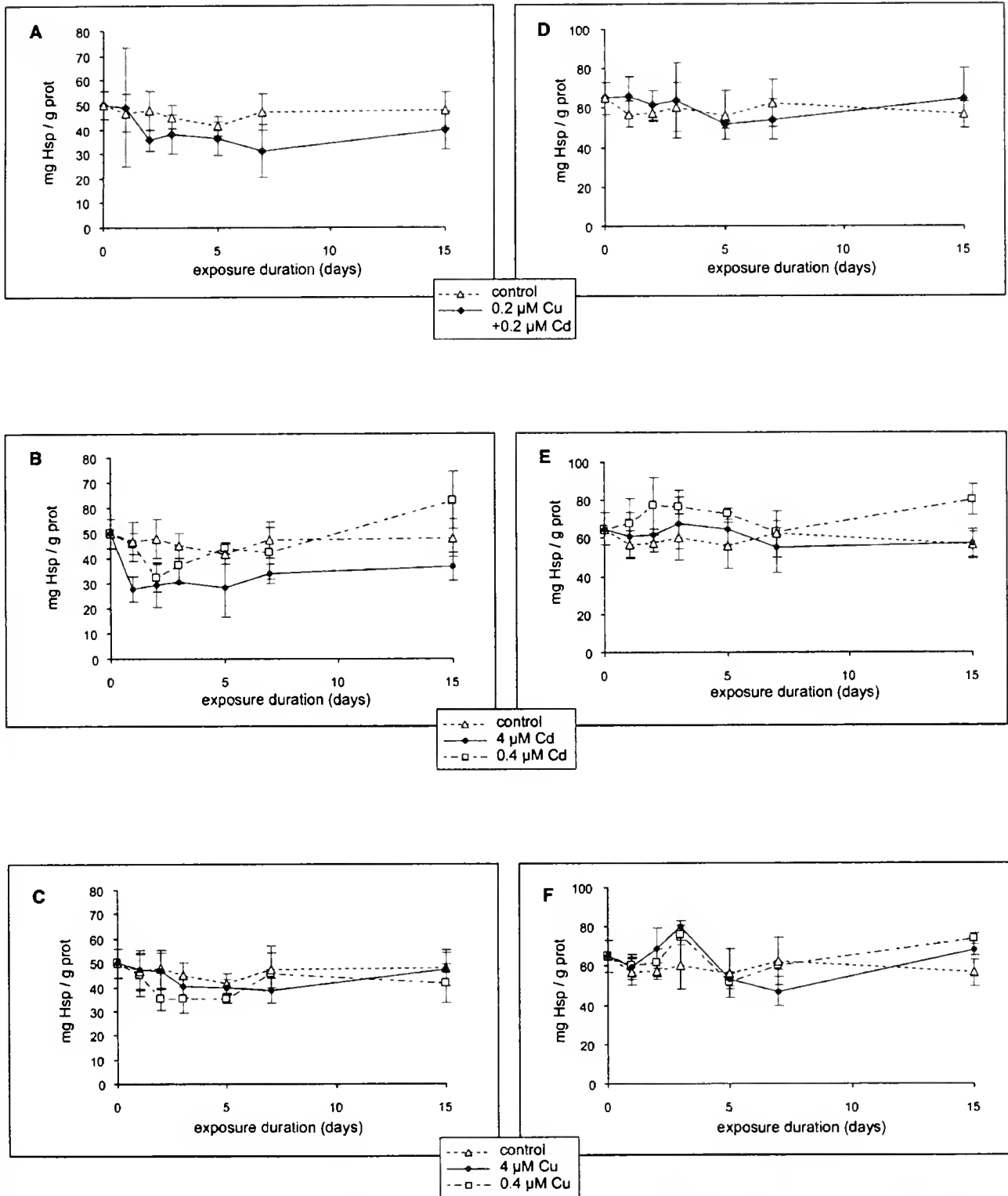
Figure 2. Quantification of HSP70 (mean ± SE) by ELISA in the gills (A, B, and C) and in the digestive gland (D, E, and F) of *O. edulis* exposed to copper and cadmium (A and D), cadmium (B and E), and copper (C and F).

in the seaweed *Enteromorpha intestinalis*. These authors observed that high levels of copper appeared to damage protein synthesis, therefore impairing the HSP70 response. In our experiment, a decline of HSP70 was observed in cadmium- and a cadmium-copper mixture exposed oysters. A similar HSP70 synthesis inhibition was

observed in earthworms, *Lumbricus terrestris*, exposed to a variety of metals (lead, cadmium, and copper; Nadeau et al. 2001). When exposure approaches lethal levels, such as 4 μM in our experiments, the average degradation rate of HSP70 will exceed its synthesis rate because of cytopathologic damage, such as ruptured

membranes, in many cells (Triebskorn & Köhler 1996, Quig 1998). The fact that the gills are the first barrier to metals could explain why this organ was more affected by the toxic effect of metals and showed a higher decrease in HSP70 concentration. At sub-lethal levels, our work showed an increase of HSP70 in response to exposure, in agreement with results described by Snyder et al. (2001). These authors showed a significant increase of HSP70 in cadmium-contaminated mussels, *M. edulis*, and limpets, *Collisella pelta*, and in heat-shocked and oil-exposed mussel, *M. galloprovincialis*, and abalone, *Haliotis rufescens*.

The ELISA developed in a previous study in *C. gigas* allowed us to specifically and rapidly quantify HSP70 proteins in tissues from marine mollusks. This immunologic method has the advantage of quantifying the protein of interest, unlike the commonly used Western blot analysis (Clegg et al. 1998, Nadeau et al. 2001), which gives only a semi-quantitative estimation of HSP70

amounts. Furthermore, this study showed that *O. edulis* displayed a differential response to the level of metal contamination. According to the present study and a previous work on metallothionein in this species (Tanguy et al. 2003), the oyster *O. edulis* do not seem to be an appropriate indicator for studying environmental contamination.

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IS BEAUTY IN THE EYE OF THE BEHOLDER? DEVELOPMENT OF A SIMPLE METHOD TO DESCRIBE DESIRABLE SHELL SHAPE FOR THE PACIFIC OYSTER INDUSTRY

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ABSTRACT Shell samples of Pacific oysters (*Crassostrea gigas*) were evaluated from three different U.S. West coast farms. Industry experts described each shell sampled as being either desirable (good) or undesirable (bad). There were slight differences in the categorization of good and bad oysters among farms, but common trends were evident. The ratio of greatest shell depth to greatest shell length (D/L) was found to be more effective in categorizing good and bad shell shapes compared with other descriptors. Good oysters had a mean D/L of 0.316, whereas the bad oysters had a significantly lower mean of 0.219 ($P < 0.001$). Using the threshold value of D/L > 0.25 for good oysters, 85.6% of all sampled oysters were correctly assigned to good and bad categories, as defined by industry participants. The use of D/L and greatest shell width to greatest shell length (W/L) may be beneficial in distinguishing shell shape quality and allow for the rapid assessment of many sampled oysters. These findings have implications in the development of industry standards for shell shape; furthermore, such standards would be useful in designing oyster breeding programs to improve shell shape.

KEY WORDS: shell, shape, oyster, *Crassostrea gigas*, standards, industry

INTRODUCTION

Product quality is becoming more important as production of Pacific oysters (*Crassostrea gigas*) increases and competition for lucrative markets rise. Shell morphology often provides consumers with their first impression of product quality. Many shellfish industries recognize shape as a valuable marketing tool. For the Atlantic Canadian oyster industry, Section 65 of the Canadian Food Inspection Agency's Fish Inspection Regulations outlines four different shape classes (differentiated by length to width ratios) by which Eastern oysters, *Crassostrea virginica*, are to be sold. In certain regions of France, growers have to sign a contractual agreement with the Shellfish Professional Organization in which they agree to not sell oysters (*Crassostrea gigas*) of a certain shape (determined by a previously reported formula; Galtsoff 1964). In exchange, these growers are able to market oysters using that region's trademark (Goulletquer, personal communication). Such industry quality control has provided successful and favorable product label identification within the marketplace. In contrast, classification of desirable and undesirable shell shapes has not been objectively defined by the U.S. West coast oyster industry.

The development of industry standards by which shape can be objectively defined would be of use to the West coast oyster industry in assessing the effects of different culture practices and genetic stocks on shell shape. In addition, shell shape may become increasingly important in product label identification and industry quality assurance. The objective of this research was to use simple linear measurements to characterize the shape of Pacific oyster (*Crassostrea gigas*) shells, classified as being either desirable (good) or undesirable (bad) in appearance by industry experts.

MATERIALS AND METHODS

Experimental oysters (commercially farmed oysters) were sampled from three (A, B, and C) commercial oyster farms and divided into two groups (approximately 50 per group) of either good or bad shell shape by each farmer. Oysters provided by farms A and C were grown intertidally in mesh oyster bags, while samples provided by farm B were grown subtidally in lantern nets. All shell measurements were performed at the Hatfield Marine Science Center in Newport, OR. Sample oysters were shucked to obtain the left valve, or "halfshell." Greatest shell length, greatest shell width, and greatest shell depth were measured for all oysters. All size measurements were made using Vernier calipers to the nearest 0.1 mm.

Analysis of variance was used to determine whether good- and bad-shaped oysters differed for any of the three linear measurements (greatest shell length, width, and depth). Analyses were performed using data from within each farm site. Normality was tested using the Kolmogorov-Smirnov method (SPSS Inc., 2000, Chicago, IL). These analyses were repeated for data pooled across all farms.

Absolute measures, such as greatest shell length, width, or depth, are less useful in categorizing oysters by shape because of variation in oyster size at harvest. To eliminate the confounding effects of size on shape it is usual to apply proportional measures (Reist 1985). As a result, two descriptors were generated to characterize shell shape: ratios of depth to length (D/L) and width to length (W/L). Normality of D/L and W/L values were determined using the Kolmogorov-Smirnov method (SPSS Inc., 2000, Chicago, IL). The ability of each variable to discriminate good from bad oysters was determined using data collected from each farm site separately and with data pooled across all farm sites. A comparison of the total percentage of correct assignments of sampled oysters (into good or bad groups) was then used to determine which of the descriptors (D/L, W/L, and three previously described formulae: Wada (1986); Galtsoff (1964); Imai and Sakai (1961) resulted in the most accurate discrimination between good and bad shaped oysters.

RESULTS

All variables were normally distributed after log transformation (Kolmogorov-Smirnov, $P > 0.05$), except for shell depth measured in the sample from farm B ($P = 0.014$). However, due to robustness of ANOVA and the large sample size ($n = 99$), this departure from normality may be ignored for the purpose of analysis (Ramsey & Schaffer 2002). Good and bad shaped oysters differed in length, width, and depth at all farm sites ($P < 0.05$) except at farm

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C, where the two groups of oysters only differed in length (Table 1). When significant differences occurred, good oysters tended to be both deeper and wider than bad oysters. Good oysters were significantly shorter in length ($P < 0.05$) than bad oysters at farms B and C; however, good oysters were significantly longer than bad oysters at farm A. Table 2 lists the means and standard deviations of D/L and W/L ratios for each individual farm, and for the pooled good and bad oyster samples. Based on the ratio D/L, good oysters were significantly deeper than bad oysters at all farms ($P < 0.05$; Table 2). Good oysters were 1.35, 1.88, and 1.36 times as deep, per unit length, as bad oysters at farms A, B, and C, respectively. Good oysters were 1.06, 1.10, and 1.22 times as wide, per unit length, as bad oysters at farms A, B, and C, respectively ($P < 0.05$). Although there were differences in mean D/L and W/L ratios among farms, the trends (larger values for good oysters than bad oysters) within sites were similar. To obtain a robust sample that would best represent different farms across industry, the data from all farms were pooled to evaluate the average differences between good and bad shell groups across all sites. Good oysters had a mean W/L of 0.689 and D/L of 0.316, whereas bad oysters had significantly lower means of 0.597 and 0.219 respectively ($P < 0.001$).

Table 3 lists reported shape descriptors for assignment of oysters into good and bad groups. Previously used thresholds for separating good and bad shapes, as well as the values maximizing the percent correct assignment in the current study are given. Correct assignment was maximized for D/L at 0.25, with 92.6% of all good oysters falling above the value of 0.25 (depth was at least 0.25 of shell length), while 78.8% of all bad oysters fell below this value. Using the Atlantic Canadian shell shape guidelines, shells with a value of length / width <1.75 would be termed as either "fancy" or "choice", the top two of four possible categories of oyster shells. Table 3 shows the percentage of oysters correctly assigned to their proper good or bad groups by using D/L > 0.25, W/L, and the Atlantic Canadian threshold of good >1.75 (as well as the value for which percent correct assignment was maximized, 1.12). In addition, a formula (Galtsoff 1964) used as a standard for the Irish and French industry (using a threshold value for good shell shape of >3, and the value of maximum correct assignment of 3.5) was compared along with a previously described formula for shell convexity (Wada 1986). Percent correct assignment for convexity was maximized at a value of 0.315 when this descriptor was applied to the current data. The index of shell depth described by Imai and Sakai (1961) maximized the percentage of oysters correctly assigned to both good and bad groups at a value of 31.6.

TABLE 1.

Shell length, width, and depth measurements of good- and bad shell-shaped oysters from three commercial U.S. West coast oyster farms.

Farm		Length (mm)		Depth (mm)		Width (mm)	
		Good	Bad	Good	Bad	Good	Bad
A	Mean	101.15*	95.99	30.41*	21.64	71.50*	61.56
	SD	7.10	8.35	3.39	3.89	5.78	7.09
B	Mean	77.62*	90.86	27.15*	17.29	52.82*	50.61
	SD	5.00	13.32	3.96	3.41	4.71	6.49
C	Mean	90.66*	117.12	27.07	26.57	60.37	61.33
	SD	7.134	23.58	4.33	6.52	8.58	6.35

* Means were significantly different ($P < 0.05$; ANOVA).

TABLE 2.

Observed ratios of depth/length (D/L) and width/length (W/L) of good- and bad shaped-oyster shells from three commercial U.S. West coast oyster farms.

Farm	Sample Type	D/L		W/L	
		Mean	Standard Deviation	Mean	Standard Deviation
A	Good	0.302	0.037	0.711	0.084
	Bad	0.226	0.040	0.646	0.092
B	Good	0.351	0.052	0.683	0.072
	Bad	0.194	0.046	0.571	0.125
C	Good	0.301	0.055	0.669	0.097
	Bad	0.235	0.079	0.547	0.136
Pooled	Good	0.316	0.053	0.689	0.087
	Bad	0.219	0.057	0.597	0.123

All mean good-shaped samples within a site were statistically larger than bad-shaped samples ($P < 0.05$, ANOVA).

A threshold value of D/L > 0.25 (for good oysters) was the most effective at correctly assigning oysters to their respective categories, with 85.6% of all oysters being correctly assigned (Table 4). This threshold was more effective at correctly assigning good oysters to good groups (90.9% correct) than bad oysters to bad groups (77.8% correct). The index of shell depth (Imai & Sakai 1961) was also effective at correctly assigning good oysters (84.2%) and bad oysters (82.0%), correctly assigning 84.4% of the total oysters sampled. The ratio of W/L, using the threshold value of >0.63 (for good oysters) correctly assigned 70.6% of all oysters. When the ratios of D/L and W/L were applied simultaneously, only 30.0% of all oysters were correctly assigned by both ratios (Table 4, Fig. 1). The Atlantic Canadian guideline was less effective in discriminating good from bad oysters using the threshold value for good oysters of >1.75 (65.7%), resulting in an overall maximum correct assignment of 70.6%. The measure of convexity (Wada, 1986) was effective at correctly assigning good oysters (86.1%), but not bad oysters (35.9%), correctly assigning 61.8% of the total oysters sampled. Using the previously applied threshold and the value of percent maximum assignment, the Galtsoff formula was effective at correctly assigning bad oysters (96.4% and 98.8% respectively), but ineffective at assigning good oysters (0% and 0%), and only assigned 49.2% and 50.4% of all oysters correctly.

DISCUSSION

Members of the U.S. West coast oyster industry have subjectively identified shell depth and width, relative to length, as the two most important factors in determining the quality of an oyster halfshell. Most oyster growers identified a long and skinny shape (typically called "rabbit ears") as being undesirable, with a deep and wide halfshell being more desirable. These distinctions, when relative shell size is considered, are described by the ratios of D/L and W/L.

Previous work has shown the ability to categorize bivalve shape regardless of absolute size. Day et al. (2000) used stepwise discriminant analysis and principal component analysis to show that relative size of the umbo cavity was the most useful character for identification of sympatric *Saccostrea* species. The authors reported success in identifying species using the non-lethal mea-

TABLE 3.

Descriptors used in the current study to compare percent correct assignment of oysters into farm-specified good and bad shell-shape groups.

Descriptor	Expression	Species	Previously Employed Threshold	Value Maximizing Percent Correct Assignment	Reference
D/L	Depth/length	*Crassostrea gigas*	NA	0.25	Current study
W/L	Width/length	*Crassostrea gigas*	NA	0.63	Current study
Atlantic Canadian	Length/width	*Crassostrea virginica*	1.75[a]	1.58	Section 65 of CFIA's Fish Inspection Regulations
Galtsoff	(Length depth)/width	*Crassostrea gigas, Crassostrea virginica*	3	3.5	Galtsoff 1964, Heath & Wilson 1999
Convexity	Width/(length width depth)	*Pinctada fucata martensii*	NA	0.315	Wada 1986
Index of Shell Depth	(Depth/mean of width and length) × 100	*Crassostrea gigas*	NA	31 6	Imai & Sakai 1961

[a] Separates top two of four possible shape categories; details in results section.

sures of total oyster depth, right valve length, and right valve width. In addition, Wilding et al. (1998) reported that hinge length was the only one of four investigated shell measures that provided a clear distinction among groups of the scallop, *Pecten maximus*.

The various formulae (Tables 3 and 4) used to separate good from bad oysters confirm the importance of depth in determining optimal oyster shape. The two relationships that were most effective at correctly assigning good and bad oysters (D/L and an index of shell depth; Imai & Sakai 1961) had only depth in the numerator. These descriptors were more effective than the ratio of W/L, a previously described shell convexity assessment method (Wada 1986), the formula used as a guideline in the Irish and French industry (Galtsoff 1964), and the Atlantic Canadian guideline (Table 3).

Culture environment has an important effect on shell shape (Carriker 1996, Boulding & Hay 1993, Seed 1968). It is commonly believed by growers that oysters subjected to movement by frequent disturbance tend to grow deeper halfshells. The outer edge of the shell is repeatedly broken off and subsequently grown back

with the net result being that the oyster grows more quickly in terms of depth than length. This process is commonly referred to as "pruning."

Few attempts have been made to investigate genetic effects on shell shape or to improve bivalve shell shape using selective breeding in aquaculture. Wada (1986) reported on first, second and third generation responses to selection for shell width and shell convexity for the Japanese pearl oyster, *Pictada fucata martensii*. Selection for shell convexity and shell width was effective and Wada obtained a realized heritability of 0.467 for shell convexity after two generations of selection, providing evidence that shell shape in the Japanese pearl oyster may be improved by selective breeding.

The current study suggests that width may not be as important as depth in determining quality. It is conceivable, however, that if a breeding program were to select oysters without consideration of width, the result could be a less desirable deep and narrow oyster. As both depth and width have been anecdotally described as being important (by industry) to the quality of a halfshell, it may be prudent to consider both relationships to determine the true quality

TABLE 4.

Percent correct assignment of good and bad oysters based on D/L, W/L, and Atlantic Canadian Measure, the Galtsoff measure, an index of shell depth, and a measure of shell convexity using values for maximum percent correct assignment, unless stated otherwise.

Sample Type	Percent Correct Assignment								
	Depth/Length (D/L)	Index of Shell Depth	Width/Length (W/L)	D/L + W/L[a]	Atlantic Canadian[b]	Atlantic Canadian	Convexity	Galtsoff[b]	Galtsoff
Good oysters	90.9	84.2	74.5	56.4	89.1	74.5	86.1	0	0
Bad oysters	77.8	84.5	64.7	3.1	42.0	64.7	35.9	96.4	98.8
Good + bad oysters	85.6	84.4	70.6	30.0	65.7	70.6	61.8	49.2	50.4

A description of the measures and the maximum percent correct assignment values for each are given in Table 3.

[a] Maximum percent correctly assigned by both D/L and W/L.

[b] Threshold value described in the literature; not the value maximizing percent correct assignment given in Table 3.
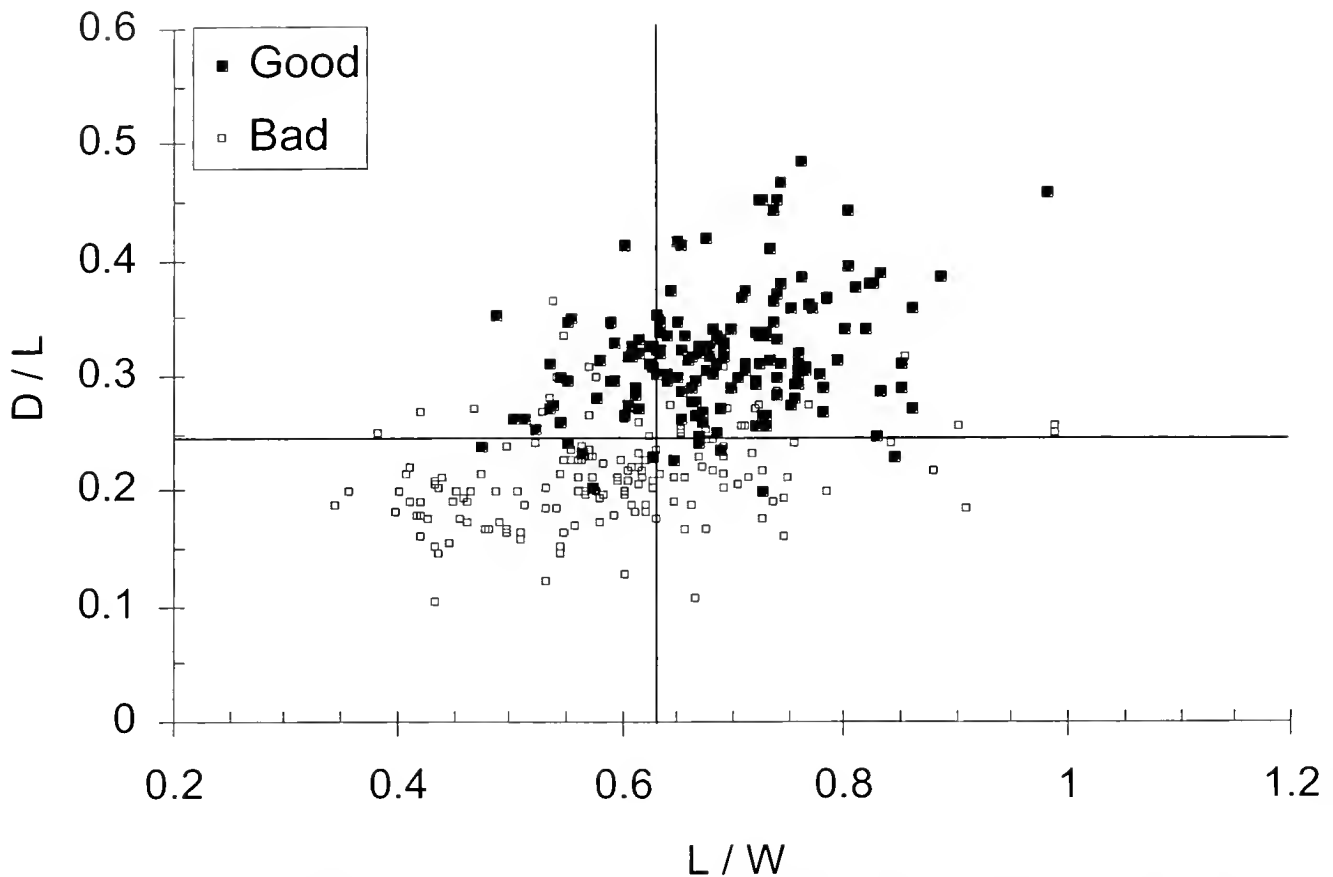
Figure 1. Ratios of depth/length (D/L) and width/length (W/L) of good- and bad-shaped oyster shells from three commercial West coast U.S. oyster farms. Lines represent threshold values of W/L > 0.63 and D/L > 0.25 maximizing percent correct assignment.

of an oyster halfshell. The use of the D/L and W/L thresholds simultaneously was ineffective in correctly assigning oysters to good or bad groups. The index of shell depth (Iamai & Sakai 1961) might be useful in this regard as it incorporates shell width, and should therefore exclude any abnormally deep and narrow oyster shells.

A method to evaluate shell shape quality that is both simple and reliable could be of great value to the U.S. West coast oyster industry. Growers could objectively compare practices to find which culture methods tend to influence shell shape in a positive way. Oysters grown in different areas commonly have different shape characteristics. Using an objective method, site differences could also be assessed, with a grower being able to determine whether particular sites produce oysters with a better shape. Another possible long-term benefit of having an objective comparison could be the establishment of industry shape standards. This would allow producers and consumers to use a common scale of shell shape measurement. The Atlantic Canadian oyster industry has realized the benefits of such a set of standards. For example, "fancy" oysters are defined in section 65 of the Canadian Food Inspection Agency's Fish Inspection Regulations as having a length not exceeding one and one-half times its greatest width, and as not being abnormally flat, thin-lipped, or malformed. Consumers can therefore go to several different farms or retailers and purchase "fancy" oysters, knowing that they share a common shape.

An important consideration in a method to characterize shell shape is the practicality of the methodology. Heath and Wilson (1999) used computer assisted image analysis to assess shell shape and size in *Crassostrea gigas*. Although they demonstrated that this method could be used to separate oysters into categories according to general shape specifications, the required equipment might be cost prohibitive and this method does not allow for assessment of oysters in the field. The ratio of D/L and the index of shell depth used in the current study would, therefore, be more practical to separate good from bad shells compared with using image analysis.

In summary, industrial-scale assessment and selective breeding programs both require methods to efficiently determine the value of an oyster in terms of shell shape. The D/L ratio and the index of shell depth show promise in this regard. The D/L threshold of <0.25 separates most of the good and bad oyster halfshells, while requiring only two simple linear measurements. The index of shell depth (Iamai & Sakai 1961) was nearly as effective and has the advantage of incorporating width, which might eliminate any abnormally deep and narrow oysters. The current data suggests that depth is likely the most important measure to evaluate oyster shell shape quality. Consideration of W/L might also be prudent in shell shape assessments to avoid narrow deep oysters. The current study only investigated differences between good and bad shell samples from three farms; therefore, future work should include samples from more industry participants.

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SHOULD SLOW GROWING PEARL OYSTER (*PINCTADA MARGARITIFERA*) SPAT ("RUNTS") BE DISCARDED?

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ABSTRACT   In this laboratory, hatchery-produced *Pinctada margaritifera* juveniles are routinely graded at 3.5 mo of age, when spat of <5 mm ("runts") are generally discarded. This article reports on an experiment to assess the relative growth rates of three size classes (<5, 5–10, and >10 mm) of hatchery-produced blacklip pearl oyster (*P. margaritifera*) spat from the same cohort. The three size classes were classified as runts, normal growers, and fast growers, and had mean (±SE; $n = 30$) dorso-ventral shell heights (DVHs) of 4.5 ± 0.1, 8.6 ± 0.3, and 12.8 ± 0.2 mm, respectively, at the start of the 4-mo experiment. The mean DVH at completion of the study for each initial size class (<5, 5–10, and >10 mm) was 24.6 ± 0.4, 32.3 ± 0.4, and 35.6 ± 0.4 mm, respectively. All differed significantly from each other ($P < 0.001$). The mean incremental increases in DVH for each size class (<5, 5–10, and >10 mm) over the 4-mo period was greatest in oysters from the 5–10-mm size class (mean DVH 23.3 ± 0.4 mm) and lowest in oysters from the <5-mm size class (mean DVH 20.0 ± 0.5 mm). Incremental increases in DVH were significantly different between oysters from the <5-mm size class and those from the larger size classes. The mean (±SE) percentage increase in DVH was greatest in oysters from the <5-mm size class (448 ± 17%) and lowest in oysters from the >10-mm size class (178 ± 7%). A number of oysters in the <5-mm size class grew very rapidly during the experiment and reached the same DVH as oysters in the larger size classes. This study shows that, given appropriate conditions, runts are capable of similar growth rates as larger spat. It may therefore be inappropriate to discard pearl oysters, which are classed as runts (<5 mm) at grading (3.5 mo). Furthermore, it is suggested that grading be delayed until 5 to 6 mo when a greater proportion of oysters are likely to be in the larger size classes.

KEY WORDS: pearl oyster, *Pinctada margaritifera*, spat, runts, growth

INTRODUCTION

The growth of cultured bivalve molluscs is highly variable during hatchery and nursery culture, and variation in growth can occur among individuals of the same age reared under identical conditions (Newkirk 1981). Small differences in the size of spat can become large differences in juvenile size (Mason et al. 1998), and the greater the time required by slow growers to reach commercial size increases costs and reduces profitability (Askew 1978). Pearl oysters need to reach a minimum shell size before being used for pearl production. This size is generally reached at approximately 2 y of age. As such, maximizing growth rate and minimizing growth variation are important factors in pearl oyster cultivation.

A large variation in growth rate is evident for pearl oysters reared under identical conditions. For example, 43-day-old blacklip pearl oyster (*Pinctada margaritifera*) spat have been reported to range in size from 1 to 5 mm in dorso-ventral shell height (DVH) (Pit & Southgate 2000), and from <2 to 23 mm DVH at 3.5 mo of age (Southgate & Beer 1997). To minimize the size variation in pearl oyster spat, Rose (1990) recommended continual grading to separate fast growers from slow growers. Slow-growing pearl oyster spat are often discarded. In this laboratory, hatchery-produced *P. margaritifera* are routinely graded at 3.5 mo of age, when spat <5 mm ("runts") are generally discarded. "Runting" may result from unfavorable culture conditions, and, if this is the case, runts may be capable of good growth rates if provided with appropriate culture conditions. Given the high cost of hatchery production and the high value of pearl oyster spat, it is in the interest of pearl oyster farmers to maximize the number of spat from a given cohort that are eventually used for pearl production. The aim of this study was to determine whether slow-growing *P. margaritifera* spat remained as runts or whether they are capable of similar growth rates as normal spat when provided with appropriate conditions.

MATERIALS AND METHODS

This study was conducted at the Orpheus Island Research Station of James Cook University, north Queensland, Australia (180°35′146°29′E), and larvae and spat were cultured according to the methods described by Southgate and Beer (1997) and Pit and Southgate (2000). At 43 days of age, when spat had a mean (±SE) DVH of 2.8 ± 0.1 mm (range 1–5 mm), they were transferred from the hatchery to the ocean where they were held in suspended mesh trays at a depth of 6 m (Southgate & Beer 1997).

Spat were graded at 3.5 mo of age into three different size classes, <5, 5 to 10, and >10 mm, which, for the purpose of this study, were classified as runts, normal growers, and fast growers, respectively. The mean DVH ($n = 30$) of *P. margaritifera* in the <5-, 5-to-10- and >10-mm size classes were 4.5 ± 0.1, 8.6 ± 0.3, and 12.8 ± 0.2 mm, respectively, and these differed significantly from each other ($F_{2,87} = 285.42; P < 0.001$). Thirty *P. margaritifera* spat from each size class were individually fixed to the bottoms of each of three replicate plastic mesh trays (60 × 35 × 10 cm) using a waterproof cyanoacrylate adhesive (Loctite 454 gel, Loctite Australia, Caringbah, New South Wales, Australia). This minimizes oyster aggregation (Friedman 1999, Pit 1998), which can significantly affect growth (Friedman & Southgate 1999). To minimize the disturbance to spat and to maximize growth rates, oysters were not measured during the 4-mo study; however, trays were cleaned *in situ* every month to remove external fouling organisms (Pit & Southgate in press). Cleaning involved the manual scrubbing of the outside surfaces of the trays. Trays were not cleaned internally, but were moved gently up and down in the water column to remove any silt and mud that had accumulated inside the trays. Oysters from each tray were measured for DVH at the end of the study.

Data were analyzed using a one-way analysis of variance to

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determine whether *P. margaritifera* from different size classes differed in size (DVH) at the completion of the study. Assumptions of homogeneity and normality were met (Zar 1984). The rates of growth among the three size classes were also assessed using nonparametric analyses to determine whether differences existed. Significant differences were identified using the Tukey's test and the Dunnett's T3 for the parametric and nonparametric tests, respectively (Zar 1984).

RESULTS

On completion of the study, the mean (±SE) DVH for each initial size classes (<5, 5–10, and >10 mm) were 24.6 ± 0.4, 32.3 ± 0.4, and 35.6 ± 0.4 mm, respectively. All differed significantly from each other ($F_{2, 87} = 167.67$; $P < 0.001$) (Fig. 1). The mean incremental growth in DVH ($n = 30$) for each size class (<5, 5–10, and >10 mm) over the 4-mo period was greatest in oysters from the 5-10-mm size class (23.3 ± 0.4 mm) and was lowest in oysters from the <5-mm size class (20.0 ± 0.5 mm). Incremental shell growth was significantly greater in the two larger size classes ($F_{2, 87} = 15.99$; $P < 0.001$) (Fig. 2). Weekly growth rates averaged 1.25, 1.42, and 1.48 mm, respectively, for the <5-, 5-10-, and >10-mm size classes. However, the mean percentage increases in DVH for each size class (<5, 5–10, and >10 mm) over the 4-mo period was greatest in oysters from the <5-mm size class (448 ± 17%) and lowest in oysters from the >10-mm size class (178 ± 7%), while oysters in the 5-10-mm size class increased by 308 ± 12%. A number of oysters in the <5-mm size class grew very rapidly and achieved DVH measurements within the ranges of those shown by oysters in the two larger size classes.

DISCUSSION

P. margaritifera used in this study were hatchery-reared animals of the same age that were cultured under identical conditions. However, when oysters were transferred from the hatchery to the nursery at 6 wk of age, their DVH ranged from 1 to 5 mm (mean 2.8 ± 0.1 mm). It is unclear whether such size variation resulted from environmental factors, genetic factors, or a combination of both. Factors that have previously been suggested to cause such size variation in bivalves include fluctuations in water quality and food quality (environmental), as well as egg and larval quality (genetic) (Gallager & Mann 1986, Rose 1990, Mason et al. 1998, Devakie & Ali 2000, Nicolas & Robert 2001).

Size variation was also evident during early nursery culture prior to grading when oysters ranged in size from 2 to 23 mm. Again, it is unclear whether size variation at grading reflected a continuation of the size variability observed in the hatchery, or
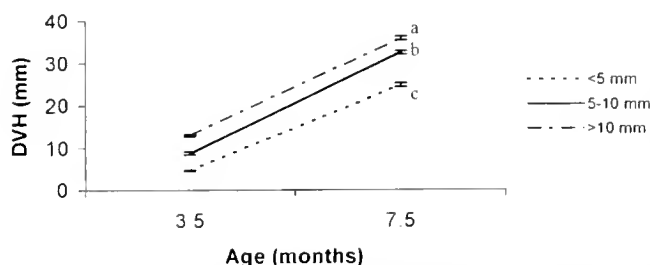

Figure 1. Changes in mean (±SE; $n = 30$) DVH of *P. margaritifera* juveniles in different size classes (<5, 5–10, and >10 mm) cultured for 4 mo at Orpheus Island. Means with the same superscript are not significantly different ($P > 0.05$).

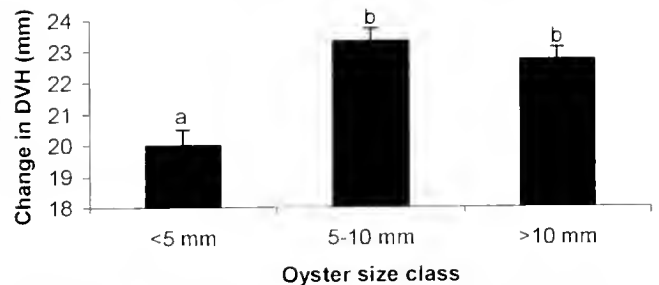
Figure 2. Mean (±SE) change in DVH of *P. margaritifera* juveniles in different size classes (<5, 5–10, and >10 mm) cultured for 4 months at Orpheus Island. Means with the same superscript are not significantly different ($P > 0.05$).

whether subsequent environmental factors were also involved. The negative impacts of poor growing conditions on pearl oyster growth rates during nursery culture are well documented. For example, pearl oysters aggregate to form clumps in culture units (Southgate & Beer 1997, Friedman & Southgate 1999). This results in a greater size range of individuals within a cohort and a higher proportion of smaller oysters when compared with oysters grown in conditions that prevent clumping (Friedman & Southgate 1999, Southgate & Beer 2000). The smaller oysters in the former group are thought to be those that are bound into clumps of oysters, and, as a result, have impaired access to good water flow and food availability (Friedman & Southgate 1999).

The growth rates of *P. margaritifera* spat recorded in this study were clearly influenced by initial size class, suggesting that genetic factors were more influential on initial spat size than were environmental factors. In a similar study with Pacific oysters, Collet et al. (1999) demonstrated a positive relationship between larval and postmetamorphic growth, indicating a genetic rather than environmental basis for slower growth in postmetamorphic bivalves. In contrast, Mason et al. (1998) reported that growth variation in Sydney rock oyster spat was not affected by initial size class and suggested that initial differences in size resulted from "temporary environmental stunting" rather than from genetic factors. Similar findings have been reported for edible oysters (Newkirk 1981, Newkirk & Haley 1982)

Hatchery production of pearl oysters is expensive, and it is clearly in the interest of pearl oyster farmers to maximize the number of spat from a given cohort that can be used for pearl production. However, the use of smaller spat, which take a longer time to reach a size suitable for pearl production, becomes an economic issue. Pearl farmers must consider the benefits of maximizing the number of usable oysters from a cohort of spat, against the increased time required for slower growers to reach pearl production size. Prior research at the culture site used in this study reported growth rates for *P. margaritifera* during nursery culture ranging from 3.66 mm mo^{-1} (in trays) to 4.86 mm mo^{-1} (in pocket nets) (Southgate & Beer 2000). Assuming similar subsequent growth rates for the three size classes of oysters used in this study, it is possible to estimate the time required for each size class to reach a pearl production size of 110 mm DVH. On this basis, oysters in the 5-10- and >10-mm size classes would reach 110 mm at 19 to 24 mo and 19 to 23 mo of age, respectively. However, oysters in the <5-mm size class would require 21 to 27 mo to reach this size (110 mm DVH). The costs involved in culturing oysters from the smaller size class for this additional time, however, may outweigh the costs of increasing oyster numbers by additional

hatchery production or the purchase of juveniles. In a similar study with *Crassostrea virginica*, O'Beirn and Luckenbach (2000) noted that the use of runts for the oyster industry would be feasible, given good growing conditions, but that it may not warrant the investment of extra time and resources.

When provided with good growing conditions, oysters in the <5-mm size class grew at a significantly slower rate than those in larger size classes. Nevertheless, certain individuals from the <5-mm size class did attain sizes within the overall size ranges of oysters in the larger size classes. This suggests that some runts may not always remain runts and indicates that such individuals are likely to have been affected by environmental stunting. Clearly, at first grading (3.5 mo of age), it is not possible to identify those *P. margaritifera* individuals in the <5-mm size class that are capable of growth rates allowing them to catch up to larger individuals within a cohort. Culling runt oysters at this stage would result in the loss of oysters that could subsequently be used for pearl production. A second grading at approximately 5 to 6 mo of age, however, would allow such individuals to be identified. This would maximize the number of oysters used for pearl production from a given cohort of juveniles. A similar outcome might also be achieved through more appropriate spat collector design. Spat are generally transferred from the hatchery to the field on spat collectors and remain on them until grading (Southgate & Beer 1997). Spat collectors that provide more uniform environmental conditions are likely to result in a more uniform size range of spat at grading.

Hatchery production of *P. margaritifera* in many developing Pacific nations is often constrained by limited resources (Southgate & Beer 1997) and cannot be conducted on a routine basis. In these cases, it is preferable to use as many oysters as possible from each cohort of hatchery-produced spat. The results of this study indicate that modifications to the current protocols may allow increases in the number of *P. margaritifera* from a given cohort that can be used for pearl production.

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CORROSION CASTING OF THE DIGESTIVE DIVERTICULA OF THE PEARL OYSTER, *PINCTADA FUCATA MARTENSII* (MOLLUSCA: BIVALVIA)

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ABSTRACT We examined corrosion casting as a means of studying the digestive organ in the pearl oyster *Pinctada fucata martensii* and other molluscs. The cast was made with resin that mixed hardener (Mercox MA) and prepolymerization methyl methacrylate (MercoxCL-2R). In pearl oysters, the resin was injected through the polyethylene tubing within 3 min, after the animal sufficiently relaxed in 0.4 mM $MgCl_2$ solution. It was left at least 1 h in the seawater and hardened. Then, it was treated with 20% NaOH for 1 day at room temperature. As a result, it was possible to cast from the mouth to the anus, including the ducts and tubules of the digestive diverticula. Using the same method, the castings of digestive organ in other molluscs, *Scapharca broughtonii, Crassostrea gigas, Meretrix lusoria* (Bivalvia), and *Haliotis discus* (Gastropoda), were completed as well as the pearl oyster.

KEY WORDS: corrosion cast, digestive organ, digestive diverticula, duct, tubule

INTRODUCTION

Molluscs absorb food and nutrients, secrete digestive enzymes, and store the nutrients in the digestive diverticula that develops at the circumference of the stomach. The digestive diverticula is connected with the stomach by ducts (Owen 1955a, 1995b, Purchon 1957, 1958, 1960). The structure of the tip of the digestive diverticula is shown as a terminal vesicle (Owen 1955a, 1995b, Nakajima 1956). Yonge (1926) demonstrated the structure of the stomach and the main ducts of the digestive diverticula with a cast made in gelatine in the Pacific oyster, *Crassostrea gigas,* but the secondary ducts and tubules were not cast.

Corrosion casting is well suited to study the three-dimensional structure of the cardiovascular–respiratory system; however, there is little information provided on the structure of the branch and connection in the secondary ducts and tubules with casting. Information on the structure of digestive diverticula will be useful for the research of taxonomy and the function of digestive diverticula.

This study examined corrosion casting as a means of characterizing the whole digestive organ, especially digestive diverticula, with prepolymerization methacrylate in some molluscs with special emphasis on the pearl oyster, *Pinctada fucata martensii.*

MATERIALS AND METHODS

Pearl oysters were obtained from a farm in Tsushima, Nagasaki prefecture. After cleaning the shell valves, they were reared for 5–10 days in running seawater filtered to remove particles >0.5 μm. The experiments were conducted in 60 pearl oysters (mean shell length: 64.4 ± 5.8 mm (SD), shell height: 72.8 ± 4.3 mm, shell width: 24.6 ± 1.6 mm, and total wet weight: 40.3 ± 6.3 g).

The resin used was red prepolymerization methyl methacrylate (Mercox CL-R, Oken Shoji) and hardener (Mercox MA, Oken

Shoji). When both reagents were mixed at 5–20%, the resin started to gradually solidify after about 5 min. Therefore, they were mixed just before injection to the digestive organ. After the pearl oyster was relaxed enough in 0.4 mM $MgCl_2$ solution (Namba et al. 1995), the left shell valve was removed and the mantle was dissected to expose the labial palp. Polyethylene tubing (1 mm in outer diameter, 20 cm length, Hibiki No. 3), which inflated the tip spherically in order to prevent the counterflow of the resin, was inserted about 5 mm from the mouth to the esophagus. Then, 4 mL of resin were injected within 3 min with a plastic syringe of 5 mL capacity. The tubing was sealed with the flame to stop the resin overflowing, and the injected pearl oyster was returned to the seawater. After it was left at least for 1 h and the resin hardened, the pearl oyster was immersed in 20% NaOH solution for 1 day at room temperature, and then washed with tap water. The completed corrosion castings were preserved in the 0.1% sodium azide.

We also examined the injection of the resin from the anus, the casting to the pearl oyster which was preserved in formalin, and the addition of methyl methacrylate (Nisshin EM) in order to lower the viscosity of the resin. We also cast other mollusks: ark shell, *Scapharca broughtonii,* Pacific oyster *C. gigas,* clam, *Meretrix lusoria* (Bivalvia), and abalone, *Haliotis discus* (Gastropoda) using this method.

RESULTS AND DISCUSSION

The cast was easily made from the mouth to the anus (Fig. 1). Within the digestive diverticula, various features of the casting were observed, such as the tubule that surrounded the stomach (Fig. 1A), the main duct (Fig. 1B), the ducts of digestive diverticula without tubules (Fig. 1C), and the ducts and tubules (Fig. 1D, E). The cast was also showed the stomach and the orifice of the ducts of the digestive diverticula that were illustrated by Yonge (1926). The ducts and the tubules, which Owen (1955a, 1955b) and Nakajima (1956) showed, were also observed (Fig. 1E).

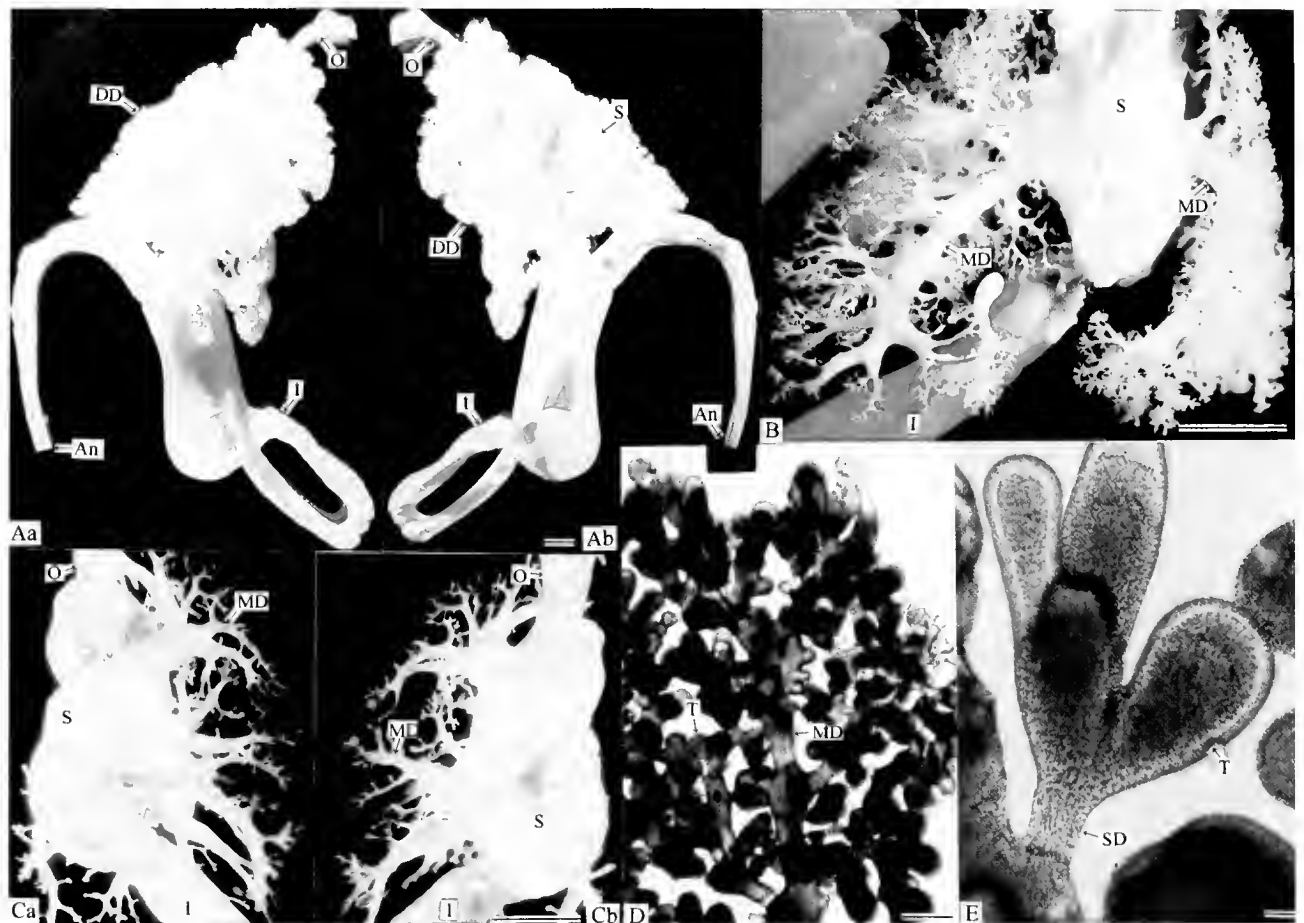*Corresponding author. E-mail: handat@fish-u.ac.jp

Figure 1. The corrosion cast of the digestive organ in the pearl oyster, *Pinctada fucata martensii*. A, the whole of digestive organ; B, the main duct; C, the stomach and the main duct without the tubule; D, the main duct and the tubule; E, the secondary duct and the tubule. The a and b represent the right and left aspects, respectively. Esophagus (O), digestive diverticula (D), stomach (S), intestine (I), anus (AN), main duct (MD), secondary duct (SD), and tubule (T). Bars in A, B, and C = 1 mm, bar in D = 100 μm, and bar in E = 1 μm.

Injecting the resin which contained the hardener at 20%, it always filled the ducts and tubules, terminal spaces (Fig. 1A). In low concentration at 5%, the resin reached the ducts (Fig. 1B and C). After casted, the observation was not often easy because there was hardly contrast on the castings. Then, they were immersed in 20% NaOH solution at 60°C. As a result, many contrasts (e.g., red to pink) emerged (Fig. 1B).

When the methyl methacrylate was added to the resin to lower the viscosity, the castings come apart to pieces in 20% NaOH solution for the proteolysis and could not be completely made.

Previous studies have demonstrated the opening and closing of the tubule in the digestive diverticula synchronized with tidal periods (Morton 1956, Morton 1970, Owen 1972), circadian rhythms (McQuiston 1969, Morton & McQuiston 1974, Robinson & Langton 1980), and food intake (Morton 1969, McQuiston 1969, Morton 1979, Robinson & Langton 1980). In this study, the castings seems to be not influenced the conditions of the tubule, because they were made regardless of them.

The food particles are transported to the tubules by the ciliary movement in the stomach (Owen 1955a, 1955b, Purchon 1957, 1958, 1960), and ciliary and/or muscular movement of the digestive diverticula (Owen 1955a, 1995b). Castings were similar

whether resin was injected from the anus or the mouth. It was not possible to make casts with animals preserved in formalin. Thus the resin is probably transported to the tubules by not only the injected pressure but also similar functions of sending the particles from the stomach to tubules.

We also examined the corrosion casting of the digestive organs in 4 molluscs. As the results, it was possible to cast and observe the ducts and tubules in ark shell *S. broughtonii* (Fig. 2A), Pacific oyster *C. gigas* (Fig. 2B), clam *M. lusoria* (Fig. 2C), and abalone *H. discus* (Fig. 2D). Therefore, these methods are applicable to cast the digestive organ in molluscs.

The detailed structure of digestive diverticula is suggested by the histological method, but it is very difficult to indicate the distribution of ducts and tubules, or to grasp a sense of the three-dimensional aspects of the structures. This casting method accurately shows the whole structure of digestive diverticula, for example, the positional relation to the stomach, and the features of branch and connection of ducts and tubules. Information on the digestive diverticula map will be very important for the criteria for classification, and also useful to investigate the function of digestion and absorption through the digestive canal, especially in the digestive diverticula and stomach.
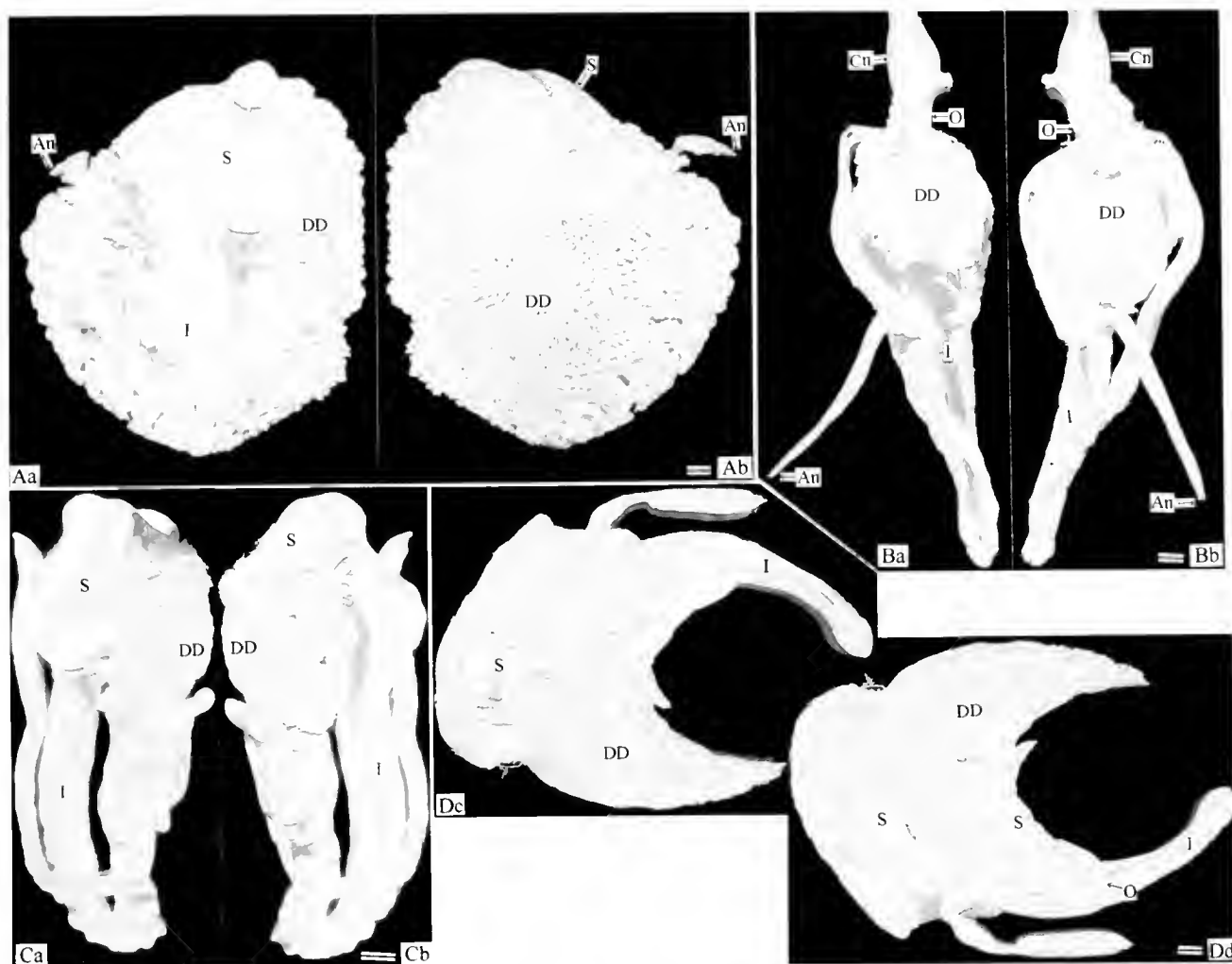
Figure 2. The casting preparation of the digestive organ of four molluscs. A, ark shell *Scapharca broughtonii*; B, pacific oyster *Crassostrea gigas*; C, clam *Meretrix lusoria* (Bivalvia); D, abalone *Haliotis discus* (Gastropoda). The a and b represent the right and left aspects, respectively. The c and d represent the dorsal and ventral views, respectively. Anus (AN), stomach (S), digestive diverticula (DD), Oesophagus (O), intestine (I). The cannula (Cn) is polyethylene tubing which was used for the injection of resin. Bars = 1 mm.

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MITOCHONDRIAL DNA REVEALS GENETIC DIFFERENTIATION BETWEEN AUSTRALIAN AND INDONESIAN PEARL OYSTER *PINCTADA MAXIMA* (JAMESON 1901) POPULATIONS

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ABSTRACT A total of 234 individual silver-lipped pearl oyster (*Pinctada maxima*) from six populations in Australia and two populations in Indonesia were analyzed for genetic variation within a 680-base pair region of the mitochondrial DNA COI gene using restriction fragment length polymorphism analysis. The Indonesian populations were markedly different from all Australian populations examined, and the differences were greater than that expected on the basis of their geographical separation. In contrast with this broader regional pattern of genetic differentiation, the Australian populations sampled were not significantly differentiated from one another, and a high degree of connectivity was observed among Western Australian pearl oyster populations. In addition, these genetic data show that Western Australian *P. maxima* populations have a closer tie to those from the northern Australian coast than with populations in Indonesia. This regional pattern of genetic separation is evident despite the proximity of Indonesia to the eastern Indian Ocean locations sampled and the potential for dispersal afforded by the southward currents of the Indonesian throughflow.

KEY WORDS: aquaculture, biogeography, fisheries management, Indo-Pacific, mitochondrial DNA, pearl oyster, *Pinctada maxima*, population genetics.

INTRODUCTION

The silver-lipped pearl oyster, *Pinctada maxima* (Jameson 1901) is found in Southeast Asia and northern Australia and provides the basis for the strong south sea pearling industry (Shirai 1994). Although there is increasing use of hatchery stock, the industry in Western Australia is still dependent upon the collection of wild shell and upon the effective management of wild stocks. Early work by Johnson and Joll (1993) showed marked differences in allozyme frequencies in pearl oyster populations collected from northern and Western Australia (WA), suggesting that these needed to be managed separately. Despite significant genetic differences detected between two northern populations of *P. maxima*, which are separated by as little as 320 km, Johnson and Joll (1993) found no differentiation between the two WA populations that were sampled some several hundred kilometers apart. On the basis of this limited sampling of just two populations, the pearl oyster stocks within this important pearl producing region were considered essentially panmictic.

Significant genetic differences have also been detected between populations of several other species of pearl oyster over a range of spatial scales. *Pinctada fucata* (Gould 1850), *Pinctada albina* (Lamarck 1819), and *Pinctada maculata* (Gould 1850) were shown to be differentiated between sites less than 100 km apart (Wada 1982). Small genetic differences have also been observed between both widespread (Durand & Blanc 1986, 1989) and geographically closer populations (Benzie & Ballment 1994) of the black lip pearl oyster *Pinctada margaritifera* (Linnaeus 1758). Finally a genetic study of *Pinctada radiata* (Leach 1814) revealed significant differentiation between sites less than 33 km apart (Beaumont & Khamdan 1991). This collection of studies suggests the potential for population substructure within the *P. maxima* pearl oyster stocks, which extend for thousands of kilometers along the WA coast.

The maternally inherited mitochondrial DNA (mtDNA) ana-lyzed in the present study has a smaller effective population size than the allozyme genetic markers used by Johnson and Joll in their 1993 study and, as such, is more sensitive to the effects of genetic drift and consequently often affords greater sensitivity for detecting genetic differences in population studies. Given the importance of the WA pearl oyster stocks to the Australian pearling industry, a sensitive genetic study of WA population substructure with more extensive spatial coverage than that of Johnson and Joll (1993) was undertaken. In addition, the analysis of collections from both Indonesia and northern Australia, two potential long-distance sources of recruits, allowed larger scale connectivity to be assessed.

Populations of a number of marine species from northwest Australia have closer genetic affinities with Pacific rather than Indian Ocean populations despite being situated geographically in the Indian Ocean (Benzie 1999). These results are consistent with a connection via the strong currents of the Indonesian throughflow which move south from Indonesia towards Australia. Given that *P. maxima* is a broadcast spawner with a larval life of 2 to 3 wk (Shirai 1994), this species is potentially capable of dispersal over long distances. For this reason the present study examines the extent to which WA populations may derive recruits from both Indonesia and northern Australia.

The present article reports the genetic structure of *P. maxima* stocks using mtDNA to determine local population structure within WA and the extent of connectivity to both Indonesian and northern Australian populations.

MATERIALS AND METHODS

Sample Collection

Between 27 and 30 adult *P. maxima* were analyzed from each of six populations in Australia and two populations in Indonesia. Samples of adductor muscle were collected from *P. maxima* oysters aboard pearling industry vessels between February 1998 and November 1999. Samples were obtained in Northern Australia to the west of Darwin and in Western Australia from the Lacepede Islands, 80 Mile Beach (shallow water), 80 Mile Beach (deep

water), Port Hedland, and Exmouth Gulf (Fig. 1). The 80 Mile Shallow collections were made inshore at less than a 10 m depth from the Northern end of 80 Mile beach. The 80 Mile Deep collections were made at a similar latitude but from a more offshore site at ~30 m depth. The two Indonesian populations, Madura and Sumbawa Island, were collected in November 1999. Live animals were delivered by road to Gondol Fisheries Station and held in flowing sea water tanks before dissection. Adductor muscle samples were immediately snap frozen in liquid nitrogen after collection.

DNA Extraction and Polymerase Chain Reaction (PCR)

DNA was extracted from using a CTAB extraction procedure modified from Adamkewicz and Harasewych (1996) in which small cubes of frozen muscle (~0.5 cm^3) were ground in pre-warmed (60°C) CTAB extraction buffer (2% CTAB, 2% polyvinylpyrrolidone, 100 mM Tris-HCl pH 8.0, 1.4 M sodium chloride, 20 mM EDTA) to which proteinase K was added to a final concentration of 0.5 mg/mL. After overnight incubation at 60°C, samples were heated to 90°C for 20 min before addition of RNase A (0.1 mg/mL) and a 1-h incubation at 37°C. DNA was then extracted and precipitated using standard phenol:chloroform: isoamyl alcohol methods as per Sambrook et al. (1989).

Echinoderm universal primers for the Cytochrome Oxidase 1 (COI) gene (CoI$_3$ fwd: 5' ATA ATG ATA GGA GGR TTT GG 3' and CoI$_e$ Rev: 5' GCT CGT GTR CTA CRT CCA T 3' (Williams

1997) were used to amplify a 680-base pair segment of that gene. PCR reactions were conducted with 2 ng/µL DNA in a 1X PCR buffer containing 1.5 mM MgCl$_2$, 0.03 units/µL Taq DNA polymerase (Qiagen, Australia), 200 µM dNTPs, and 0.5 µM each primer. Thermocycler conditions were 94°C for 1 min (one cycle), followed by 94°C for 1 min, 45°C for 1 min, 72°C for 1 min, 30 s (30 cycles), with a final 4°C hold. Fifty-microliter PCRs were performed in a Perkin–Elmer 9700 thermocycler.

Restriction Fragment Length Polymorphism (RFLP) Analysis

Of the 39 restriction enzymes tested, only five (DpnII, Eco0190 I, FokI, HaeIII, and NlaIV) produced polymorphic fragment patterns, and these were used to survey RFLP variation within the amplified region of the COI gene. Overnight digest reactions contained 5 µL of PCR product and ~0.03 units/µL restriction enzyme (New England Biolabs, Beverley, MA) in a 15-µL reaction with 1X buffer as per the enzyme manufacturer's instructions. Digest fragments were separated on 3% agarose gels (2% GibcoBRL agarose-1000, 1% Progen DNA grade agarose) at 4–5 volts/cm for up to 5 h with repeated photography of ethidium bromide-stained gels throughout the running period. Fragment sizes were estimated by regression against standard size markers and for each restriction enzyme the unique fragment patterns were given an alphabetical assignation (Table 1). The position of each restriction enzyme site producing the unique fragment patterns was identified by DNA sequencing of several individuals and a composite profile of the
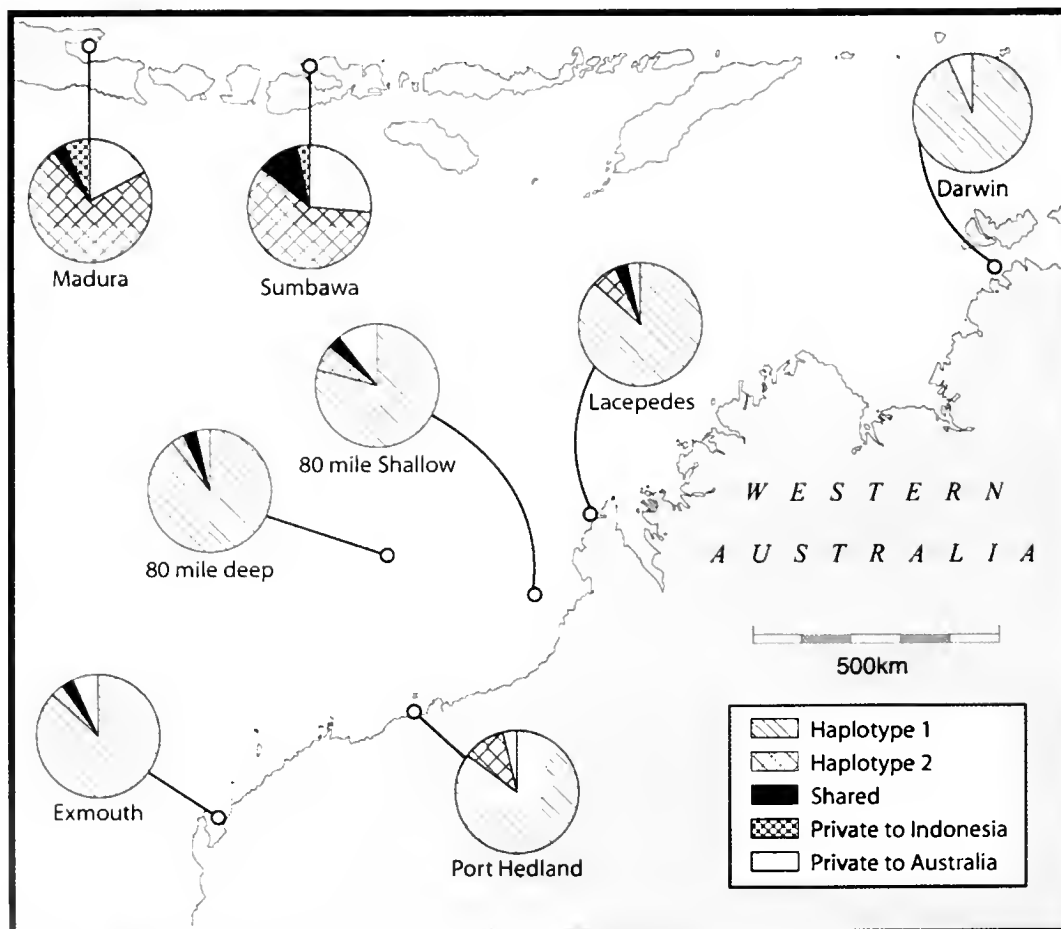

Figure 1. Pie diagrams illustrating the frequencies of the major haplotype or haplotype groups differentiating the eight *P. maxima* populations.

TABLE 1.

Mitochondrial DNA restriction fragment sizes observed among 234 *Pinctada maxima* from Australia and Indonesia

Enzyme	Haplotype	Fragment Sizes (bp)
*Dpn*II	a	454,226
	b	263,226,145
	c	226,158,145,105,45
	d	408,226,45
*Eco*01091	a	680
	b	590,93
*Fok*I	a	603,77
	b	680
	c	353,250,77
	d	448,155,77
*Hae*III	a	299,291,51,42
	b	590,51,42
	c	632,51
*Nla*IV	a	388,249,43
	b	388,155,94,43
	c	431,249
	d	543,94,43

presence/absence of each site was constructed for each animal for all restriction enzymes (Table 2).

Statistical Analyses

The DA program in REAP (McElroy et al. 1992) was used to estimate haplotype diversity (h) and nucleotide diversity (π) within populations and nucleotide divergence (d_{XY}) among populations (Nei & Tajima 1981). Spatial structuring of the populations was investigated using programs in ARLEQUIN (Schneider et al. 2000). AMOVA (Excoffier et al. 1992) was used to calculate ϕ_{ST} (analogous to F_{ST}), $N_e m$, and to perform hierarchical analysis of ϕ_{ST}. The MXCOMP program in NTSYS (Rohlf 1997) was used to calculate the Mantel test (Mantel 1967) to measure the degree of association between the matrix of pairwise ϕ_{ST} comparisons and the geographic distance between populations. Significance levels for simultaneous multiple tests were adjusted following Rice (1989). Further analysis, such as mismatch distributions, tests of neutrality, and timing of population expansion were not conducted because the small number of sites covered by the RFLP data would result in large errors and low statistical power.

A character state matrix showing the presence or absence of presumptive restriction sites created using programs in REAP (McElroy et al. 1992) was used to construct unrooted, phylogenies using the maximum likelihood method in the RESTML program in PHYLIP, which assumes a Jukes-Cantor model of evolution (Felsenstein 1993), and the parsimony method implemented in PAUP (Beta Version 4.0b2; Swofford 1990). RESTML was set to find the best tree with global rearrangement of subtrees and input order of the haplotypes jumbled three times. In PAUP, restriction sites were treated as relaxed Dollo characters with gains weighted twice as heavily as losses (McMillan & Bermingham 1996). One thousand optimal trees were found using a heuristic search with the tree bisection and reconnection branch swapping algorithm and the 50% majority rule consensus was applied to obtain a single consensus tree.

RESULTS

The survey identified 16 composite haplotypes among the 234 samples (Table 2, Fig. 1). Two haplotypes (12.5%) accounted for 91% of the individuals assayed (213 individuals). Nine haplotypes (56.3%) were unique, accounting for 3.9% of the animals. The other five haplotypes (31.3%) were each represented by only two or three animals. At the population level, nine haplotypes were private (i.e., occurred in only one population) while at the regional level, eight haplotypes were private to Australia (62% of all haplotypes found in Australia) and three private to Indonesia (38%).

Genetic Diversity Within Populations

On average, the Indonesian populations had higher levels of genetic diversity than the Australian ones, with Darwin having the lowest level of all (Table 3). The pattern was seen most clearly in the data for haplotype diversity (h), which was two times greater in the Indonesian populations (mean h = 0.520) than in the Australian populations (mean h = 0.246). Nucleotide diversity (π) was also two times greater in the Indonesian populations (mean π = 0.0097) than in the Australian populations (mean π = 0.0046).

Genetic Differentiation Among Populations

The most common haplotype (1) was more frequent in Australian populations, where it comprised 79–93% of the individuals assayed compared with 17–27% in the Indonesian populations. The next most common haplotype (2) was more frequent in Indo-

TABLE 2.

Composite mtDNA haplotypes observed among 234 *Pinctada maxima* from Australia and Indonesia

Composite Haplotype Number	*Dpn*II	*Eco*01091	*Fok*I	*Hae*III	*Nla*IV
	abcd	e	fgh	ijk	lmn
1	1011	0	001	111	011
2	1011	1	001	111	111
3	1011	1	000	111	111
4	1011	0	001	101	011
5	1011	0	011	111	011
6	1111	1	001	111	111
7	1011	0	001	111	111
8	1011	0	000	111	011
9	0011	0	001	111	011
10	0001	0	001	111	011
11	1011	1	101	111	111
12	1011	0	001	111	010
13	1011	1	001	101	111
14	1011	1	001	111	101
15	1011	1	001	111	011
16	1011	0	001	100	010
	1110	**1**	**112**	**031**	**211**

Presence (1) or absence (0) of restriction sites was inferred from banding patterns obtained from single digestions of extracted total DNA with each of the five restriction enzymes (Table 1). Bold numbers at the base of the table indicate the number of times a cutting site was lost in the maximum likelihood phylogeny. The 16 haplotypes represented 14 putative cutting sites, two of which were present in all animals surveyed. In the maximum likelihood phylogeny one site (j) was lost three times and two (h, l) were lost two times. The remainder were lost once (nine sites).

TABLE 3.

Measures of genetic diversity within populations: number of haplotypes (n_h), the ratio of (n_h) to the number of individuals sampled (n_i): $[(n_h/n_i)]$, haplotype diversity (h), and nucleotide diversity (π) within each of eight populations of the pearl oyster *Pinctada maxima*

Population	n	n_h	n_h/n_i	h ($\pm$ SE)	π
Madura	29	5	0.17	0.458 ($\pm$ 0.102)	0.0086
Sumbawa	30	5	0.17	0.582 ($\pm$ 0.079)	0.0107
Darwin	30	2	0.07	0.129 ($\pm$ 0.079)	0.0026
Lacepedes	30	4	0.13	0.251 ($\pm$ 0.102)	0.0051
80 Mile Deep	29	4	0.14	0.200 ($\pm$ 0.098)	0.0028
80 Mile Shallow	29	6	0.21	0.374 ($\pm$ 0.113)	0.0078
Port Hedland	27	3	0.11	0.271 ($\pm$ 0.105)	0.0048
Exmouth	30	5	0.17	0.253 ($\pm$ 0.104)	0.0045
Average		4.25 ($\pm$ 0.49)	0.15 ($\pm$ 0.02)	0.315 ($\pm$ 0.056)	0.0059 ($\pm$ 0.0011)
All populations	234	16	0.07		

nesian populations where it comprised 60–72% of individuals compared with 3–11% in Australian populations. These data, and the fact that three haplotypes were private to Indonesia and eight were private to Australia, suggest considerable regional differentiation among populations (Fig. 1). There were highly significant pairwise ϕ_{ST} values between the two Indonesian populations and all the Australian populations, the mean ϕ_{ST} being 0.562 (Table 4). There was no significant differentiation among populations within Australia (with the exception of Darwin and some Western Australian sites) or among populations within Indonesia. The significant differentiation of Darwin and some Western Australian populations (mean ϕ_{ST} = 0.038) was an order of magnitude less than that for the Indonesian-Australian comparisons. A hierarchical AMOVA analysis, partitioning variation within populations, between populations within regions (Indonesia and Australia), and between regions, confirmed that all of the genetic variation occurred within populations (47%), and between regions (53%; $P < 0.05$).

When pairwise ϕ_{ST} values were plotted as a function of the geographical separation of the populations, the Australian-Indonesian comparisons formed a separate group whose degree of genetic differentiation was far greater than those comparisons within regions (Fig. 2). The Mantel test of ϕ_{ST} against distance in km for the total data set was significant ($r = 0.69$, $P < 0.001$). However, when the data were decomposed into comparisons either between or within regions, there was no significant relationship between ϕ_{ST} and geographical separation among Australian populations ($r = 0.56$, $P = 0.156$). There was not enough data to allow

a test within Indonesia. The pattern of connectivity among populations (using the effective number of migrants per generation (N_{em}) as the measure of exchange) emphasizes the strong connection within regions and the limited exchange between regions (Fig. 3).

Haplotype Phylogeny

The 50% consensus tree based on parsimony analysis showed little structure and no deep relationship between haplotype groupings related to their geographical distribution (Fig. 4). The maximum likelihood network (not illustrated) was dominated by two star-like nodes each centered on one of the two most common haplotypes (haplotypes 1, 2). Both stars included haplotypes found in either Australian and Indonesia or both regions, and most haplotypes differed by only one restriction site change from the dominant haplotypes.

DISCUSSION

RFLP analysis of a portion of the mitochondrial COI gene has provided strong evidence for high levels of dispersal among WA populations of *P. maxima* confirming the findings of Johnson and Joll (1993) based on nuclear markers (allozymes). The analysis also showed clearly that the WA populations were more closely connected to northern Australian populations than to Indonesian ones. There has been movement of pearl oysters by the cultured pearl industry, largely from some WA wild sites to farms in the Northern Territory, but it is highly unlikely that the pattern ob-

TABLE 4.

Pairwise F_{st} among eight populations of the pearl oyster *Pinctada maxima*

	Madura	Sumbawa	Darwin	Lacepedes	80 Mile Deep	80 Mile Shallow	Port Hedland
Sumbawa	−0.023[ns]	—					
Darwin	0.684***	0.617***	—				
Lacepedes	0.569***	0.497***	0.046[ns]	—			
80 Mile Deep	0.657***	0.585***	0.000[ns]	−0.000[ns]	—		
80 Mile Shallow	0.521***	0.456***	0.050***	−0.008[ns]	0.010[ns]	—	
Port Hedland	0.560***	0.487***	0.057*	−0.028[ns]	0.005[ns]	−0.024[ns]	—
Exmouth	0.592***	0.521***	0.038[ns]	−0.022[ns]	−0.013[ns]	−0.012[ns]	−0.024[ns]

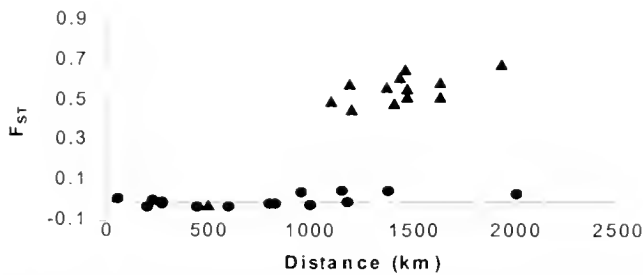* $P < 0.05$; *** $P < 0.001$; [ns] not significant.

Figure 2. F_{ST} graphed as a function of the geographical separation of the population pairs. Comparisons with Indonesian populations have been given a different symbol (triangles).

served in this study is related to those stock movements. Using allozymes, Johnson and Joll (1993) noted clear genetic differences between the WA populations and those from Oxley Island in the Northern Territory despite large numbers of WA animals having been introduced to a farm within 80 km of Oxley Island. The present study sampled wild populations in the Darwin region that were geographically more distant from the nearest farm supporting the suggestion that the connectivity observed between the Darwin and WA populations is unlikely to be a reflection of stock transfer.

The level of divergence of *P. maxima* populations using the mitochondrial COI gene RFLP data (assuming a 2% divergence per million years) suggests present day mixing between all Australian populations but isolation of Indonesian and Australian populations during the Pleistocene at least 100,000 years ago. Caution needs to be applied to these interpretations because of the limited data available in the RFLP analysis and the large errors inherent in these types of estimates in any case (Edwards & Beerli 2000, Kishino et al. 2001, Zhivotovsky 2001). Nevertheless, these data present a consistent picture of divergence between Indonesian and Australian populations well before the last low sea level stand around 12,000 years ago. Therefore, despite the apparent possibility for migration of marine invertebrate larvae from Indonesia on strong southerly flowing currents the limited gene exchange observed between Indonesian and WA populations of *P. maxima* is consistent with the strong westward deflection of the Indonesian

throughflow just south of the Indonesian arc which makes it unlikely that this current would reach the coastal regions of Australia.

In population genetic analysis of the giant tiger prawn *Penaeus monodon* (Fabricius 1798) also using RFLP analysis of mtDNA, Benzie et al. (2002) showed a closer relationship between the WA population of *P. monodon* and those from northern and eastern Australia, and a clear distinction from Indonesian and Philippines populations. The Philippines sample was to some extent intermediate between Indonesian and Australian samples, suggesting links to Southeast Asia primarily via eastern Southeast Asian and eastern Australian populations and also linkages to WA via northern Australia. There were no samples of *P. maxima* available from the Philippines or elsewhere in Southeast Asia or eastern Australia for the present study but the pattern observed in *P. maxima* over the range surveyed is consistent with that for *P. monodon*.

A strong genetic divide between the Pacific and Indian Ocean populations found in several species of marine invertebrates may involve considerable shifts in gene frequency as well as deep divisions in haplotype phylogeny (Benzie 1999, Barber et al. 2000). In contrast, genetic distances between the Pacific and western Australian populations of marine invertebrate species may be an order of magnitude less (Benzie 1999) and in mtDNA markers may involve differences in the frequency of relatively closely related haplotypes (Williams & Benzie 1997, 1998, Benzie et al. 2002). The fact that Indonesian and northern/WA populations of *P. maxima* did not show deep divergence of COI haplotypes associated with geographical region is consistent with these studies. The fact that genetic diversity is higher in southeast Asian populations of *P. maxima* than in Australian populations is also consistent with a general trend of decreasing genetic diversity outwards from southeast Asia to more geographically distant sites (Benzie et al. 2002).

Marked genetic differences between WA and Indonesian pearl oyster stocks contrasts with considerable gene exchange over thousands of kilometers among Western Australian populations. The mtDNA variation in *P. maxima* populations between Indonesia and Australia suggests a strong influence of biogeographical events at the regional scale. Future assessments of larger scale patterns of dispersal of this species should include samples from elsewhere in the Indian Ocean and from additional locations in Southeast Asia and from eastern Australia.

Figure 3. Map illustrating the levels of gene flow between *P. maxima* populations estimated from mtDNA.

	M (29)	S (30)	D (30)	L (30)	8D (29)	8S (29)	PH (27)	E (30)	All (234)	Rank
9						3.5			0.4	5
10			6.7						0.8	4
1	17.2	26.7	93.3	86.7	89.7	79.3	85.2	86.7	70.5	1
4						3.5	3.7		0.8	4
5				3.3					0.4	5
8	3.5				3.5	3.5			1.3	3
12								3.3	0.4	5
16						3.5			0.4	5
2	72.4	60.0		6.7	3.5	6.9	11.1	3.3	20.5	2
3		3.3						3.3	0.8	4
6		6.7		3.3					1.3	3
7	3.5								0.4	5
11	3.5								0.4	5
13						3.5			0.4	5
14		3.3							0.4	5
15								3.3	0.4	5

☐ Haplotype shared by Australia and Indonesia
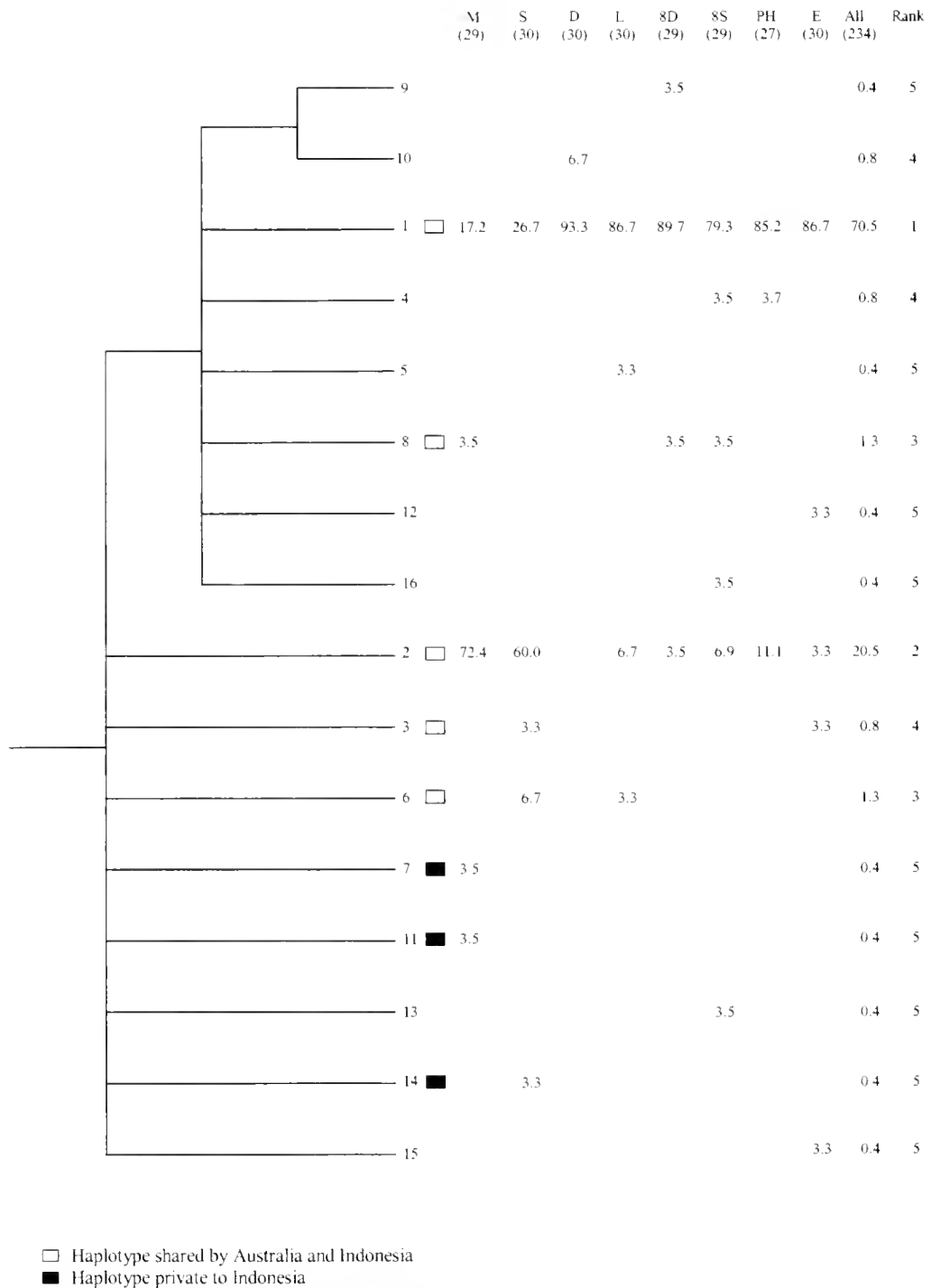■ Haplotype private to Indonesia

Figure 4. Percentage frequencies of the 16 composite mtDNA haplotypes observed among *P. maxima* individuals, and the 50% majority rule consensus tree (from 1000 maximum parsimony networks) indicating the relationships among the haplotypes. Numbers in parentheses immediately below the location codes indicate the number of individuals assayed. The column of numbers immediately to the right of the tips of the branches of the tree is the composite haplotypes listed in Table 2. The columns on the far right give the percentage of each haplotype in the total population, and their rank abundance, respectively. Locations are as follows: M, Madura; S, Sumbawa; D, Darwin; L, Lacepedes; 8D, 80 Mile Beach deep; 8S, 80 Mile Beach shallow; PH, Port Hedland; E, Exmouth Gulf.

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SHALLOW-WATER DISTRIBUTION AND POPULATION CHARACTERISTICS OF *STROMBUS GIGAS* AND *S. COSTATUS* (GASTROPODA: STROMBIDAE) IN BOCAS DEL TORO, PANAMA

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ABSTRACT   Extensive visual surveys for the economically and ecologically significant queen conch (*Strombus gigas*) and milk conch (*Strombus costatus*) were conducted within the Bocas del Toro archipelago. Overall population densities are among the lowest recorded in the region (*S. gigas* 1.43 conch ha^{-1}; *S. costatus* 1.27 conch ha^{-1}), and are likely the result of overexploitation by both commercial and subsistence fishing. The very low adult densities (*S. gigas* 0.30 conch ha^{-1}) and the lack of reproductive behaviors observed are a serious concern when one considers the "Allee effect" and the resultant negative *per capita* population growth rates reported elsewhere in the literature. This information has provided some of the rationale for establishing the recently announced 5-y ban on conch exploitation on the Caribbean coast of Panama.

KEY WORDS: queen conch, stock assessment, overfishing, Panama, Allee effect, *Strombus*

INTRODUCTION

Strombus gigas Linnaeus, 1758, and *Strombus costatus* Gmelin, 1791, are two herbivorous gastropods of the family *Strombidae* that inhabit shallow seagrass meadows (SGs), sand beds, and algal flats throughout the Caribbean. Queen conchs have long been valued for their meat and shell, and were first harvested in the Caribbean by the Lucayans and Arawaks during pre-Columbian times (Brownell & Stevely 1981, Berg & Olsen 1989). Local commercial and subsistence use of both conch species has continued to this day and on occasion still provide a primary source of protein in some fishing communities.

During the last 30 y, the overall harvest of queen conch has increased substantially, driven largely by international export as well as growing resident populations and increasing tourism in the Caribbean region (Berg & Olsen 1989, Tewfik 1997). Conch is commercially exploited in at least 22 countries throughout the region, and is often consumed only as a luxury food item due to its relative rarity and high market value (Mulliken 1996, Theile 2001). The shell products of several strombids are also sought after and are well recognized in the tourist industry of many Caribbean nations. Present landings of conch meat in the region are now in excess of 13,000 metric tons (Food and Agriculture Organization of the United Nations 2000). However, it should be noted that Food and Agriculture Organization landings are for all "Strombid conchs" and may therefore include several species. Significant landings of other strombids, including *S. costatus*, are likely to be occurring in places such as Mexico (Gil 1994, Theile 2001). The fear of the disappearance of commercial Queen conch fisheries has prompted *S. gigas* to be included under appendix 2 of the Convention for the International Trade of Endangered Species (CITES) in 1992. Most recently, CITES has initiated a "significant trade review" for the species (Theile 2001).

The San Blas and Bocas del Toro archipelagos are the main areas of conch fishing in Panama (Martans 1997). Limited data are available for the total number of conch landings in Panama, such landings being considered incidental to the spiny lobster harvest, with the latest figure being 116 metric tons in 1998 (Martans 1997, Autoridad Marítima de Panamá 1999). No specific regulations exist for the harvest of either *S. gigas* or *S. costatus* in Panama,

however, the use of scuba gear is prohibited for the harvest of any marine resource (Martans 1997). Aside from the role that conchs serve in both local and regional economies, their populations provide critical links between primary producers and higher-level consumers within near-shore marine communities throughout their range (Stoner & Waite 1991, Stoner et al. 1995).

The following article will describe the abundance, population structure, morphology, and spatial distribution of *S. gigas* and *S. costatus*, which have been heavily exploited over the last few decades in the Bocas del Toro archipelago. The consequences of this exploitation on future recruitment will also be discussed. Finally, some brief comments will be made regarding the potential interaction that may exist between the two strombids defined here, with special attention to the spatial partitioning of these species over shallow, near-shore seagrass-sand-algal complexes that are typical of many areas of the Caribbean.

MATERIALS AND METHODS

The study was conducted over a 47,158-ha area of shallow water (<10 m) habitats in the Bocas del Toro archipelago between February and September 2000. A comprehensive description of the sea bottom topography, climate, geology, and reef distribution of the archipelago are available in several other publications (Rodríguez et al. 1993, Greb et al. 1996, Guzman & Guevara 1998). The entire shallow (<10 m) coastal zone, irrespective of habitat type, was divided into 240 2 × 2-km grid squares, of which 120 grids or sites were randomly selected and surveyed. Within each site, three replicate belt transects (100 × 6 m) were surveyed by two divers (width 3 × 3 m each) at each of two different depth strata (0.5–5 and 5–10 m). In total, each site had 1800 m^2 per depth strata or 3600 m^2 in total area surveyed.

All strombids located within a transect were counted and measured for total shell (siphonal) length (SL), maximum shell width, and lip thickness (at mid-lateral region approximately 40 mm from the edge) to the nearest millimeter using a caliper. Adult status was assigned to all conchs with a lip thickness >4 mm (Appeldoorn 1988). The depth and major substrate/habitat type where the strombids were located was also noted. The substrate/habitat types were classified according to a predefined typology that included only the most common habitats: algal plain (AP); SG; sand plain (SP); and coral rubble (CR) (Table 1). All data sets were analyzed using

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TABLE 1.

Substrate/habitat categories used in characterizing all sites surveyed within the Bocas del Toro archipelago, Panama.

Habitat	Code	Description
Algal plain	AP	Fine mud, coarse sand, rubble, shell bottom dominated by benthic algal cover (*Penicillus* spp., *Caulerpa* spp., *Dasycladus* spp., *Halimeda* spp., *Udotea* spp., *Padina* spp., *Laurencia* spp.)
Seagrass meadow	SG	Coarse sand bottom dominated by Turtle (*Thalassia testudinum*.) and Manatee (*Syringodium filiforme*.) grass.
Sand plain	SP	Coarse sand bottom with sparse or no benthic algae or seagrass cover.
Coral rubble	CR	Rubble, shell fragment bottom with sparse cover of macro and encrusting algae.

parametric statistics in SYSTAT, version 10.2 (Systat Software Inc., Richmond, CA).

The density distribution of the two species was mapped using Geographical Information System. A digital classification for the area of study was based on a combination of digital images from three sources: topographic maps at a scale of 1:50,000; color aerial photographs at a scale of 1:25,000; and LANSAT TM-5 satellite images (Guzman & Guevara 2002). Density data were integrated using the programs MIP (Micro Images Inc., Lincoln, NE), version 3.1 (Map and Image Processing System), and ArcView, version 3.0.

RESULTS

Shallow marine environments (<10 m) covered approximately 47,158 ha of the archipelago. A total of 432,000 m^2 (43.2 ha) was surveyed during the course of the study using 720 transects. A total of 45 *S. gigas* (80% juveniles) and 48 *S. costatus* (4.2% juveniles) were found during the entire 8-mo survey (February–September 2000). SL and shell lip thickness distributions occurred over the

normal ranges reported in the literature for both species (Fig. 1a, b). Significant correlations were made between total SL and shell width (*S. gigas* R^2 = 0.933, *P* < 0.05; *S. costatus* R^2 = 0.874, *P* < 0.05) (Fig. 2a). Although correlation coefficients were much lower for total SL versus lip thickness (*S. gigas* R^2 = 0.202; *S. costatus* R^2 = 0.533), the relationships were still significant (*P* < 0.05) (Fig. 2b). The lower correlation coefficients were to be expected, given the cessation of SL growth at sexual maturity (3.5–4.5 y old) followed by only lip-thickness growth during adulthood, which is typical for this group of mollusks (Alcolado 1976, Appeldoorn 1988).

S. gigas occurred at 20% of the 120 sites surveyed, with densities ranging from 0 to 27.8 conch ha^{-1}, a median of zero, and a mean (±SE) total density of 1.43 ± 0.37 conch ha^{-1} (adults 0.30 ± 0.11 conch ha^{-1}; juveniles 1.13 ± 0.31 conch ha^{-1}) (Fig. 3). *S. costatus* occurred at 11.7% of sites surveyed, with densities ranging from 0 to 58.3 conch ha^{-1}, a median of zero, and a mean total density of 1.27 ± 0.55 conch ha^{-1} (adults 1.23 ± 0.53 conch ha^{-1}; juveniles 0.05 ± 0.03 conch ha^{-1}) (Fig. 3). The highest densities for *S. gigas* (21–30 conch ha^{-1}) were observed in two regions
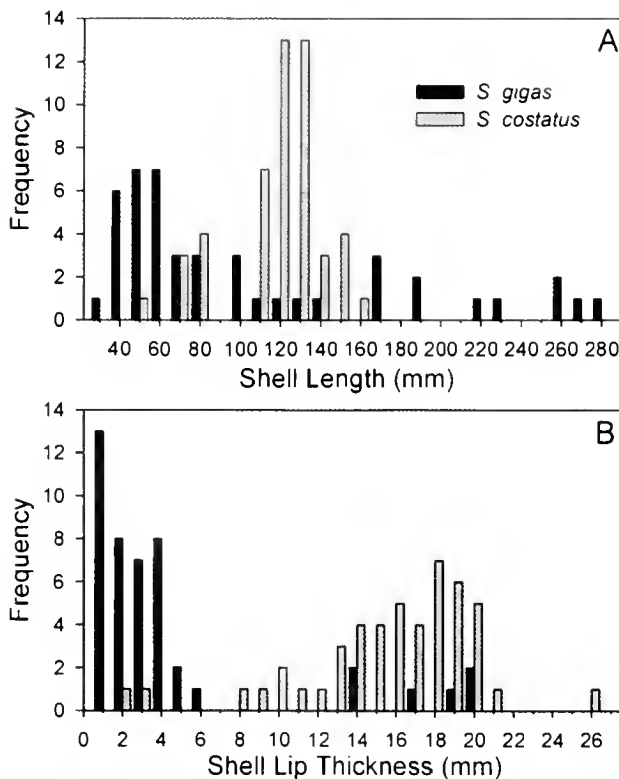

Figure 1. SL (A) and shell lip (B) frequency distributions of *S. gigas* (*n* = 45) and *S. costatus* (*n* = 48) in Bocas del Toro, Panama.
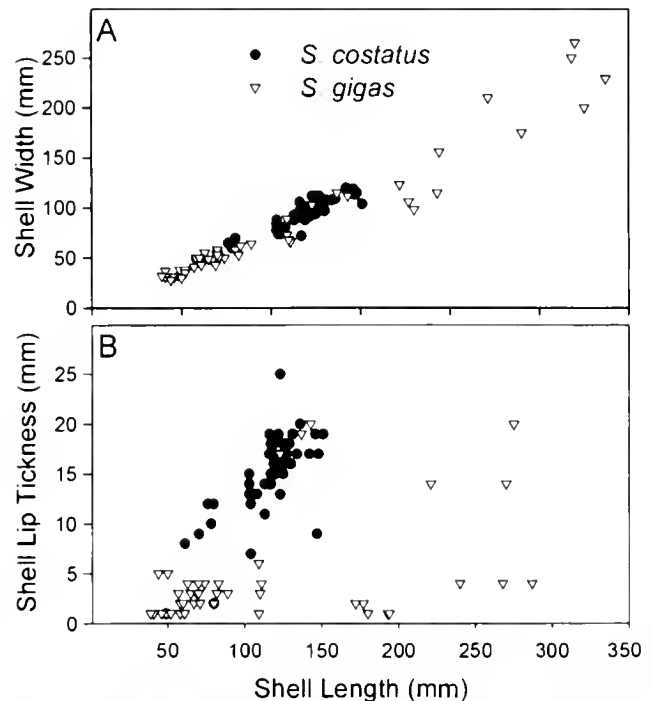

Figure 2. SL versus shell width (A) of *S. gigas* (R^2 = 0.933) and *S. costatus* (R^2 = 0.874), and SL versus shell width (B) of *S. gigas* (R^2 = 0.202) and *S. costatus* (R^2 = 0.533) in Bocas del Toro, Panama.
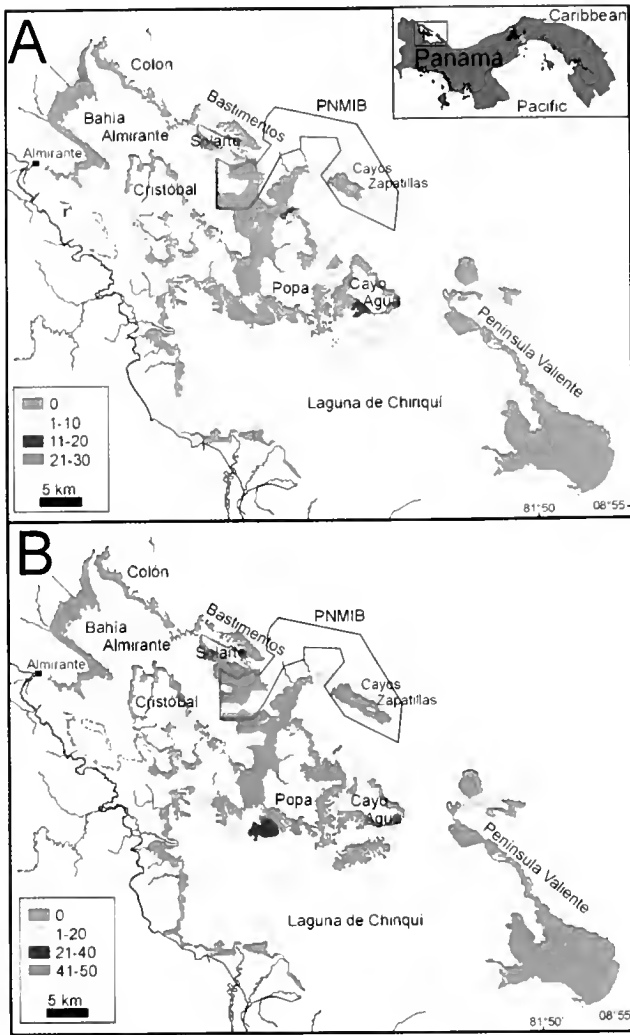
Figure 3. Density distribution (individuals/ha) of *S. gigas* (A) and *S. costatus* (B) over the shallow water (<10 m) in Bocas del Toro, Panama. The MPA is denoted by the polygon.

Figure 4. Habitats occupied by *S. gigas* and *S. costatus* in Bocas del Toro, Panama. See Table 1 for habitat descriptions.

DISCUSSION

The long-term, heavy exploitation of strombid populations within the shallow water habitats of the Bocas del Toro archipelago have likely contributed to the overall densities of *S. gigas* (1.43 conch ha^{-1}), which are among the lowest reported in the region (Table 2). Considerably less information is available for *S. costatus* in the literature, however, the densities observed here (1.27 conch ha^{-1}) are considered low when compared with that of Bermuda (2.6 conch ha^{-1}) (Berg et al. 1992) and the Southwest Dominican Republic (50–200 conch ha^{-1}) (Tewfik, unpubl. data). It is suspected that the densities of *S. costatus* began to decline only after the populations of the larger and more valuable fisheries species, *S. gigas*, were already at low levels.

Although this study has no information available on conch densities of <10 m, it did intensively survey habitats that are known to be important for conch as nursery and breeding areas throughout the region (Randall 1964, Stoner & Ray 1996, Tewfik et al. 1998, Stoner 2003). We suspect that areas down to ≥20 m may also have low densities, given the considerable capabilities of artisanal free divers that have been observed in Panama and other areas of the Caribbean (Martans 1997, Béné & Tewfik 2001), this despite the refuge that deeper waters might provide for adults. The low densities of conch and the lack of reproductive activity observed during this study become quite serious when one considers the "Allee effect," as described by Stoner and Ray-Culp (2000). Negative rates of *per capita* population growth were shown to occur below critical population levels. Specifically, mating (pairing and copulating) never occurred when adult densities fell below 56 conch ha^{-1}, and spawning never occurred with densities below 48 conch ha^{-1}. Again, no such reproductive activities were observed during the entire 8 mo (February–September) of this study, which covered the intense spring and summer reproductive period for conch (Randall 1964, Buckland 1989, Stoner et al. 1992, Tewfik et al. 1998). This has serious implications for the future levels of local recruitment and rebuilding of depleted populations, even with the establishment of MPAs and strict enforcement of fisheries regulations.

The spatial distribution of the two-strombid species was concentrated in the shallow (<5 m) SGs and is slightly surprising, given that these areas are the most accessible to local fishers. Another interesting element of the spatial distribution is that there was relatively little overlap (5 sites) out of the 33 sites occupied by either species (Fig. 3). This begs the question of whether there may

encompassing 1.7% (805 ha) of the total area. The lowest densities (1–10 conch ha^{-1}) were found in 19 scattered areas: two inside the marine protected area (MPA), Cayos Zapatillas (474 ha with 6 conch ha^{-1}), and near the southwest side of the park (480 ha with 3 conch ha^{-1}) (Fig. 3a). The highest densities for *S. costatus* (41–50 conch ha^{-1}) were located northwest of Bastimentos Island in an area of 125 ha (0.3%) (Fig. 3b). The lowest densities for this species (1–20 conch ha^{-1}) were observed in 11 relatively small areas (7.5%), one of which occurred inside the MPA (761 ha with 14 conch ha^{-1}) (Fig. 3b).

The distribution of strombids by the two depth strata vastly favored the shallower of the two (0–5.0 m) with 84% and 98%, respectively, of *S. gigas* and *S. costatus* being found in these areas. The most favored habitat/substrate type for both species was SGs (>70%), with a relatively even distribution of the remaining individuals among AP, CR, and SP areas (Fig. 4). When examining site occupation among the two species, *S. gigas* appears to have a broader distribution than *S. costatus* (24 vs. 14 sites), and the number of co-occupied sites was limited to just 4.2%, or 5 sites of the total 120 sites surveyed (Fig. 3).
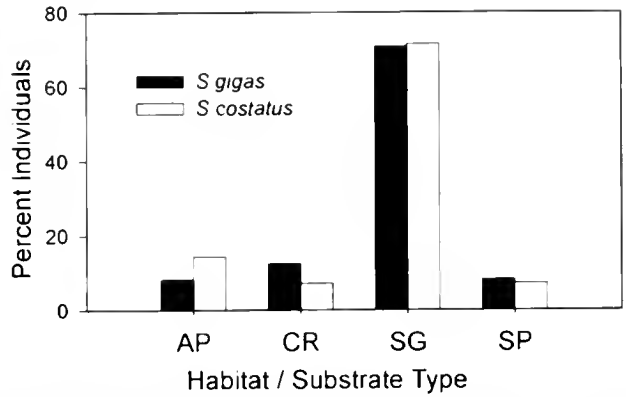
TABLE 2.

Comparison of mean densities of *S. gigas* in the Caribbean determined by visual surveys.

Location		Conch ha^{-1}	Reference
Antigua and Barbuda	Juveniles	13.5	Tewfik et al. (2001)
	Adults (lip >4 mm)	3.7	Tewfik et al. (2001)
Bahamas			
Little Bahamas Bank	1983/83	28.5	Smith & Neirop (1984)
Great Bahamas Bank	Unprotected Bank (1983/1983)	20.8	Smith & Neirop (1984)
	Protected Bank (1991/1994)	53.6	Stoner & Ray (1996)
	Protected Shelf (1991/1994)	96.0	Stoner & Ray (1996)
Bermuda	1988	0.5	Berg et al. (1992)
	1989	2.9	Berg et al. (1993)
Belize	Sub-legal (<15 cm)	14.4	Appeldoorn & Rolke (1996)
	Legal (>15 cm)	14.9	Appeldoorn & Rolke (1996)
Dominican Republic	Juvenile (del Este 1996)	283.0	Delgado et al. (1998)
	Adults (del Este 1996)	4.5	Delgado et al (1998)
	Juvenile (del Este 1997)	22.5	Delgado et al. (1998)
	Adults (del Este 1997)	1.6	Delgado et al. (1998)
	Juvenile (del Este)	14.4	Torres & Sullivan-Sealy (2000)
	Adults (del Este)	0.6	Torres & Sullivan-Sealy (2000)
	Juvenile (Jaragua)	53.0	Posada et al. (1999)
	Adults (Jaragua)	0.6	Posada et al. (1999)
Florida Keys	1987–1988	2.4	Berg & Glazer (1995)
	1990	1.5	Berg & Glazer (1995)
Haiti	Juveniles (Gonave Island)	10.0	Haitian Fisheries Division (pers. com.)
	Adults (Gonave Island)	0.0	Haitian Fisheries Division (pers. com.)
	Rochelios Bank	15.0	Haitian Fisheries Division (pers. com.)
	Western end	160.0	Haitian Fisheries Division (pers. com.)
Honduras	Juveniles	7.3	Tewfik et al. (1998)
Cayos Cohinos	Adults	7.3	Tewfik et al. (1998)
Jamaica			
Pedro Bank (1994)	Juveniles (Artisanal Zone)	15.0	Tewfik (1996)
	Adults (Artisanal Zone)	73.6	Tewfik (1996)
	Juveniles (10–20 m)	51.2	Tewfik (1996)
	Adults (10–20 m)	152.3	Tewfik (1996)
	Juveniles (20–30 m)	73.7	Tewfik (1996)
	Adults (20–30 m)	202.9	Tewfik (1996)
Pedro Bank (1997)	Juveniles (Artisanal Zone)	221.0	Tewfik & Appeldoorn (1998)
	Adults (Artisanal Zone)	93.0	Tewfik & Appeldoorn (1998)
	Juveniles (10–20 m)	466.0	Tewfik & Appeldoorn (1998)
	Adults (10–20 m)	48.0	Tewfik & Appeldoorn (1998)
Morant Bank (1996)	Juveniles (0–10 m)	482.1	Stephens (1997)
	Adults (0–10 m)	10.9	Stephens (1997)
	Juveniles (10–20 m)	59.9	Stephens (1997)
	Adults (10–20 m)	101.1	Stephens (1997)
	Juveniles (20–30 m)	31.8	Stephens (1997)
	Adults (20–30 m)	214.5	Stephens (1997)
Mexico	Cozumel (1989)	89.0	Martínez Vasquez (1995)
	Cozumel (1995, after closure)	830.0	Martínez Vasquez (1995)
Panama	Bocas del Toro (0–10 m)	1.4	This study
Puerto Rico	Southwest (1985/1986)	8.1	Torres Rosado (1987)
	West (1995)	4.2	Mateo et al. (1998)
	East (1996)	7.2	Mateo et al. (1998)
US Virgin Islands	St. Croix (1981)	7.6	Wood & Olsen (1983)
	St. Thomas/St. John (1981)	9.7	Friedlander et al. (1994)
	St. Thomas/St. John (1990)	12.3	Friedlander et al. (1994)

be a true partitioning of suitable habitats and resources between the two congeneric herbivores due to some form of competitive interaction. Berg et al. (1992) explained the differences in population distribution between the two species as being due to differences in habitat preference, and to the processes of larval dispersion, reten-

tion, and recruitment (see Stoner 2003). However, a true competitive interaction (exploitative or interference) may also be possible, as has been investigated for other groups of trophically similar benthic plants (Williams 1987) and animals (Williams 1981, Tegner & Levin 1982, Keller 1983).

In summary, this study concentrated on the population characteristics of two common strombids over their critical shallow water nursery and breeding habitats. Both species appear to be severely overexploited within the archipelago. The present low densities, combined with the suspected Allee effect, ultimately resulting in decreased recruitment levels, could severely restrict recovery. Information from this study will be combined with other surveys of macrophyte (see Stoner 2003), algal, and other invertebrate distributions to begin to understand the overall benthic community dynamics within the archipelago and elsewhere. Finally, it is hoped that this baseline information may also be useful in assessing the success of the nationwide 5-y ban on conch harvest that is under consideration by the Panamanian government.

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WHEN IS THE ABALONE *HALIOTIS DISCUS HANNAI* INO 1953 FIRST ABLE TO USE BROWN MACROALGAE?

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ABSTRACT The dietary value of microscopic algal stages (gametophyte and juvenile sporophyte) of a brown alga *Laminaria japonica*, Areschoug 1851 and of the benthic diatoms *Cylindrotheca closterium* (Ehrenberg) Reimann and Lewin 1964, and *Achnanthes longipes* Agardh 1824 were examined for different developmental stages of *Haliotis discus hannai* Ino 1953 [0.4–2.9 mm shell length (SL)] to determine the size at which abalone begin to use macroalgae efficiently. Most individual abalone showed active feeding behavior, but there was considerable variation in growth of abalone between different algae and developmental stages of abalone. The growth rates of smaller post-larvae (0.4–1.2 mm SL) fed gametophytes and juvenile sporophytes of *L. japonica*, or *A. longipes* were significantly lower than those fed *C. closterium*. In contrast, juvenile sporophytes of *L. japonica* and *A. longipes* produced significantly faster growth in larger postlarval abalone (>1.8 mm SL) than gametophytes of *L. japonica* or *C. closterium*. Postlarvae in all developmental stages fed *C. closterium* actively grazed and efficiently ingested diatom cells. However, the relative dietary value of *C. closterium* decreased as abalone grew, probably because feeding efficiency on this diatom decreased because of its low cell volume and thin film-like colonies. Smaller post-larvae (0.4–1.2 mm SL) grazed repeatedly on the same surface of gametophytes, juvenile sporophytes of *L. japonica*, or on *A. longipes* without detaching these algae, whereas larger post-larvae (>1.8 mm SL) detached and ingested large amounts of whole cells of these algae. Postlarval abalone (>1.8 mm SL) began to use *L. japonica* gametophytes and juvenile sporophytes at approximately the same size at which morphologic changes occurred in their radulae, which enabled the ingestion of macroalgae.

KEY WORDS: benthic diatom, brown alga, dietary value, gametophyte, growth, *Haliotis discus hannai*, postlarval abalone, sporophyte

INTRODUCTION

Survival and growth rates in early life stages of abalone *Haliotis discus hannai* Ino 1953 are considerably affected by food type and the ability of individuals to use available food (Kawamura & Takami 1995, Kawamura et al. 1995, Seki 1997, Takami et al. 1997a, 1997b. Takami et al. 2000, Sasaki & Shepherd 2001, Takami 2002). Understanding the abalone's early life feeding habits considered to be important in improving the rearing techniques in abalone hatcheries and also in understanding the factors controlling natural recruitment (Kawamura et al. 1998a, Sasaki & Shepherd 2001, Takami 2002).

As young of *H. discus hannai* grow, the main food sources shift from benthic diatoms to macroalgae (Kawamura et al. 1998a, Takami 2002). For postlarval abalone, benthic diatoms are the principal foods. The dietary value of diatoms for postlarvae is significantly different between diatom species or strains and is controlled largely by the ingestibility and digestibility of diatoms. Limited diatoms produce high digestion efficiencies and thus relatively rapid postlarval growth (Kawamura & Takami 1995, Kawamura et al. 1995, 1998a, 1998b, Roberts et al. 1999a). Attachment strength of diatoms is one of the factors that affects diatom digestibility for postlarval abalone (Kawamura et al. 1995, 1998a, 1998b, Roberts et al. 1999a). Very tightly attached diatoms, such as *Cocconeis* spp. and *Achnanthes* spp., require considerable force to be detached from substrata and are usually ruptured if dislodged. In contrast, many diatoms with low adhesive strength are ingested without cell rupture, and the majority of ingested cells pass

through the gut alive and unbroken. There are some exceptional diatom species, such as *Cylindrotheca closterium* (Ehrenberg) Reimann and Lewin 1964, which has low attachment strength but is subject to high digestion efficiencies and supports rapid growth of postlarvae, probably because of its weak silica frustule, which is easily broken (Kawamura et al. 1995, 1998a, 1998b). *Cocconeis* spp., which have a high attachment strength and a relatively high dietary value for postlarval *H. discus hannai* larger than ~0.8 mm shell length (SL; Kawamura et al. 1995, Takami et al. 1997a), are often dominant in the habitat of postlarval abalone in the natural environment (Kawamura et al. 1992, Takami 2002) and are used for rearing postlarvae in abalone hatcheries (Joriya & Suzuki 1987, Suzuki et al. 1987). Benthic diatoms, such as *Cocconeis* spp., are probably one of the important diets for postlarval abalone in their natural habitat. In contrast, it has been suggested that juvenile abalone of more than 10 mm SL do not graze *Cocconeis* species if more favorable foods are available (Joriya & Suzuki 1987, Suzuki et al. 1987). This is because *Cocconeis* spp. are not efficient food sources for these larger juveniles because their low-volume cells and prostrate growth form provides little energy (Takami et al. 1996).

Large juveniles (>10 mm SL) and adult *H. discus hannai* prefer to feed on brown macroalgae especially *Laminaria* spp. (Sakai 1962, Kikuchi et al. 1967, Uki 1981) and show rapid growth rates when fed these algal species (Kikuchi et al. 1967, Uki 1981, Uki et al. 1986). Evidence from natural habitats suggests that the diet of abalone becomes dominated by macroalgae as juveniles grow (Tomita & Tazawa 1971, Shepherd & Cannon 1988). However, it is not clear at what size *H. discus hannai* begin to use macroalgae. Moreover, most of the food value experiments of brown macroalgae for abalone have been conducted with mature algae whose

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tolerance to herbivory may be different from juvenile algae (Van Alstyne et al. 1999, 2001). From the standpoint of physical aspects, small abalone may be able to ingest juvenile macroalgae more easily than mature macroalgae.

In this study, we compared the dietary value of microscopic algal stages (gametophyte and juvenile sporophyte) of *Laminaria japonica* Areschoug 1851 and benthic diatoms for different developmental stages of *H. discus hannai* to determine the size at which abalone begin to use macroalgae efficiently.

MATERIALS AND METHODS

Reproductive fronds of *L. japonica* were collected from the subtidal zone, Hokkaido Japan in October 2000. To obtain zoospores, fragments (2–3 cm^2) of reproductive fronds were rinsed with sterilized seawater and placed separately in glass culture vessels containing sterilized seawater. To obtain zoospores, fertile fragments (2–3 cm^2) of the desired algae were rinsed with sterilized seawater and placed separately in 200-mL glass beakers containing sterilized seawater. Newly liberated zoospores were pipetted to 50-mL polystyrene or 200-mL glass beakers containing PESI medium (Tatewaki 1966). Beakers were kept in a growth chamber at 15°C and 43–113 μE/m^2/s on a 12:12 LD cycle, and zoospores were allowed to settle on to the surface of the beaker. The settlement density was 25–30 zoospores/mm^2. Any diatom contaminants were not observed in the beakers. After 5–8 days of incubation, morphologic differences were observed between female and male plantules. Two types of microscopic algal stages of *L. japonica* (haploid gametophytes and diploid sporophytes) were used for the experiments. Gametophytes were kept in a growth chamber at 25°C to inhibit maturation, whereas the sporophytes were kept at 15°C to promote maturation (Yabu 1964). Juvenile sporophytes were allowed to grow until the size of thalli reached 0.5–1 mm in length. Gametophytes grew prostrate across the surface of the vessel, whereas juvenile sporophytes grew erect and formed three-dimensional colonies.

Benthic diatoms *Cylindrotheca closterium* and *Achnanthes longipes* Agardh 1824 were also used as food items for abalone. These benthic diatoms were isolated from an abalone nursery tank at Tohoku National Fisheries Research Institute, Miyagi Japan, and were grown following the methods of Kawamura et al. (1995).

Larval abalone were hatched in May and October 2000 at the Yamagata Sea Farming Association (Yamagata, Japan) and reared using the method of Uki and Kikuchi (1984). Four days after fertilization at 20°C, the veliger larvae were transported to Tohoku National Fisheries Research Institute within 4 h. Competent larvae were transferred to 200-mL glass beakers with 150 mL of autocaved filtered (0.45 μm; Millipore HA) natural seawater (FSW) containing 150 μg/mL each of penicillin G sodium and streptomycin sulphate BP. These larvae were induced to metamorphose by the addition of 1 μM γ-amino butyric acid (Takami et al. 2000). Four days after metamorphosis induction, an adequate number of *C. closterium* cells were added as a food supply. The rearing beakers were incubated in light at 31–53 μE/m^2/sec on a 12:12 LD cycle. These abalone were maintained as a source of experimental animals, by adding supplementary *C. closterium* cells and replacing the water every 3–4 days with new FSW without antibiotics. All chemicals were obtained from Wako Pure Chemical Industries (Osaka, Japan).

Six experiments were conducted using different size classes of abalone. Detailed information on the experiments is presented in Table 1. Before each experiment, abalone were dislodged with a fine needle from the stock beakers and placed into a 50-mL polystyrene dish with 25 mL of FSW containing 6 mg/L of GeO$_2$ without food for a period of 2 days in the dark. GeO$_2$ effectively inhibits the proliferation of diatoms attached to abalone and does not affect the survival and growth of animals (Takami et al., 1997b). Most *C. closterium* cells ingested by abalone were digested, so any contamination by live diatom cells from abalone feces was negligible (Kawamura & Takami 1995, Kawamura et al. 1995, Roberts et al. 1999a).

Active postlarval abalone were placed into 50-mL polystyrene (Exp. I–IV) or 200-mL glass beakers (Exp. V, VI) in which each algal diet was available (Table 1). Beakers were submerged in a 35-L tank. Beakers containing experimental animals and algal di-

TABLE 1.

Details of experimental treatments.

Exp. No.	Algal Species	Algal Type	Initial Shell Length of Abalone (μm, mean ± SE)	Rearing Temperature (°C)	Duration of Experiment (days)	Number of Rearing	Number of Abalone per Beaker
I	*Laminaria japonica*	Gametophyte	458 ± 6.4	20 ± 1	7	3	5
	Cylindrotheca closterium	Benthic diatom	447 ± 6.9	20 ± 1	7	3	5
II	*Achnanthes longipes*	Benthic diatom	674 ± 5.4	20 ± 1	7	5	10
	Cylindrotheca closterium	Benthic diatom	657 ± 5.5	20 ± 1	7	5	10–12
III	*Laminaria japonica*	Gametophyte	898 ± 21	20 ± 1	8	3	5
	Laminaria japonica	Juvenile sporophyte	860 ± 17	20 ± 1	8	3	5
	Cylindrotheca closterium	Benthic diatom	854 ± 23	20 ± 1	8	3	5
IV	*Laminaria japonica*	Gametophyte	1092 ± 14.7	20 ± 1	7	3	5
	Laminaria japonica	Juvenile sporophyte	1152 ± 28.0	20 ± 1	7	3	5
	Cylindrotheca closterium	Benthic diatom	1121 ± 21.2	20 ± 1	7	3	5
V	*Laminaria japonica*	Gametophyte	2008 ± 51.6	17 ± 1	10	3	5
	Laminaria japonica	Juvenile sporophyte	2106 ± 81.4	17 ± 1	10	3	5
	Cylindrotheca closterium	Benthic diatom	1874 ± 78.7	17 ± 1	10	3	5
VI	*Laminaria japonica*	Juvenile sporophyte	2894 ± 153	17 ± 1	10	3	2
	Achnanthes longipes	Benthic diatom	2809 ± 137	17 ± 1	10	3	2
	Cylindrotheca closterium	Benthic diatom	2711 ± 90	17 ± 1	10	3	2

ets were covered with a 200 (Exp. I–IV)- or a 600 (Exp. V, VI)-μm nylon mesh to allow water exchange. Incoming filtered (1 μm) natural seawater was maintained at a flow rate of approximately 2.4 L/min into the tanks. The rearing temperatures were set at a temperature that abalone of specific developmental stages encounter in the Miyagi coast. Because the spawning season of *H. discus hannai* in the area is from late summer to mid autumn, postlarvae encounter decreasing temperature as they age. SL of live individuals in each experiment was measured to the nearest 10 μm using a monitor and video camera system with an image analyzer, connected to an inverted microscope (Exp. I–VI) or a dissecting microscope (Exp. V, VI) at the beginning and at the end of the experiment. The feeding behavior of abalone was observed at intervals of 1–3 days using an inverted microscope.

The differences between survival and growth rates of treatments were tested using Student's *t* test (Exp. I, II) or Tukey-Kramer multiple comparison test (Exp. III, VI). Survival data were arcsine-transformed before analysis to normalize the data.

RESULTS

In all experiments, considerable variation was found in the growth rates of abalone between both algal types and developmental stages of abalone (Fig. 1), even though most individuals were observed actively feeding. The growth rates of postlarval abalone of 0.4–1.2 mm SL that were fed gametophytes and juvenile sporophytes of *L. japonica* were significantly lower (14–17 μm/day)
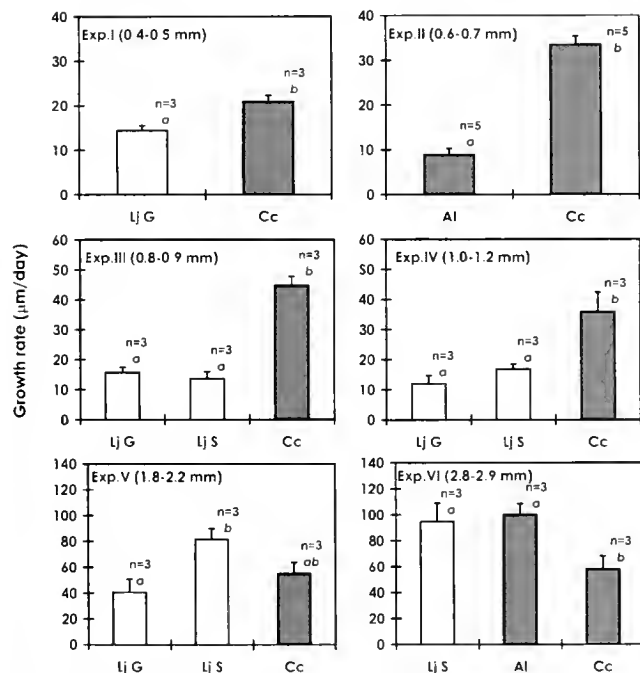

Figure 1. Growth of six developmental stages of postlarval *Haliotis discus hannai* (μm per day) fed gametophytes of *Laminaria japonica* (Lj G), juvenile sporophytes of *L. japonica* (Lj S), the benthic diatom *Achnanthes longipes* (Al), or the benthic diatom *Cylindrotheca closterium* (Cc). Numbers in parentheses indicate the range of initial shell length of abalone used for each experiment. Each bar represents mean ± SE with the number of replicates. Letters on the top of each column indicate the results of Student's *t* test (Exp. I, II) or Tukey Kramer multiple comparison (Exp. III–VI) tests; columns with different letters represent means that are statistically different (*P* < 0.05).

than those of postlarvae fed the benthic diatom *C. closterium* (21–44 μm/day; Fig. 1; Exp. I, III, IV, *P* < 0.05). Differences in growth rates between postlarvae fed *C. closterium* and gametophytes or juvenile sporophytes were larger for postlarvae of 0.8–1.2 mm SL (Fig. 1; Exp. III, IV) than of 0.4–0.5 mm SL (Fig. 1; Exp. I). Growth rates were not significantly different between postlarvae fed gametophytes and juvenile sporophytes in Exp. I, III, and IV (*P* > 0.05). For larger postlarvae (> 1.8 mm SL), juvenile sporophytes produced significantly faster mean growth (81–95 μm/day) than gametophytes (41 μm/day, Exp. V, *P* < 0.05) or *C. closterium* (58 μm/day, Exp. VI, *P* < 0.05).

Postlarvae of 0.6–0.7 mm SL (Fig. 1; Exp. II) fed *A. longipes* showed significantly lower growth rates (9 μm/day) than those fed *C. closterium* (33 μm/day, *P* < 0.05). In contrast, the mean growth rate of post-larvae of 2.8–2.9 mm SL (Fig. 1; Exp. VI) fed *A. longipes* was significantly higher (100 μm/day) than that of post-larvae fed *C. closterium* (58 μm/day, *P* < 0.05).

The postlarvae that were fed *C. closterium* actively grazed and efficiently ingested diatom cells in all the experiments. Smaller postlarvae <1.2 mm SL (Exp. I–IV) grazed repeatedly on the same area of gametophytes, juvenile sporophytes, or *A. longipes* without detaching these algae. We could not directly observe the ingestion of algal diets by larger postlarvae (>1.8 mm SL; Exp. V, VI) ingested algal diet or not because most of the abalone stopped feeding when we tried to observe them under the microscope. However, we concluded that larger postlarvae ingested large amounts of gametophytes and sporophytes of *L. japonica* because these algae were almost completely cleared from the substratum and many feces remained. Ruptured cells of *A. longipes* were observed in the fecal pellets of postlarvae in Exp. VI but not in Exp II.

Significantly lower survival rates were detected when smaller postlarvae were fed *L. japonica* gametophyte (Exp. I) and *A. longipes* (Exp. II; Fig. 2, *P* < 0.05) rather than *C. closterium*. In Exp. III–VI, the survival rates of individuals were generally high (80–100%) except for Exp. IV (Fig. 2) when many contaminant protozoans were observed in all rearing beakers.

DISCUSSION

The results of this study show that dietary values of gametophytes and juvenile sporophytes of a brown alga, *L. japonica*, vary depending on the developmental stage of abalone. Most smaller postlarvae (<1.2 mm SL) could not efficiently detach either gametophytes and sporophytes when feeding. In contrast, larger postlarvae (> 1.8 mm SL) detached and ingested these brown algae and showed comparable or faster growth rates than those fed a benthic diatom, *C. closterium* (Fig. 1).

In the experiments using smaller postlarvae < 1.2 mm SL (Exp. I–IV), abalone fed *C. closterium* showed the highest growth rates. This diatom species has a weak silica frustule and low attachment strength; therefore, abalone can ingest and break the diatom cells resulting in high ingestion and digestion efficiencies of abalone. The differences in growth rates between postlarvae fed *L. japonica* and *C. closterium* were more marked on animals of 0.8–1.2 mm SL (Exp. III, IV) than those of 0.4–0.5 mm SL (Exp. I). These results correspond to the changes in abalone feeding (Kawamura et al. 1998a). The energy source of postlarvae is gradually transferred from yolk supply to particulate food after metamorphosis at a size of ~0.4–0.5 mm SL. Young postlarvae can grow using mucus materials secreted from diatoms (Kawamura & Takami 1995) and
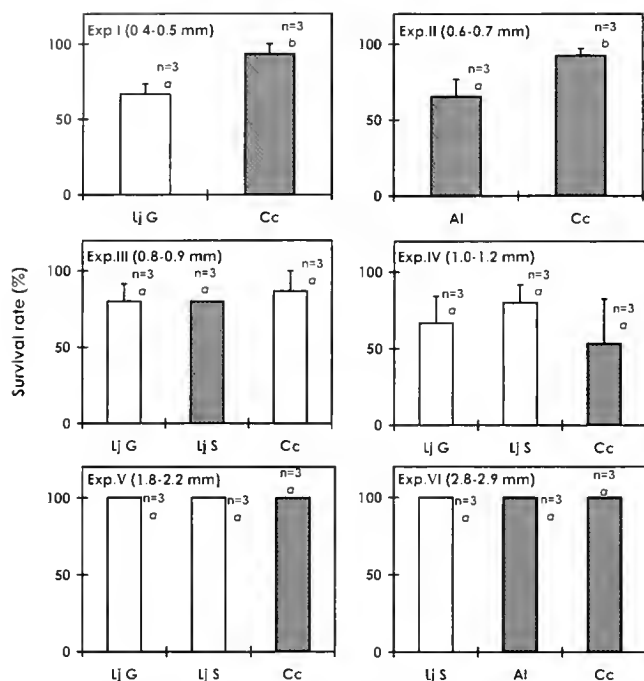
Figure 2. Survival rates of six developmental stages of postlarval *Haliotis discus hannai* fed gametophytes of *Laminaria japonica* (Lj G), juvenile sporophytes of *L. japonica* (Lj S), the benthic diatom *Achnanthes longipes* (Al), or the benthic diatom *Cylindrotheca closterium* (Cc). Numbers in parentheses indicate the range of initial shell length of abalone used for each experiment. Each bar represents mean ± SE with the number of replicates. Letters on the top of each column indicate the results of Student's *t* test (Exp. I, II) or Tukey Kramer multiple comparison (Exp. III–VI) tests; columns with different letters represent means that are statistically different ($P < 0.05$).

macroalgae, such as crustose coralline algae (CCA; Daume et al. 1997, Kitting & Morse 1997, Takami et al. 1997b) supplemented by residual yolk supply (Takami et al. 2000, Roberts et al. 2001) and possibly absorption of dissolved organic matter (Shilling et al. 1996). At around 0.6–0.8 mm SL, postlarvae become responsive to the digestibility of diatom diets and grow more rapidly on efficiently digested diatoms (Kawamura et al. 1995, Takami et al. 1997a, Kawamura et al. 1998b, Roberts et al. 1999a). Dietary benefits are size dependent in postlarval abalone (Roberts et al. 1999a). Postlarvae of 0.6–1.2 mm SL who were fed gametophytes, juvenile sporophytes, and *A. longipes* could not get adequate energy sources for rapid growth because of the difficulty in ingestion of these algae and possibly insufficient amount of secreted mucus (Kawamura & Takami 1995, Kawamura et al. 1998a).

The growth of postlarval *H. discus hannai* of 1.3 mm SL fed thinly sliced fronds of brown alga *Undaria pinnatifida* was comparable to that of abalone fed diatoms (Sakai 1976). *H. discus discus* of 3–4 mm SL fed softened fronds of *U. pinnatifida* (Fujii & Yotsui 1989) and germlings of macroalgae (Maesako et al. 1984) also showed good growth rate (72–110 μm/day). Takami et al. (1998) reported that postlarval *H. discus hannai* of 1 mm SL had a suite of enzymes useful for digesting brown algal polysaccharides and these enzyme activities increased rapidly from approximately 2 mm SL, suggesting they could use macroalgae if they could ingest the algal fronds. The ingestion efficiency of postlarvae on algal diets is determined by the radula morphology (Roberts et al. 1999a, 1999b, Kawamura et al. 2001). The major

ontogenetic changes in the radula structure occur around 1–2 mm SL for postlarval *H. discus hannai* (Kawamura et al. 2001). For example, the adult complement of five pairs of lateral teeth was completed by 1.9 mm SL. A rapid increase in the clearance angle of the radula (Padilla 1985) was observed in postlarval *H. discus hannai* between 1–2 mm SL (Kawamura et al. 2001). Postlarvae >1 mm SL develop radula teeth with positive clearance angles that are more suitable for cutting rather than just sliding across the substratum. Larger post-larvae have well-developed outer lateral teeth (L3–L5 teeth), which appear to be used to cut the elastic macroalgae and three-dimensional growth forms of benthic diatom such as *A. longipes*. In *H. discus hannai* larger than 1.5 mm SL, the L3–L5 teeth become longer and more pointed (Kawamura et al. 2001). The results of this study show that postlarval *H. discus hannai* begin using gametophytes and juvenile sporophytes almost at the same size at which major morphologic changes in radula occur.

The relative dietary value of *C. closterium* decreased as postlarvae grew (Fig. 1). Three-dimensional *A. longipes* colonies and juvenile *L. japonica* sporophytes provide a much higher biomass per unit area than low volume, two-dimensional *C. closterium* films, once post-larvae are able to detach and ingest them. The significant difference in growth rate between post-larvae fed juvenile sporophytes and gametophytes (Fig. 1; Exp. V) might be caused by differences in the algal growth forms, because gametophytes also show prostrate growth form.

In Exp. I and II, the survival rates of postlarvae fed gametophytes of *L. japonica* (66.7%) and *A. longipes* (65.6%) were significantly lower than those fed *C. closterium* (92.7–93.3%; Fig. 2). This low survival was not considered to be caused directly by starvation because abalone at these stages could survive more than 15 days of food deprivation (Takami & Kawamura, unpubl.). There is a possibility that the diffusive boundary layer (DBL), where diffusion dominates molecular transport, severely affects survival of post-larval abalone because oxygen concentrations in the dark may be reduced whereas algal secondary metabolites increase affecting water quality (Searcy-Bernal 1996, Roberts et al. 2000). The DBL "water quality" probably depends upon culture condition, and the algal strains and species used. Algal species with three-dimensional growth forms have a thicker DBL than the diatoms that form flat film-like colonies (Roberts et al., 2000); therefore, the DBL produced by *A. longipes* may have had reduced "water quality" and affected survival of the smaller postlarvae. Another possibility is that the combination of nutritional stress and water quality stress causes mortality more quickly than seen from starvation in clean containers.

Larval *H. discus hannai* settle preferentially on CCA in the natural environment, and grow on CCA for several months (Saito 1981, Sasaki & Shepherd 1995, 2001, Takami 2002). Food sources from CCA include the alga's surface polysaccharides and epithelial cell contents, which can keep postlarvae alive but are not adequate to support rapid growth (Garland et al. 1985, Daume et al. 1997, Kitting & Morse 1997, Takami et al. 1997b). CCA rely on grazing by herbivores to prevent their surfaces from being covered with competitively superior algae (Paine 1980, Steneck 1982). Grazing-resistant algae with strongly adhesive prostrate forms such as benthic diatoms *Cocconeis* spp. tend to dominate under high grazing pressures by relatively large gastropods (Kesler 1981, Ioriya & Suzuki 1987, Suzuki et al. 1987, Steinman et al. 1989, Kawamura et al. 1992). Because grazing gastropods occur at high densities on CCA (Ayling 1981, Choat & Schiel 1982, Kawa-

mura et al. 1992, Takami, 2002), *Cocconeis* spp. are often dominant and appear to be the main food sources for early life stages of abalone on CCA (Kawamura 1994, Takami 2002). However, *Cocconeis* films probably become energetically inadequate as juvenile grow, and juvenile abalone come to rely on three-dimensional algal populations for food (Takami et al. 1996, Kawamura et al. 1998a).

The germling or juvenile stage of macroalgae is generally susceptible to grazing by herbivores (Lubchenco 1978, Robles & Cubit 1981, Lubchenco 1983, Dayton 1985, Dean et al., 1989, Asano et al. 1990, Paine 1992, Martinez & Santelices 1998, Van Alstyne et al. 1999, 2001). Therefore, newly recruited juvenile algae may find it difficult to grow on CCA surfaces. However, northern Japanese Laminarian species have prodigious reproductive output, consequently they have considerable potential for dense recruitment if grazing pressure is low (Yendo 1911, 1919). The season of sexual reproductive in *L. japonica* in Miyagi is from late autumn to mid winter when grazers' activities are relatively low due to the low water temperature. By this time, most of the 0-y-old abalone are more than 2 mm SL (Sasaki & Shepherd 1995, 2001, Takami 2002), a size at which they can efficiently ingest juvenile sporophytes of *L. japonica*. Juvenile *L. japonica* may be an important food source for these abalone at this early life stage.

ACKNOWLEDGMENTS

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PROCEEDINGS OF
WORKSHOP ON REBUILDING TECHNIQUES FOR ABALONE IN
BRITISH COLUMBIA

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CANADA

PREFACE

The following 6 papers and 11 abstracts published in this issue of the Journal of Shellfish Research are part of 17 presentations delivered at an international workshop on rebuilding techniques for abalone in British Columbia (BC) held at Nanaimo, BC, Canada, January 14 to 16, 2003 (Campbell & Heimstra 2003). The decline of northern (pinto) abalone (*Haliotis kamtschatkana*) stocks since the late 1970s has prompted fishery closure since 1990, listing this species as "threatened" by the Committee on the Status of Endangered Wildlife in Canada in 1999, two international workshops (Campbell 2000, Campbell & Heimstra 2003), and development of a national recovery strategy for *H. kamtschatkana* in BC (Toole et al. 2002).

Over exploitation and declines in wild abalone populations have occurred in many parts of the world and the methods for successfully rebuilding wild stocks are still in the developmental stage. The workshop discussed community stewardship projects, aquaculture, out planting and restocking, wild stock manipulation, and monitoring tools and evaluation performance indicators as methods for abalone rebuilding. The 6 papers published in this volume represent some of the presentations at the workshop. The papers underwent the stringent refereeing and review process required by this journal. I thank the authors and the many referees for their efforts and co-operation for reviewing and revising the manuscripts.

ALAN CAMPBELL
Editor

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UPDATE ON EMERGING ABALONE DISEASES AND TECHNIQUES FOR HEALTH ASSESSMENT

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ABSTRACT This article presents a review of new diseases and additional information on known pathogens of abalone that were encountered in the last few years as a result of increasing efforts towards the culture of abalone around the world and concurrent investigations into abalone health. A novel haplosporidian was associated with high mortalities (82.5–90%) of cultured juvenile paua (*Haliotis iris*) in New Zealand. Disease outbreaks among cultured abalone in Tasmania, Australia were associated with two species of *Vibrio* (*V. harveyi* and *V. splendidus* I) and a *Flavobacterium*-like bacterium with stress factors precipitating the diseases in most cases. The agent of withering syndrome responsible for mass mortalities of black abalone (*H. cracherodii*) in southern California was identified as the Rickettsiales-like prokaryote "*Candidatus* Xenohaliotis californiensis". The exotic sabellid polychaete that seriously impacted abalone culture in California was named *Terebrasabella heterouncinata* and experimentally found to reproduce at low temperatures but with a significantly temperature-dependant generation time (a developmental cycle of 298 days at 11.2°C in comparison to 165 days at 15.6°C). To assess the health of cultured abalone, histologic examinations are essential. For histology, tissue samples (less than 1 cm thick) should be fixed in Davidson's solution or 10% formalin in seawater such that there is at least 10 parts fixative to 1 part tissue. Histopathology will not only indicate the presence of infectious agents but can be useful for monitoring the suitability of the diet and aquaculture environment. These assessments will benefit abalone aquaculture and provide assurance that only healthy animals are used in stock rehabilitation programs.

KEY WORDS: disease, parasites, abalone, *Haliotis*

INTRODUCTION

The decline of wild stocks of abalone around the world and the increasing demand for this product in the market place has increased efforts in the culture of various abalone species and interest in rehabilitating wild stocks. Concurrent with this increased attention to abalone, awareness of the various infectious diseases of abalone has arisen. Prior to 2000, six severe diseases associated with mortality in various species of abalone had been reported in the literature (Table 1 Bower 2000). Since that time, knowledge of some of these diseases has increased and other infectious diseases have been detected.

Various investigations have revealed that infectious diseases can have a significant negative impact on the aquaculture of abalone (Elston & Lockwood 1983, Oakes & Fields 1996, Bower 1987a, Li et al. 1998, Lizárraga–Partida 1998, Nishimori et al. 1998, Ruck & Cook 1998, Kuris & Culver 1999, Cáceres Martínez et al. 2000, Moore et al. 2000b, Diggles et al. 2002). Because infectious diseases can be equally disastrous if inadvertently introduced into new locations by stock rehabilitation efforts that involve the translocation of abalone, it is important to be aware of the available information on diseases to circumvent complications (Sinderman 1988). The current low abundance of wild stocks of northern abalone (*Haliotis kamtschatkana*) in British Columbia has stimulated the culture of this species and the development of plans for rehabilitation. In conjunction with this effort, abalone will be examined for infectious diseases. Thus, it is prudent to have information on diseases that have affected abalone around the world and on procedures used to assess abalone health at hand. This paper presents information on abalone diseases that was published since the review by Bower (2000) and describes techniques that can be implemented to examine abalone for infectious disease. Although directed towards concerns for northern abalone, the information provided herein is directly applicable to all abalone species regardless of location.

UPDATE ON ABALONE DISEASES

Since 2000, new significant diseases of abalone have been encountered during efforts to culture abalone in various parts of the world and new information has been published on previously known diseases.

In New Zealand, a novel haplosporidian was associated with high mortalities (82.5–90%) of cultured juvenile paua (*Haliotis iris*) at one farm in the eastern Bay of Plenty (Diggles et al. 2002). Runts were more severely affected but, all infected abalone showed weak adherence to the substrate, had a shriveled foot with pale blister-like lesions on the foot and mantle, and failed to right themselves when turned over. In lightly infected abalone, uni- to multinucleate plasmodia (up to 13.5 µm in length) occurred in the connective tissue surrounding the gut, amongst glial cells adjacent to the nerves of the mantle and foot and within the gill lamellae. In heavy infections, numerous plasmodia were present in the hemolymph, gills, heart, kidneys, mantle, foot, epipodium, and connective tissue of the digestive gland. Spore formation was not observed but sporocyst-like bodies were found amongst plasmodia in the right kidney of an adult paua collected from the wild (Hine et al. 2002, Diggles et al. 2002). Research into this new pathogen is on going in New Zealand.

Various bacteria have also been isolated from cultured abalone experiencing disease and mortalities. In Tasmania, Australia, disease outbreaks among cultured abalone (*Haliotis rubra, H. laevigata* and their hybrids) were associated with two species of *Vibrio* (*V. harveyi* & *V. splendidus* I) and *Flavobacterium*-like bacterium. In most cases, stress factors (e.g., high temperatures, grading trauma, anesthetics, gradual increase in salinity in the recirculation system, etc.) were reported to have precipitated the diseases (Handlinger et al. 2001, Handlinger et al. 2002). In Kanagawa Prefecture, Japan, *Vibrio carchariae* (possibly a junior synonym of *Vibrio harveyi*) was isolated from cultured abalone (*Haliotis* (=*Sulculus*) *diversicolor supratexta*) experiencing a mass mortal-

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Category	Pathogen/Disease	Known Distribution
1. Cause severe disease and mortality	*Vibrio fluvialis* II/pustule disease	vicinity of Dalian, China
	Labyrinthuloides haliotidis	British Columbia, Canada
	Perkinsus olseni	South Australia
	Sabellid polycheate	California, USA; Baja California, Mexico; southern Africa
	Withering foot syndrome	California, USA
	Amyotrophia	western Japan
2. Parasites of lesser concern	Ciliates	Global-specific studies from southern Africa
	Margolisiella haliotis	California, USA
	Echinocephalus pseudouncinatus	Southern California, USA; Gulf of California, Mexico
	Trematode parasitism	Global
3. Detrimental under adverse conditions	Ubiquitous opportunistic organisms	Global
	Shell-boring organisms	Global

ity. In this case, white spots consisting of necrotic muscle fibers and bacteria on the abalone foot accompanied by high mortalities were characteristic of the disease (Nishimori et al. 1998). *Vibrio carchariae* was also identified as the probable cause of mass mortalities of *Haliotis tuberculata* in the natural environment along the Brittany and Normandy coasts of France and in a land-based abalone farm in Normandy (Nicolas et al. 2002). Dixon et al. (1991) reported that exposure to ozonated water and treatment (bath and injection) with a broad spectrum antibiotic (sulphadimidine sodium) was effective against bacterial infections (caused by *Clostridium lituseberense* or *Vibrio alginolyticus*) in some abalone (*Haliotis midae*) in a South African experimental facility. However, Handlinger et al. (2002) found antibiotic use to give equivocal results on bacterial infections in Tasmanian farmed abalone.

In addition to the newly encountered diseases, new information has been published on previously known diseases (Table 1). Taiwu et al. (2000) reported that phage particles isolated from *Vibrio fluvialis*-II and inoculated into *Haliotis discus hannai* suffering from pustule disease caused by this bacterium in China raised abalone survival rates by up to 50%. In Australia, field studies using molecular detection techniques indicated that infections with the protistan *Perkinsus olseni* in wild *Haliotis rubra* at Taylor Island, South Australia, was positively correlated with both water temperature and size of abalone. Also, the parasite was being maintained by *H. rubra* with negligible contributions from other susceptible abalone species or other mollusks (Lester et al. 2001). Subsequent data and analysis by Hayward et al. (2002) indicated that the transmission of *P. olseni* among the wild *H. rubra* appeared to be reduced and infections were less severe in 2002. This apparent reduction in disease was attributed to lower maximum summer sea surface temperatures (cooling of almost 3°C to below 20°C).

The exotic sabellid polychaete that seriously impacted abalone culture in California was named *Terebrasabella heterouncinata* by Fitzhugh and Rouse (1999). Experimental studies indicated that one *T. heterouncinata* is capable of self-fertilization for the production of fully functional organisms and that generation time was highly temperature dependent. Although this polychaete is capable of completing its life cycle at cold temperatures (11.2°C), the complete developmental cycle was slow requiring 298 days in comparison to 165 days at 15.6°C and 111 days at 20.9°C (Finley et al. 2001). Also, *T. heterouncinata* is capable of infecting a wide host of gastropod species native to California (Kuris & Culver 1999).

Shell mineral deposition in *Haliotis rufescens* was exploited by *T. heterouncinata* resulting in the formation of a protective burrow around the polychaete. Heavy infestations caused downward orientation of the shell margin, shell deformation and stunted abalone growth (Day et al. 2000). Ultrasound micro-cavitation was found to destroy the feeding crown of this sabellid and improved the growth of treated *H. midae*. However, the abalone showed severe stress behavior during the ultrasound treatments and a second treatment may be required to destroy new sabellid infestations recruited from larvae and eggs that were protected by the abalone shell during the first treatment (Loubser & Dormehl 2000).

The agent of withering syndrome responsible for mass mortalities of *Haliotis cracherodii* in southern California was identified as a Rickettsia-like prokaryote in the class Proteobacteria and was given the provisional name of "*Candidatus* Xenohaliotis californiensis" because of the inability to culture the organism *in vitro* (Friedman et al. 2000a). A polymerase chain reaction (PCR) assay and *in situ* hybridization (ISH) test have been developed for the detection of this pathogen (Andree et al. 2000, Antonio et al. 2000). "*Candidatus* Xenohaliotis californiensis" can also be rapidly detected in tissue squashes of infected gastrointestinal epithelium using a nucleic acid fluorochrome stain (Moore et al. 2001a). In surveys of abalone from Baja California, Mexico, this pathogen was detected in high prevalences in symptomatic and non-symptomatic cultured and natural populations of *H. rufescens, H. fulgens, and H. corrugata* (Cáceres Martínez et al. 2000, Cáceres Martínez & Tinoco–Orta 2001, Álvarez–Tinajero et al. 2002). Also, infected *H. rufescens* have been detected as far north as San Francisco, California (Finley & Friedman 2000). An unidentified Rickettsia-like prokaryote was also detected in the digestive gland of *H. midae* from South Africa (Mouton 2000).

Warm temperatures (>18°C) seem to be required for the development of withering syndrome in abalone exposed to "*Candidatus* Xenohaliotis californiensis" (Moore et al. 2000a, 2000b). This pathogen was transmitted between abalone by injection and bath exposure to post-esophagus homogenates prepared from infected abalone and by cohabitation with abalone exhibiting withering syndrome (Moore et al. 2001b, Friedman et al. 2002). Examination of hemocyte activity indicated that the hemocytes of infected *H. cracherodii* were more chemotactic but were less able to engulf and destroy foreign particles, which may contribute to the mortality associated with withering syndrome (Friedman et al. 2000b). Intramuscular injection and oral administration of oxytetracycline was effective in reducing the losses of infected abalone

(Friedman et al. 2003). However, other antimicrobials (chloramphenicol, clarithromycin, and sarafloxicin) had no measurable affect on the disease (Friedman et al. 2000a).

In addition to the new information on severe diseases of abalone, observations on other parasites were also published since the previous review by Bower (2000). A renal coccidia was reported from *H. midae* in South Africa (Mouton 2000) and *Margolisiella haliotis* was detected in *H. rufescens* from Baja California, Mexico (Cáceres Martínez & Tinoco-Orta 2001). Two species of spionid polychaetes (mudworms), *Boccardia knoxi* and *Polydora hoplura*, were associated with severe blistering in the shell and 50% or greater mortality among cultured abalone at several sea-based facilities in southern Tasmania, Australia (Lleonart et al. 2003). Three species of shell boring clams (*Lithophaga aristata*, *Lithophaga plumula*, and *Penitella conradi*) were found boring in the shell of *H. fulgens* and two of these species (*L. aristata* and *L. plumula*) were also observed infesting the shells of *H. corrugata* from the vicinity of Isla de Cedros on the west coast of Baja California, Mexico (Álvarez–Tinajero et al. 2001). Nollens et al. (2002) reported that endoscopy applied to anesthetized *H. iris* was more accurate than radiography and ultrasonography for the detection of the shell lesions caused by the invasion of a fungus (described by Grindley et al. 1998). Although endoscopy was invasive, apparently no discernible effects on survival of the abalone, attributable to the procedure, were observed 8 months after screening (Nollens et al. 2002).

ABALONE DISEASE CONCERNS IN BRITISH COLUMBIA

Although some and possibly all of the diseases of abalone detected in other parts of the world have the potential of occurring in British Columbia, to date, only one infectious disease of concern has been detected. The protistan *Labyrinthuloides haliotidis* was involved in the demise of an attempt to culture northern abalone,

H. kamtschatkana, in British Columbia in the early 1980s (Bower 1987a, 1987b). This parasite is only known to be lethal for abalone smaller than 5 mm in shell length. Because of the relatively large size of this parasite (about 10 μm in diameter) and the translucent nature of the tissues of small abalone (<5 mm shell length), infected abalone can easily be detected by examination with a compound light microscope.

Detection of *L. haliotidis* in small abalone begins when the culture containers are being cleaned. Moribund abalone that are either weakly attached to the substrate or have fallen to the bottom and are not attempting to right themselves should be sampled. The foot and head of infected abalone will have lost tissue integrity and appear swollen (look puffy). Squash the abalone between a glass slide and coverslip, and examine squashed tissues under a compound microscope. In comparison to normal/health abalone (Fig. 1a), the tissues of infected abalone will be filled with stationary spherical protists (~10 μm in diameter, see Fig. 1b) many of which may be undergoing binary fission (semispherical specimens with a clear central dividing line, see Fig 1c). If infection with *L. haliotis* is suspected, samples should be submitted to a competent authority for confirmation. Prior to submission, abalone should be preserved for histologic examination as described later. Also, immediate steps should be taken to mitigate potential spread of the disease.

The spread of *L. haliotidis* within an abalone culture facility or between facilities can be mitigated by applying good husbandry techniques. Essentially, abalone and equipment should not be transferred between tanks, water exchange between tanks should be avoided and personnel must be careful to not facilitate cross contamination. Although *L. haliotidis* is resistant to many disinfectants, it can be destroyed by a 20 min exposure to 25 mg sodium hypochlorite (chlorine) per liter of sea water (Bower 1989). Treatments applied to infected abalone in the past (Bower 1989) have proven problematic. If this parasite should again appear in an
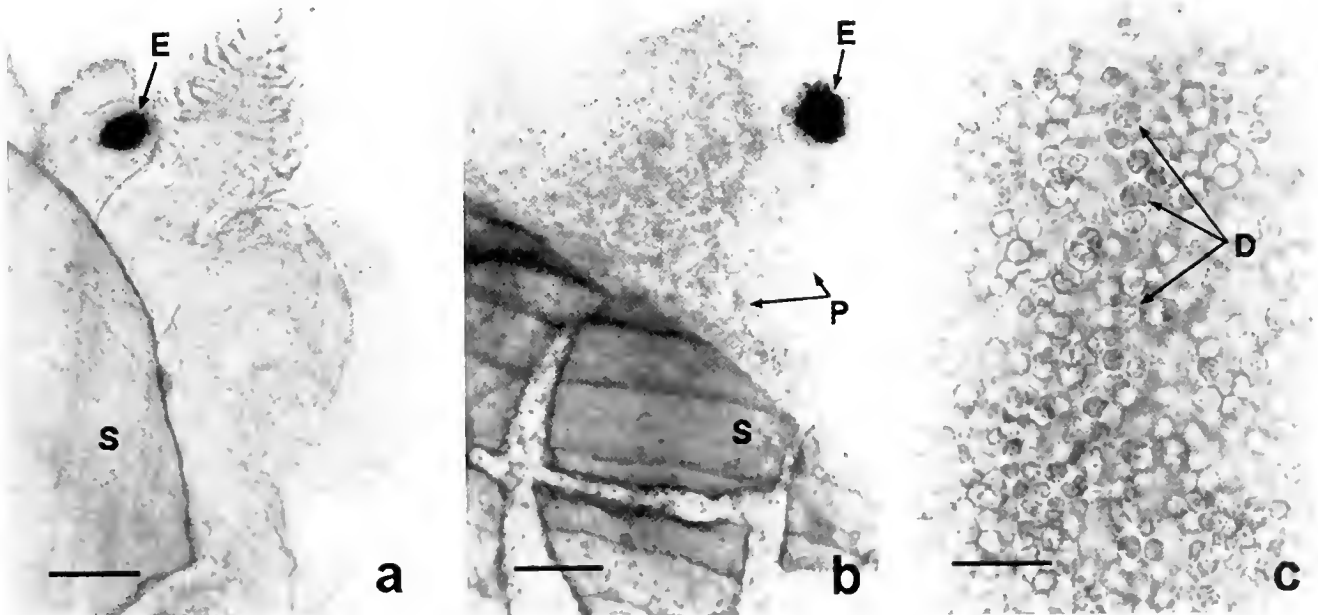

Figure 1. Unstained wet mount squashes of juvenile abalone, *Haliotis kamtschatkana*, about 2 mm in shell length. (a) Head region of a normal uninfected specimen showing the eye (E) and shell (S). Scale bar = 100 μm. (b) Same magnification of a specimen heavily infected with numerous *Labyrinthuloides haliotidis* (P) liberated from and embedded in tissues of the head region—the eye is swollen due to loss of tissue integrity caused by the parasite. Scale bar = 100 μm. (c) Magnification of infected foot muscle showing numerous *L. haliatidis* some of which are in the process of dividing by binary fission (D). Scale bar = 25 μm.

abalone culture facility, research will be required to identify efficacious methods of control.

ASSESSMENT OF ABALONE HEALTH

As for all other mollusks, infectious diseases of abalone are becoming more evident with increased efforts towards the culture of various species of abalone around the world. Disease can often be circumvented in the culture environment by implementing knowledge gained from research on the cause of the disease. Also, when cultured abalone are to be used in rehabilitation efforts, specimens placed into the natural environment must be in good health for the endeavor to have a chance of success. Because few specific diagnostic tools are available for detecting diseases of abalone and assessing abalone health, standard procedures of histologic examination must be used. Although the microscopic examination and interpretation of histologic preparations of abalone tissues requires extensive specific knowledge and experience, the preparation of the tissues for histology can be performed with minimal training and equipment. Following is a brief description of the procedures and materials required to prepare abalone for histologic examination.

Abalone for histologic examination should first be examined fresh, all abnormalities noted, and shell length measured. For histology, appropriate tissue samples must be chemically preserved (Table 2). Regardless of the preservative used, it is critical that tissue samples are less than 1 cm thick, that there is at least 10 parts preservative to 1 part tissue and that the tissues are placed in the preservative as soon as possible after collection. After 24 to 48 h in the preservative, tissues should be transferred to 70% ethanol for storage until further processing or shipping to a pathologist for examination.

Abalone of small size and some organs in larger abalone should not be dissected because of damage caused to the tissue in the dissection process. As a general guideline, abalone less than 5 mm in shell length should be preserved intact. Abalone from about 5 mm to 3 cm in shell length should be removed from the shell prior to being preserved whole (ie, with no further dissection). Abalone greater than 3 cm in shell length must be removed from the shell and tissues dissected (Fig. 2). The region of the digestive gland, stomach, crop, and heart kidney complex (Fig. 2b,c) should not be dissected prior to fixation because of damage to these delicate tissues caused by the dissection process. The part of the abalone consisting mainly of the foot muscle can be discarded unless le-
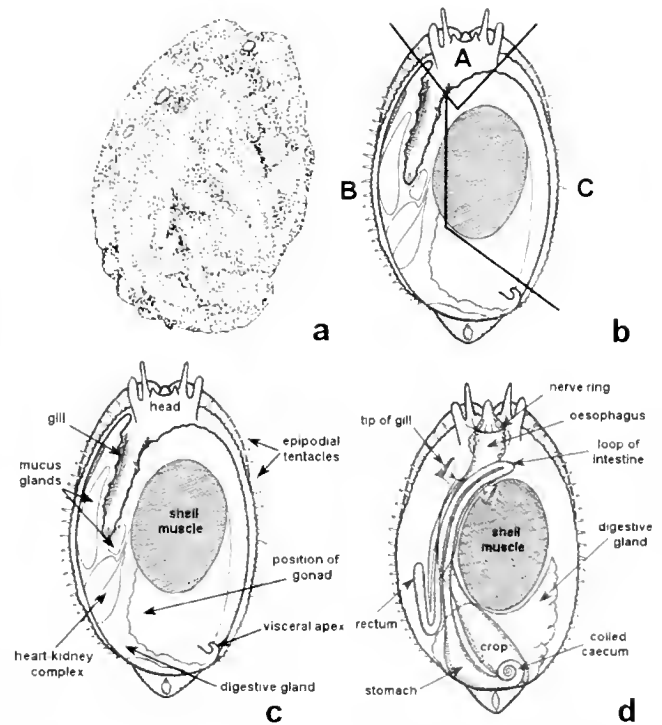

Figure 2. Dorsal views of an abalone. (a) Orientation of features on outer surface of shell. (b) Body parts A (head) and B (viscera) should be preserved for histologic examination and part C (foot) can be discarded. The lines indicate where the tissues should be cut to avoid unnecessary disruption to the delicate visceral organs. (c) Major organs underlying the shell. (d) Major organs underlying the respiratory, excretory, and reproductive organs. Images b, c and d were modified from Bullough (1958).

TABLE 2.

Formulation of two preservatives used to fix abalone tissues for histological examination. Tissues must be fixed as soon as possible after the abalone is removed from the water. Prior to fixation, record all lesions observed on each specimen and confirm that there is at least 10 parts preservative to every 1 part of abalone tissue.

Davidson's Solution (Shaw & Battle 1957)	10% Formalin in Sea Water
400 mL glycerin, and	1 part formaldehyde
800 mL formaldehyde	9 parts sea water
1200 mL 95% ethanol	
1200 mL sea water	
Just prior to use add 1 part glacial acetic acid to every 9 parts of the above mixture.	

sions are detected or withering syndrome is suspected. Lesions or other tissue abnormalities should be described and location noted because they are usually not evident after the tissue has been chemically preserved. If the lesion is on the foot (part of the abalone usually not preserved for histologic examinations), a representative sample, which is no greater than a 1 cm cube, should be added to the preservative. If withering syndrome is suspected, a 3–5 mm cross section of the foot muscle should be preserved.

Preserved tissues can be stored for prolonged periods (years). However, histologic examinations should be conduced as soon as possible to expedite the use of resulting interpretations. For histologic examination, the tissues must be processed and stained for microscopic examination as described and illustrated by Howard and Smith (1983). In brief, the fluids in the tissues must be removed and replaced with paraffin wax. The wax embedded tissues are then cut into about 6-μm thin slices (sections) and the sections mounted on a glass slide and stained. The cells in the resulting stained histologic sections are then examined microscopically for abnormalities and observations correlated with the notes taken prior to sample preservation.

Histologic examinations will usually reveal the presence of symbionts and parasites. Knowledge and experience on the identification of these organisms is used to determine which can cause diseases of concern. Once an infectious agent has been identified, steps to prevent further spread within a culture facility or to the natural environment can be implemented. In addition to detecting pathogens, histologic examinations can also be useful in assessing

the suitability of the culture environment. The morphology of tissue cells can indicate the suitability of the diet or an increased intensity of symbionts (either bacteria or protists) can signify unsuitable parameters in the habitat. Until other more specific and sensitive assays are available to detect disease agents and assess abalone health, histologic examination will serve as a valuable tool for optimizing abalone culture conditions and avoiding unnecessary losses during rehabilitation efforts.

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FECUNDITY AND SEASONAL REPRODUCTION OF NORTHERN ABALONE, *HALIOTIS KAMTSCHATKANA*, IN BARKLEY SOUND, CANADA

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ABSTRACT Fecundity, size at maturity and seasonal reproduction of northern or "pinto" abalone, *Haliotis kamtschatkana*, from exposed "surf" areas and more sheltered, productive abalone habitat were investigated in Barkley Sound. Examination of histologic sections of gonads indicated that size at maturity occurred at a smaller size for the stunted "surf" abalone than for abalone from more sheltered areas. Gonad index and stages showed that gonads were mainly ripe and that most abalone spawned during April to July. Although there were smaller abalone with ripe eggs from the "surf" area than those from the sheltered area, abalone females of comparable size from both areas had similar egg numbers. However, there were larger females with considerably higher fecundity from the sheltered areas than from the "surf" areas. Implications of transplanting "surf" abalone to productive habitats to increase growth and fecundity rates are discussed in the context of population rebuilding attempts for *H. kamtschatkana*, which is listed by the Committee on the Status of Endangered Wildlife in Canada as a "threatened" species in Canada.

KEY WORDS: abalone, *Haliotis kamtschatkana*, fecundity, reproduction, size at maturity

INTRODUCTION

The northern or "pinto" abalone, *Haliotis kamtschatkana* J. H. Jonas, 1845 (Gastropoda), which occurs from Sitka Island, Alaska, to Baja California, is found on rocky habitats from the intertidal to subtidal depths of 100 m, with most adults found at less than 10 m in British Columbia (BC) (Sloan & Breen 1988). Declines of northern abalone abundance in the 1980s resulted in an abalone fishery closure in December 1990, and with continued poor recruitment (Campbell 2000, Jamieson 2001) this species was designated as "threatened" in April 1999 by the Committee on the Status of Endangered Wildlife in Canada (COSEWIC). Reproductive characteristics of northern abalone are considered important in understanding the population biology of this species in BC. Early studies indicated that northern abalone became sexually mature at about 50 mm shell length (SL) (Quayle 1971, Paul & Paul 1981). Eggs released by captive spawning females in sea water were estimated at 2.3 million over 2.3 h by a 135 mm SL female and 33,700 over 1.5 h by a 101 mm SL female (Olson 1980, Caldwell 1981). Campbell et al. (1992) found 50% size at maturity to be 55 mm SL and fecundity to range from thousands to millions of eggs per female for northern abalone collected from southeastern Queen Charlotte Islands (QCI) in June 1990. Spawning generally occurred from April to July (Quayle 1971, Breen & Adkins 1980, Sloan & Breen 1988).

Spatial variation in demography of abalone populations is common throughout the world (Shepherd et al. 1992, Worthington & Andrew 1998). Abalone populations that have small or stunted individuals typically occur in locations having less than optimal environmental conditions. In poor habitats, individuals generally have slower growth and reach a smaller maximum size than abalone found in more optimal habitats (Shepherd 1988, Sloan & Breen 1988, Emmett & Jamieson 1988, Nash 1992, Wells & Mulvay 1995). In BC, transplanting slow growing northern abalone from high wave exposure ("surf") areas to sheltered locations was shown to increase individual growth rates in both the QCI (Breen 1986) and in Barkley Sound (Emmett & Jamieson 1988). Surf abalone in exposed areas are slower growing individuals that fail to reach sizes greater than 100 mm SL (Sloan & Breen 1988). Factors such as abalone density, genetics, quantity and quality of available food, predator activity, substrate type (rugosity), and

wave action may all influence growth and survival rates (Sloan & Breen 1988). Donovan and Carefoot (1997, 1998) found respiration activity and locomotion (mucus production) were a major part of the energy budget of northern abalone. Predator presence, high wave action, lack of food (Sloan & Breen 1988), and lack of suitable crevices (Shepherd 1986) for shelter from predators or wave action may increase abalone activity and respiration, reducing energy available for growth and reproduction.

Little comparative information on the reproductive capabilities between abalone from poor and optimal habitats is available for different areas of BC. The purpose of this article is to provide seasonal patterns of reproduction, sizes at maturity, and fecundity levels for northern abalone from both exposed "surf" and moderately sheltered abalone habitats in Barkley Sound on the west coast of Vancouver Island. Size-specific sexual maturity and fecundity are important in determining the relative potential egg production of northern abalone populations from poor and good areas.

MATERIALS AND METHODS

Northern abalone were collected by SCUBA divers at two sites in Barkley Sound: (1) an exposed area east of an unnamed island with a height of 39 m indicated on chart 3670 (Canadian Hydrographic Service) referred to in this article as Island 39 (Lat. 48°51.550′N, Long. 125°19.048′W) where "surf" abalone were found, and (2) a moderately sheltered area southwest of Willis Island (Lat. 48°54.907′N, Long. 125°20.873′W) where a large size range of abalone was found. Representative size ranges of abalone at an equal sex ratio were collected on several occasions from both sites during 1991 and 1992 (see Table 1 for sample dates). Immediately after collection, shell length (SL) in mm, total wet weight (g), sex (by gonad color: beige for males and green for females) were recorded for each abalone. Random transect surveys (for the method see Cripps & Campbell 1998, Lessard et al. 2002) to determine density and size of emergent northern abalone, were conducted along approximately 80 m of the east side of Island 39 (*n* = 3) on 4 July, 2002, and along approximately 300 m of the east side of Hankin Island (Lat. 48°55.266′N, Long. 125°21.946′W) (*n* = 5) on 6 June 2002. Substrate type for Island 39 was bedrock with a few crevices and rocks. Substrate type for both Willis and Hankin Islands was bedrock with many small and

TABLE 1.

Seasonal gonad stages of *H. kamtschatkana* sampled from Willis Island and Island 39 during 1991–1992.

Date	Gonad Stages (% of n)					
	1	2	3	4	5	n
Willis Island						
18 June, 1991	42	45	3	10	0	60
5 July	100	0	0	0	0	20
7 August	0	0	0	75	25	20
10 October	15	0	8	69	8	13
22 Jan., 1992	3	0	7	90	0	29
6 April	8	50	4	17	21	24
21 April	5	50	10	20	15	20
5 May	35	30	0	25	10	20
20 May	15	45	0	40	0	20
6 June	5	40	5	35	15	20
20 June	28	56	0	0	17	18
2 July	35	50	0	0	15	20
Island 39						
18 June, 1991	16	45	0	30	9	44
6 April, 1992	0	50	0	21	29	24
21 April	15	50	0	5	30	20
5 May	33	29	0	10	29	21
20 May	10	55	0	30	5	20
6 June	5	48	0	48	0	21
20 June	35	40	0	15	10	20
2 July	20	20	0	25	35	20

The 5 stages of mature gonads were classified as 1 = ripe; 2 = partly spawned; 3 = fully spawned; 4 = recovery stage 1; 5 = recovery stage 2. n = sample size.

large boulders. Daily sea surface water temperatures were obtained from the Amphitrite Point (north west outer tip of Barkley Sound) database during 1991 to 1992 to compare mean monthly temperatures. Mean annual and mean first 6 mo surface temperatures were calculated from monthly mean temperatures for each year.

To determine seasonal reproductive condition and maturity of each male and female abalone, the conical appendage containing the gonad sheath located over the hepatic gland (Poore 1973) was removed and preserved in Davidson's Solution (Shaw & Battle 1957). The fixed gonad was cut about midway between the apex of the shell and the tip of the conical appendage and cross sections of the gonad and hepatic gland were traced on transparent plastic. Relative areas of hepatic gland and gonad in cross section were obtained by weighing the plastic outlines. The gonad index (I) was calculated as I = 100 G/T, where G is the cross-sectional area (weight of plastic) of the gonad, and T is the total cross-sectional area (weight of plastic) of both the gonad and hepatic gland. Histologic slides were prepared by staining sections of gonad with hematoxylin-eosin. Histologic sections of gonad were classified into 6 stages. Immature individuals (Stage 0) were characterized by no differentiation in gonadal tissue, or where there was small gonadal bulk for (a) males that was comprised mostly of primary or secondary spermatocytes with no spermatozoa present and (b) for females that was comprised mostly of primary oocytes and some stalked oocytes, but no mature or degenerating oocytes present. The other stages were for mature abalone gonads that had well developed spermatazoa or oocytes usually in abundance, or where there were few mature oocytes, or degenerating unspawned oocytes were present. The 5 stages of mature gonads were: (1)

ripe, (2) partly spawned, (3) fully spawned, (4) recovery stage 1; and (5) were recovery stage 2 (Wells & Keesing 1989). This pattern of gametogenesis is similar for several different abalone species (eg. Newman 1967, Young & DeMartini 1970, Giorgi & DeMartini 1977, Mottet 1978, Tutschulte & Connell 1981).

To determine the relationship between the proportion of mature northern abalone and shell length, data were combined by 5-mm SL classes for both males and females (since the curve for each sex was similar) and was estimated for each study area using the equation:

$$P_i = L_i/(L_i + e^{(A - B L_i)}) \tag{1}$$

where P_i is the proportion of the mature abalone in the *i*th 5-mm SL interval, L_i is the shell length in the *i*th 5-mm SL interval, and the coefficients A and B were estimated with a non-linear (simplex) Marquardt least squares method (SYSTAT 2000). Only data from northern abalone collected during June 1991 and June 1992 were used in estimating proportion maturity values, since this reproductive time period provided the maximum opportunity to distinguish between immature and mature individuals.

Potential fecundity was determined from ripe gonads representing the full size range of mature *H. kamtschatkana* collected during June 1991, just prior to spawning, by placing whole females in 10% formalsaline. Internal organs of individuals more than 90 mm SL were injected with formalsaline to help accelerate fixation and hardening of the ovaries. Each ovary was then removed, the gonad index measured, the hepatic organ excised, and the drained wet weight of each ovary recorded. Three small subsamples, each weighing approximately 0.006 g (range ± 0.003 g) wet weight, were removed randomly from each ovary, weighed, and the mature eggs (oocytes) in each ovary freed from ovarian connective tissue with fine dissecting forceps and a small paint brush. Freed eggs were counted under a dissecting microscope. Diameters of 10 oocytes per female were measured from a selection of mature females over a wide range of sizes (59.8–125 mm SL and 40.5–64.4 mm SL, respectively) from Willis Island and Island 39. Mean egg density per gram was determined from the subsamples. Initial tests indicated no differences (t-test, $P > 0.05$) in mean egg density between different locations on the ovaries of five northern abalone. Other studies (eg. Giorgi & DeMartini 1977, Wells & Keesing 1989) found eggs homogeneously distributed throughout the ovaries in other abalone species. Fecundity, or total number of eggs per female, was estimated as the product of mean egg density and total ovary weight.

The relation between fecundity (F) and shell length (L) was expressed with the natural log transformed linear regression equation:

$$\log_e F = \log_e A + B \log_e L \tag{2}$$

where the coefficients A and B were estimated using the least squares method. Analysis of covariance (ANCOVA) was used to test for the homogeneity of slopes and elevation coefficients of the log transformed data regressions (Zar 1984) of fecundity of northern abalone collected from different areas. All ANCOVA comparisons for fecundity and SL, in similar size groups, between areas Willis Island, Island 39, and south east QCI (Campbell et al. 1992) indicated there were no differences ($P < 0.05$) between slopes or elevations, so size at fecundity data were combined into one equation. The relation between fecundity (F) and total abalone drained wet weight (W) was expressed with the linear equation

$F = A + B W$, where the coefficients A and B were estimated using the least squares method.

Potential total egg production per m^2 (E) was estimated as:

$$E = \sum_i F_i P_i S_i (N_i, N) \bar{d} \tag{3}$$

where F_i is the fecundity (eggs/female) at shell-length increment i (mid point of each 5 mm SL size class increment was used), P_i is the proportion mature at i, S_i is the sex ratio of mature individuals, assumed to be 0.5 for all i (this study, Sloan & Breen 1988), N_i is the number of abalone measured in the i'th size, and N is the total number of abalone measured. Estimated mean density, $\bar{d}$ (number / m^2), of all emergent abalone for a number of transects was calculated as:

$$\bar{d} = \frac{\sum_t (N_t x_t)}{\sum_t x_t} \tag{4}$$

where N_t is the number of abalone counted in transect t, x_t is the length of transect t (i.e., area in square meters since each transect was one meter wide).

Cumulated total potential egg production was estimated from *H. kamtschatkana* size frequency and mean density estimates of 0.750 and 0.404 abalone/m^2, obtained at Island 39 and Hankin Island, respectively, during June to July 2002.

RESULTS

Shell Lengths

From biologic samples collected during 1991 to 1992, the median SL was larger for abalone from Willis Island, 87.0 mm (range 44.0–125 mm SL, $n = 313$), than abalone from Island 39, 67.0 mm (range 32.4–87.0 mm SL, $n = 217$). Of the total abalone collected, 16% and 0% were 100 mm SL or greater, from Willis Island and Island 39, respectively.

From the random transect survey completed during June to July 2002, the median SL was larger for abalone from Hankin Island, 79.0 mm (range 18.0–115 mm SL, $n = 37$), than for abalone from Island 39, 61.0 mm (range 23.0–98.0 mm SL, $n = 27$). Of the total abalone collected, 13.5% and 0% were 100 mm SL or greater, from Willis Island and Island 39, respectively.

Seasonal Reproduction

The gonad index was high for *H. kamtschatkana* from both locations during May to July 1991 to 1992, but was low at Willis Island during August 1991 to January 1992 (Fig. 1). Histologic examination of gonad sections indicated a similar reproductive pattern for most abalone from both locations (Table 1). During June 1991 and April to June 1992, gonads were either ripe or partly spawned. Abalone from Willis Island had ripe gonads during July 1991 and mostly recovery stages during August 1991 to January 1992 (Table 1). Results indicated that gonads were ripe mainly during the summer months of May to early July, with spawning occurring mainly during May to July. A few abalone had ripe to spawned gonads in April 1992. A few abalone from Willis Island had ripe gonads and may have spawned from October 1991 to April 1992 (Table 1).

The mean annual sea surface temperature was 10.4°C for 1991 and 11.3°C for 1992 in Barkley Sound (Amphitrite Point). Mean sea surface temperatures were warmer (by 1.4°C) for the first 6 mo
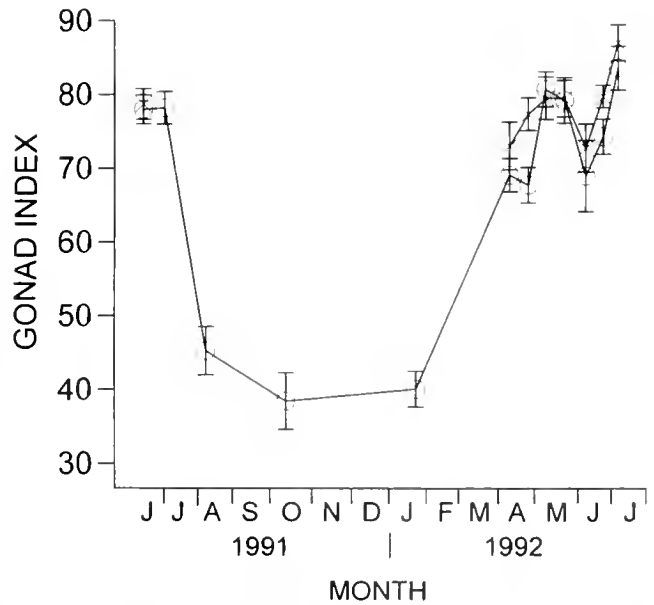

Figure 1. Seasonal changes in mean gonad index (percent) of *H. kamtschatkana* from Willis Island (O) and Island 39 (X) from June 1991 to July 1992. Horizontal line about each mean is ±1 standard error. See Table 1 for sample sizes.

of 1992 (10.7°C) than that of 1991 (9.3°C). During the main reproductive period of northern abalone, May, June, and July mean monthly sea surface temperatures were 10.7, 11.4, and 12.5°C for 1991 and 12.1, 13.0, and 14.4°C for 1992, respectively.

Size at Maturity

Although there was considerable variation, gonad indices increased with increasing SL (Fig. 2). Gonad indices were larger at smaller sizes of abalone from Island 39 than at Willis Island. Size at 50% maturity was lower for abalone from Island 39 (44 mm SL)
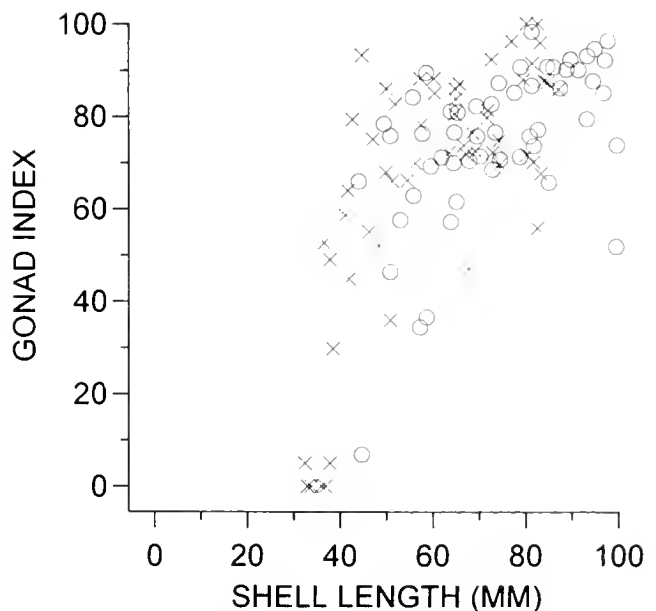

Figure 2. Size specific mean gonad index (percent) of *H. kamtschatkana* from Willis Island (O) and Island 39 (X) during June 1991.

than those from Willis Island (50 mm SL) (Fig. 3, Table 2). Examination of histologic sections indicated that the smallest mature abalone were 42.6 and 49.4 mm SL and the largest immature individuals were 50.8 and 57.0 mm SL at Island 39 and Willis Island, respectively, during June 1991 to 1992. Maturity occurred for most abalone at sizes greater than 65 mm SL. Although the color of whole gonads could be differentiated to determine sex, histologic examination indicated that not all gonads were mature in the 32 to 57 mm SL range.

Fecundity

For females with ripe gonads, both mean oocyte diameter (231 μm ± 2 SE, $n = 6$) and mean density or number of eggs per g (178,386 ± 3,360 SE, $n = 33$) did not significantly differ (two way

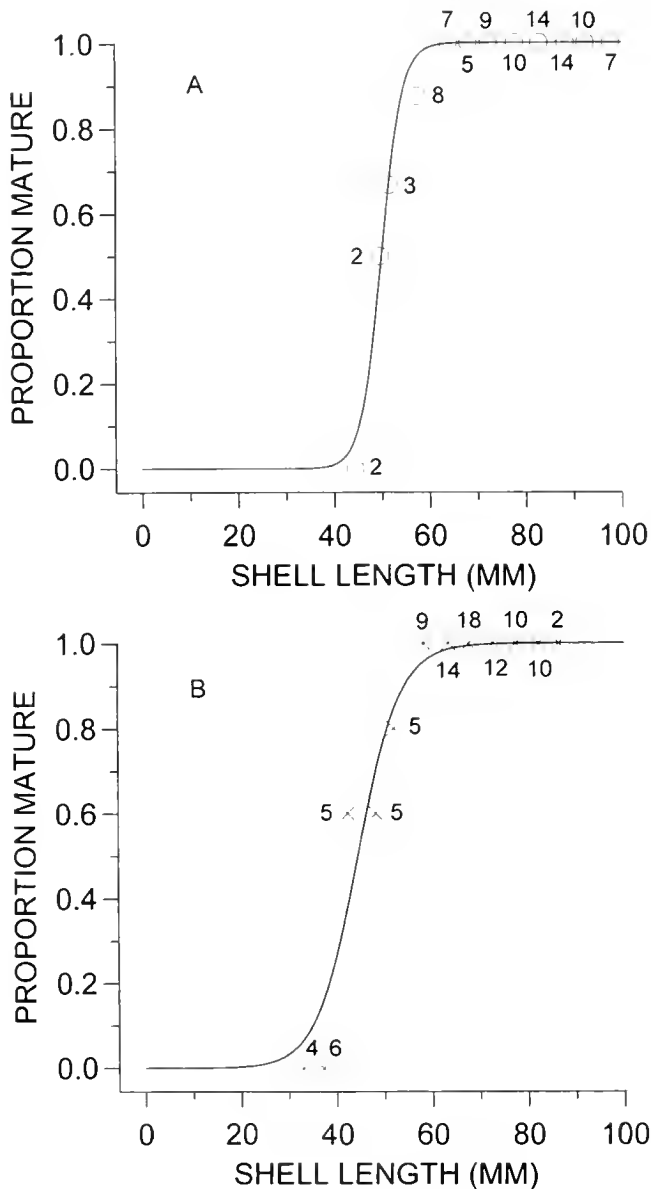

Figure 3. Proportion mature at mid point of each 5-mm shell length class of *H. kamtschatkana* (sexes combined) from (A) Willis Island and (B) Island 39. Numbers next to symbols are sample sizes. See Table 2 and text for information on predicted equations for size at maturity curves.

TABLE 2.

Equation coefficients for size at maturity equation from *H. kamtschatkana* sampled in Barkley Sound. See text for equation details and Fig. 3 for data. Values in brackets are approximate 95% confidence intervals. R^2 = coefficient of determination.

	Equation Coefficients		
Site	A	B	R^2
Willis Island	25.807 (±6.475)	0.438 (±0.129)	0.987
Island 39	12.863 (±4.973)	0.204 (±0.111)	0.943

ANOVA, $P > 0.05$) between abalone of different sizes or between those from Willis Island and Island 39. ANCOVA comparisons indicated there was no difference ($P > 0.05$) in slopes or intercepts in the linear relation of $\log_e$ transformed gonad weight (g) and SL between Willis Island and Island 39. Therefore, data from both locations were combined into one equation:

$$\log_e \text{(gonad weight, g)} = -13.4807 + 3.48 \log_e \text{(SL)}$$
$$(R^2 = 0.82, P < 0.01, n = 33)$$

estimated by the least squares method. Given that (1) egg densities were independent of abalone size and eggs were distributed homogeneously throughout the ovary, and (2) there was a relationship between the preserved ovary wet weight and SL, the fecundity of *H. kamtschatkana* could be related to SL or total abalone weight (Table 3). Although there was considerable variation in fecundity between individuals of similar size, the increase in fecundity in relation to increases in SL and weight of *H. kamtschatkana* was highly correlated (Fig. 4, see Table 3). The smallest female (40 mm SL) from Island 39 had 90,594 eggs, the largest female (125 mm SL) from Willis Island had 3.0 million eggs, and the largest female (144 mm SL) from southeast QCI had 11.3 million eggs (Campbell et al. 1992).

Potential Population Egg Production

The cumulated proportion of sizes was higher for small size classes of *H. kamtschatkana* from Island 39 than from Hankin Island (Fig. 5). This was reflected in a higher cumulated potential egg production from the 50 to 80 mm SL class from Island 39 than from Hankin Island (Fig. 6).

TABLE 3.

Equation coefficients for the fecundity (F, number of eggs per female) and shell length (L in mm) log transformed equation $\log_e F = \log_e A$ B$\log_e L$ for *H. kamtschatkana*, and fecundity and total weight (W in g) linear equation $F = A$ BW. Areas: 1 = Willis Island and Island 39 combined (this study), 2 = Willis Island and Island 39 (this study) combined with south east Queen Charlotte Islands (Campbell et al. 1992).

Independent Variable	Areas	Equation Coefficients			
		A	B	R^2	n
Shell length	1	−1.5203	3.5137	0.843	33
	2	−2.2152	3.6789	0.915	56
Total weight	1	108,463	14588.5	0.803	33
	2	−411,590	25179.5	0.910	56

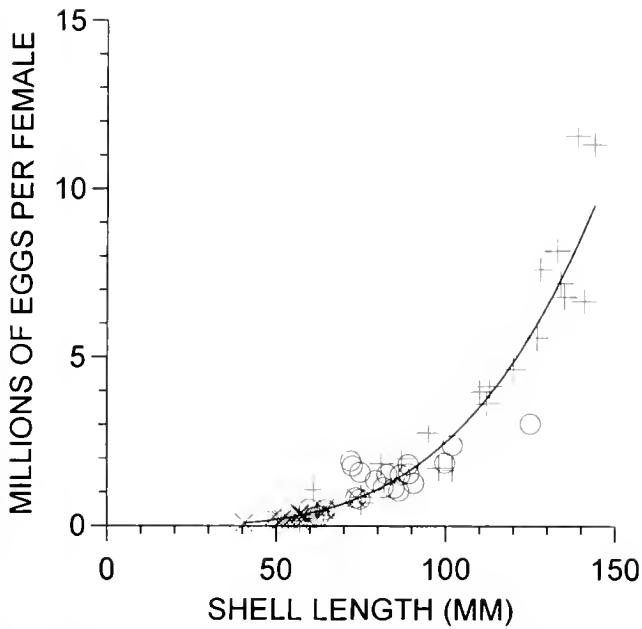R^2 = coefficient of determination. n = sample size.

Figure 4. Fecundity at shell length of female *H. kamtschatkana* from Willis Island (O) and Island 39 (X) during June 1991 (this study), and from Queen Charlotte Islands (+) during June 1990 (after Campbell et al. 1992). See Table 3 for regression equation coefficients.

Despite mean abalone density estimates being higher at Island 39 (0.750/m²) than at Hankin Island (0.404/m²) during June to July 2002, the estimated total potential egg production per unit area (millions of eggs per m²) was higher at Hankin Island (0.26) than at Island 39 (0.20) (Fig. 6) due to higher fecundity of large abalone (≥100 mm SL) present at Hankin Island. In contrast, if we assumed a similar hypothetical abalone density of 1.0/m² for both areas, estimated total potential egg production (millions of eggs
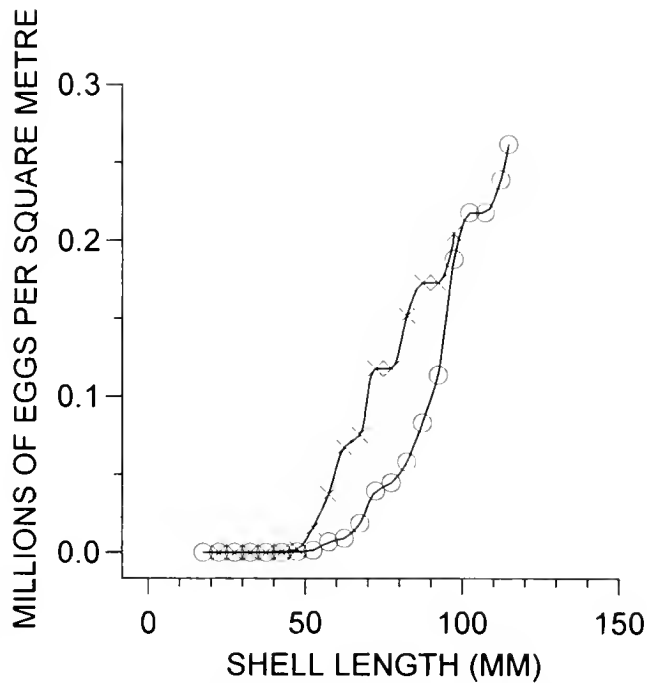

Figure 6. Cumulated total potential egg production (millions per m²) by 5-mm SL classes of *H. kamtschatkana* from Hankin Island (O) and Island 39 (X) based on mean density estimate of 0.404 and 0.750 abalone/m², respectively, during June to July 2002. See text for method to calculate potential total egg production. Lines fitted to data by a spline smoother with cubic equations (SYSTAT 2000).

per m²) would be more than twice as much for Hankin Island (0.65) than for Island 39 (0.27).

DISCUSSION

Shell lengths of *H. kamtschatkana* sampled from Island 39 were all less than 100 mm, typical of abalone from a "surf" area with less than optimal habitat conditions (Sloan & Breen 1988, Emmett & Jamieson 1989). In contrast, there was a larger range of sizes of abalone from the moderately exposed areas of Willis and Hankin Islands. The largest abalone encountered in this study was a 125 mm SL female from Willis Island. Low numbers of large abalone (≥100 mm SL) found in this study and in other recent surveys (eg, Lucas et al. 2002) are in sharp contrast to the many (51%) large *H. kamtschatkana* (maximum 146 mm SL, $n = 1305$) sampled by Quayle (1971) in Barkley Sound during 1963 to 1964.

Seasonal reproduction of *H. kamtschatkana* occurred mainly from April to June at Willis Island and Island 39 in our study, similar to that found by Quayle (1971) in other islands in Barkley Sound. Warmer conditions in January to June 1992 than in 1991 may have caused abalone to spawn earlier in 1992 than in 1991. Our study also confirms that there may be a few abalone that are ripe throughout the year that may be able to spawn (see review by Sloan & Breen 1988). Intra- and inter-specific variability in gametogenesis and annual spawning periods is common for many abalone species and spawning may partly depend on local conditions (eg, temperature, storms, food quality, and abundance) (Newman 1967, Webber & Giese 1969, Shepherd & Laws 1974, Paul et al. 1977, Hayashi 1980, Sloan & Breen 1988, Wells & Keesing 1989, Stekoll & Shirley 1993, Hooker & Creese 1995, Sasaki & Shepherd 1995, Wilson & Schiel 1995).
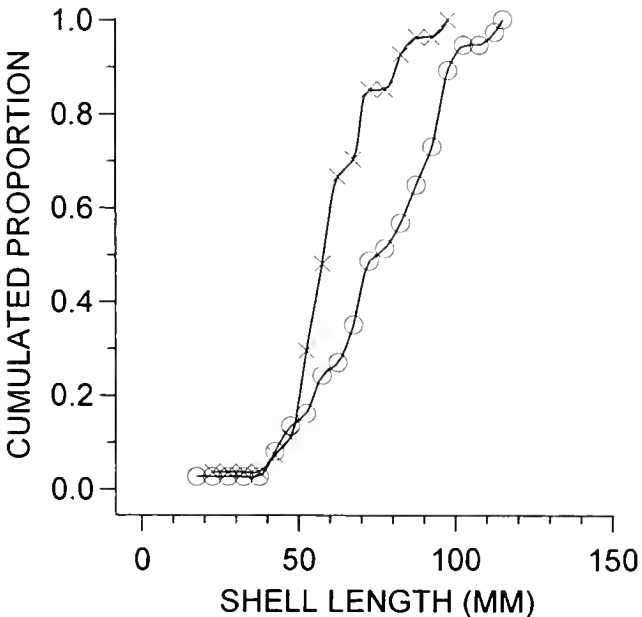

Figure 5. Cumulated proportion size frequency by 5-mm SL classes of *H. kamtschatkana* from Hankin Island (O) and Island 39 (X) during June 2002. Lines fitted to data by a spline smoother with cubic equations (SYSTAT 2000).

This study recorded the lowest 50% size at maturity (44 mm SL from Island 39) to date for wild *H. kamtschatkana*. The 50% maturity at 50 mm SL at Willis Island was similar to that found by Quayle (1971) for abalone generally in Barkley Sound. Maturity of wild *H. kamtschatkana* further north of Barkley Sound (eg, QCI and Alaska) was found to vary between 50 to 64 mm SL (Larson & Blankenbeckler 1980 referenced in Sloan & Breen 1988, Paul & Paul 1981, Campbell et al. 1992). We confirm Quayle's (1971) observation that although sexes could be differentiated by color of gonads at small sizes (e.g., 32 mm SL) the start of sexual maturity was observed at larger sizes (smallest mature individual we observed was 42.6 mm SL). Size at sexual maturity for an abalone species can vary between locations (Shepherd & Laws 1974) depending on various factors such as food quality and availability and different temperature regimes (Kikuchi & Uki 1974a, Kikuchi & Uki 1974c, Kikuchi & Uki 1975, Paul et al. 1977, Paul & Paul 1981).

Fecundity estimates were similar for *H. kamtschatkana* of equivalent shell lengths from Willis Island, Island 39 and southeast QCI, and the number of eggs increased exponentially with increases in shell size. Fecundity estimates of *H. kamtschatkana* (this study, Campbell et al. 1992) are within the range reported for other abalone species. The largest number of eggs reported for a *H. kamtschatkana* was 11.56 million eggs for a 139-mm SL female (Campbell et al. 1992). High fecundity has been reported for other abalone species—25.4 million eggs for a 175-mm SL *H. midae* (Newman 1967), 12.6 million eggs for a 190.5-mm SL *H. rufescens* (Giorgi & DeMartini 1977). Methods to estimate fecundity vary from estimating the total number of eggs in ovaries by weight (Newman 1967, Poore 1973, Giorgi & DeMartini 1977, Hayashi 1980, Wells & Keesing 1989, this study) and by volume (Sainsbury 1982, Prince et al. 1987, McShane et al. 1988) to counting the number of eggs spawned (Kikuchi & Uki 1974a, Kikuchi & Uki 1974b, Kikuchi & Uki 1974c, Kikuchi & Uki 1975, Hayashi 1980, Olson 1980, Caldwell 1981, Tutschulte & Connell 1981, Ault 1985, Clavier 1992). Although fecundity was estimated as the total eggs present in an ovary prior to spawning in this study, probably not all eggs may be spawned within a spawning event (Poore 1973, Giorgi & DeMartini 1977, Ault 1985). Caldwell (1981) found that *H. kamtschatkana* females at 101 and 135 mm SL spawned an estimated 0.03 and 2.3 million eggs, respectively, in 1.5 to 2.5 h in the laboratory, which were lower than the mean total eggs (2.6 and 7.5 million eggs) estimated in ovaries of individuals of the same size from our study. The relationship between fecundity and SL for various abalone species has been described either as curvilinear (Newman 1967, Poore 1973, Giorgi & DeMartini 1977, Hayashi 1980, Wells & Keesing 1989, Wilson & Schiel 1995, Litaay & De Silva 2001, this study) or as linear (Poore 1973, Sainsbury 1982, Prince et. al. 1987, McShane et al. 1988). The relationship between fecundity and weight of the whole abalone has been considered as curvilinear (Ault 1985, Litaay & De Silva 2001) or as linear (Newman 1967, Tegner et al. 1989, Shepherd et al. 1991, Shepherd et al. 1995).

Northern abalone from exposed "surf" areas were capable of reproducing and, at the equivalent size, potentially have similar fecundity per unit area as abalone from more sheltered areas. Size at maturity and fecundity, size composition and density are important in determining the total potential contribution of an abalone population in an area. Given similar densities, larger abalone in moderately sheltered areas have a potentially higher reproductive contribution than smaller, slower growing abalone in high "surf" exposed areas. The importance of having sufficient numbers of large abalone for reproductive output has been emphasized in the fishery context (Breen 1986, Tegner et al. 1989, Shepherd & Baker 1998) and for evaluating marine protected areas as conservation tools for abalone (Edgar & Barrett 1999, Wallace 1999, Rogers–Bennett et al. 2002). Fertilization and recruitment success of various abalone species may also be density dependent (Clavier 1992, McShane 1995a, McShane 1995b, Babcock & Keesing 1999).

Implications of transplanting abalone from "poor" to "good" habitats to increase survival, growth, and reproductive potential, as a rebuilding technique for *H. kamtschatkana* in BC, are complicated. Emmett and Jamieson (1988) concluded that transplanting large sizes of "surf" northern abalone from exposed sites to more productive areas was biologically and economically feasible if survival of the transplanted abalone was reasonably high. They did not evaluate methods to potentially enhance reproductive output or recruitment in the transplant areas. Tegner (1992, 1993, 2000) reported on a transplant of reproductively mature green abalone, *H. fulgens*, in California with subsequent strong evidence of successful local recruitment until the brood stock were poached. The long-term success of a brood stock transplant is dependent on adult survival and density for fertilization success, and local hydrodynamics for larval settlement (Babcock & Keesing 1999). Future attempts to rehabilitate *H. kamtschatkana* in BC by transplanting "surf" abalone will require pilot experiments to test transplant methods for their feasibility to determine measurable success in increased population survival, growth, reproduction, and recruitment. The choice of recipient "good" sites would include criteria such as identifying locations with complex substrates, moderate to low exposure, and availability of suitable algal food for optimal abalone growth and survival. Abundance and distribution of *H. kamtschatkana* in exposed and moderately sheltered areas are not well known and need to be estimated prior to large scale rebuilding efforts in local areas of coastal BC.

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ESTIMATING JUVENILE NORTHERN ABALONE (*HALIOTIS KAMTSCHATKANA*) ABUNDANCE USING ARTIFICIAL HABITATS

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ABSTRACT This study assesses the use of artificial concrete block habitats that provide standardized sample areas for measuring the abundance of northern abalone (*Haliotis kamtschatkana* Jonas) in comparison to 10 randomly selected 1-m^2 quadrant samples where all movable rocks were examined for cryptic abalone. A total of 278 abalone were measured within artificial structures and juvenile abalone ($\leq$50 mm shell length, SL) were the most abundant size class. Juvenile abalone used artificial structures at greater mean densities (abalone/m^2) than nearby natural habitat (1.27 ± 0.25 SE versus 0.07 ± 0.09 SE) and emergent abalone (>50 mm SL) used artificial habitats at similar densities as they did in nearby natural habitats (0.38 ± 0.09 SE versus 0.44 ± 0.10 SE). Juvenile abalone abundance was significantly different between sites but not within sites, suggesting artificial structures showed promise in their ability to detect area specific differences in recruitment and to easily measure juvenile abalone abundance.

KEY WORDS: juvenile, abalone, cryptic, artificial habitat, recruitment, *Haliotis kamtschatkana*

INTRODUCTION

Northern abalone (*Haliotis kamtschatkana*) fisheries in British Columbia (BC) remain closed to commercial, recreational, and First Nations groups since 1990 due to conservation concerns (Campbell 2000). Dive surveys conducted by Fisheries & Oceans Canada (DFO) at index sites in BC estimated that northern abalone abundance had declined by more than 75% during 1978 to 1984 and continue to remain low (Breen & Adkins 1979 1981, Winther et al. 1995, Campbell et al. 2000). In April 1999, the Committee on the Status of Endangered Wildlife in Canada (COSEWIC) listed northern abalone as "threatened", meaning likely to become endangered if limiting factors are not reversed.

The most significant factors inhibiting northern abalone recovery are illegal harvests and poor recruitment (Campbell 2000). Recruitment, defined as the number of juvenile abalone growing and surviving to the adult population each year, may be insufficient as a result of critically low adult densities (Shepherd & Brown 1993, Shepherd & Partington 1995) that reduce reproductive success due to low fertilization of gametes (Allee et al. 1949). Other processes that may reduce abalone recruitment include variation in timing and intensity of gamete production, larval predation, and post-larval mortality (McShane 1992, 1995). Recruitment processes for northern abalone are not well understood (Breen 1986, Sloan & Breen 1988).

Increasing the abundance of existing wild northern abalone populations in BC is the long-term goal of the northern abalone national recovery strategy (Toole et al. 2002). One component of the strategy is to conduct abalone research and rebuilding experiments that may lead to increased breeding success, recruitment, and population densities. To evaluate the success of various rebuilding experiments, it will be necessary to measure changes in abalone recruitment by quantifying the abundance of juveniles.

Artificial collectors have been successful at measuring the intensity of abalone larval settlement (Keesing et al. 1995, Nash et al. 1995) but require high maintenance, a considerable time investment to sort samples and appropriate larval identification expertise. Other larval settlement survey techniques such as underwater magnification (Shepherd & Turner 1985), anesthesia (Prince & Ford 1985), and suction (McShane & Smith 1988) also require great diving and sample sorting efforts. In California, Davis (1995) used artificial concrete block habitats that provided standardized sample areas to monitor juvenile abalone recruitment. Comparing

results from previous juvenile abalone surveys that required the destruction of natural habitat (Tegner et al. 1989), Davis (1995) was able to provide surrogate juvenile abalone habitat and produce an index of abalone recruitment.

This article describes the design and testing of artificial concrete block habitats over a 12-month period at 6 sites in Haida Gwaii (Queen Charlotte Islands), BC. The objectives were to determine if concrete block habitats provided surrogate habitat for juvenile northern abalone and if so, the ability of artificial habitats to quantify juvenile abalone abundance in different locations. To determine if juvenile abalone abundance within artificial habitats was representative of nearby natural habitats, invasive surveys of natural abalone habitats during the same time period were compared.

MATERIALS AND METHODS

Twenty-four artificial concrete block habitats were tested at 6 sites located at Lyell, Faraday and Murchison Islands (Fig. 1). These sites are within the Haida Gwaii Juan Perez Sound abalone stewardship area, where annual ecological assessments, abalone population surveys, and mark-recapture monitoring were conducted during 1998 to 2003 (Jones et al. 2003). The general area currently supports average densities of 0.35 emergent abalone/m^2 and 0.17 emergent youth (<70 mm shell length, SL) abalone/m^2 (Campbell et al. 2000).

The artificial habitat design used is a modification of that described by Davis (1995). Each habitat provides about 3.5 m^2 of surface area and consists of 24 concrete mini-blocks haphazardly oriented within a modified commercial crab trap (Fig. 2). Standard 20 cm × 20 cm × 40 cm concrete blocks were cut into quarters longitudinally to produce four individual mini-blocks. Discarded commercial crab traps measuring approximately 1 m in diameter and 0.3 m in height were altered by removing the central "fishing" component, leaving a structurally effective frame of corrosive resistant metal enclosed with stainless steel mesh. Diamond-shaped openings within the wire mesh frames were approximately 66 mm × 91 mm and tested with empty shells to confirm their permeability to abalone measuring less than 66 mm SL. Each structure also possessed a prefabricated entry or exit hole measuring 102 mm in diameter that was permeable to all abalone sizes and a hinged lid that allowed access to load, remove, and examine concrete mini-blocks during artificial habitat deployment and sampling.

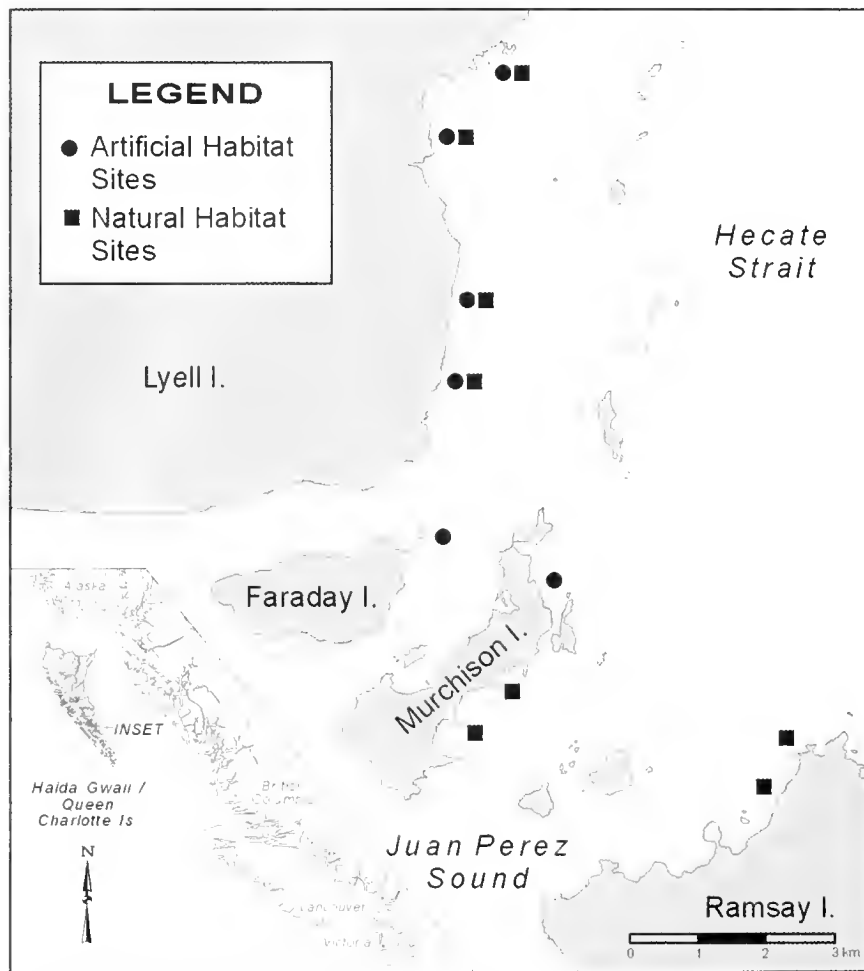In July 2001, 24 habitats were deployed by belaying each intact

Figure 1. Artificial and natural abalone habitat study sites in northern Juan Perez Sound abalone stewardship area, Haida Gwaii, British Columbia.

unit from the dive support vessel to the ocean floor. Divers repositioned each structure with an industrial airlift bag. Within a site, 4 habitats were oriented parallel to shore in depths of 4 to 9 m and from 7 to 30 m apart. The habitats were randomly located within areas dominated by small boulders and cobble encrusted with red coralline algae. No anchoring mechanisms were used to secure the units in place, because each unit weighed approximately 120 kg and possessed a stable base.

Divers visually inspected artificial concrete habitats for structural integrity in February 2002 and thoroughly surveyed each unit *in situ* during May and July 2002. A pair of divers sampled artificial habitats by removing and examining each concrete minibrick for abalone. All abalone found were measured for maximum SL to the nearest millimeter and empty abalone shells were also measured and removed. After all bricks were examined, they were haphazardly repositioned within the metal frame. No special effort

Figure 2. Artificial habitat design.

TABLE 1.

Total number of abalone found in 4 artificial habitats at each of 6 sites during surveys in May and July 2002.

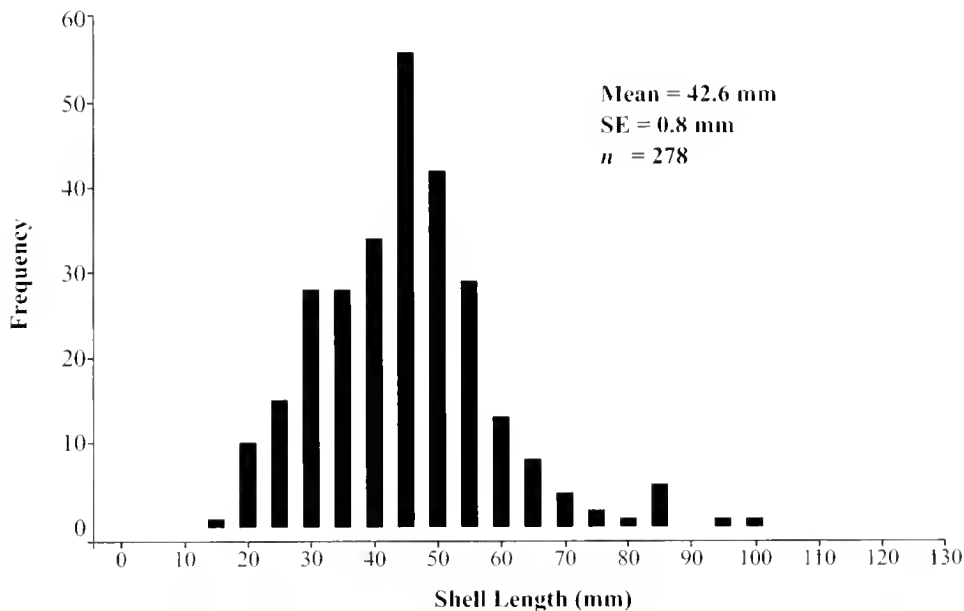|	May		July	
Site	Alive	Dead	Alive	Dead
1	17	1	24	1
2	44	1	38	4
3	5	2	3	2
4	12	0	10	1
5	37	3	31	0
6	37	3	20	1
Total	152	10	126	9

Figure 3. Size-frequency distribution of abalone measured within artificial habitats during May and July 2002.

was made to remove or monitor abalone adhering to bricks as the bricks were replaced back into the wire mesh containers.

To estimate the abundance of juvenile abalone occupying natural habitats, sampling was conducted within 10 m^2 of area at 4 artificial habitat sites and 4 additional random sites (see Fig. 1). At randomly selected locations throughout the available abalone habitat at each study site, divers invasively searched 10 1-m^2 quadrats for all hidden and exposed abalone. This method involved looking on the undersides of all movable rocks but did not include any destruction of natural habitat as care was taken to return any disturbed rocks to their original position. Diver efficiency in searching natural habitats was not measured.

RESULTS

All 24 artificial habitats contained abalone ($n = 152$, mean = 6.3 ± 0.95 SE abalone/container) during the first survey in May 2002, ten months after installation. During the second survey in July 2002, all but 2 artificial habitats contained abalone ($n = 126$, mean = 5.3 ± 0.87 SE abalone/container). There was no significant difference in mean abalone/container for either total abalone abundance (*t*-test, t = −0.84, d.f. = 46, $P > 0.406$) or total juvenile abalone (≤ 50 mm SL) abundance (*t*-test, t = −0.47, d.f. = 34, $P > 0.643$) between the two sample periods.

A total of 278 abalone and 19 empty shells were counted and measured within artificial habitats during the study (Table 1). Juvenile abalone (≤ 50 mm SL) accounted for 75.4% ($n = 224$) of all those measured, while only 3.6% ($n = 15$) were more than 70 mm SL and considered to be mature. The smallest and largest abalone found in artificial structures were 15 mm and 100 mm SL. The average abalone size was 42.6 mm SL (Fig. 3) and 56.4 mm SL for all empty shells. On average, each artificial habitat required 12.5 min for a pair of divers to completely survey.

The mean density of juvenile, mature, and all-sized abalone within artificial habitats was 1.27, 0.06, and 1.65 abalone/m^2, respectively (Table 2). Juvenile abalone densities in artificial habi-

tats were significantly different between sites (one-way ANOVA, $F = 8.409$, d.f. = 5,35, $P < 0.001$), but not within sites, suggesting differential recruitment to these locations.

A total of 82 abalone were counted and measured within natural habitat samples. Juvenile abalone accounted for 13.4% ($n = 11$) of all those measured, while 64.6% ($n = 53$) were mature. The smallest and largest abalone found in natural habitats were 14 mm and 124 mm SL. The average abalone was 79.3 mm SL (Fig. 4) and 75.2 mm SL for empty shells. Mean abalone densities during the May and July surveys were similar (ANOVA, $F = 0.819$, d.f. = 1,15, $P = 0.38$) and there was no difference in total abalone densities between artificial habitat sites (sites 1–6) and additional random sites (sites 7–10). The mean density of juvenile, mature, and all-sized abalone measured with natural habitats were 0.07, 0.33, and 0.51 abalone/m^2, respectively, (Table 3). Natural habitat samples were located at a mean depth of 3.08 ± 0.08 m datum (min = −0.4 m, max = 4.8 m).

Juvenile abalone densities measured within artificial habitats were compared with natural habitat samples. At sites 1 to 4, where

TABLE 2.

Mean number and densities (#/m^2) of abalone in 4 artificial habitats at each of 6 sites surveyed in May and July 2002.

| Site | No. of Abalone | Abalone Density (#/m^2) | | |
		≤ 50 mm SL	>70 mm SL	All Sizes
1	20.5	1.00	0.00	1.46
2	41.0	2.14	0.14	2.93
3	4.0	0.29	0.00	0.29
4	11.0	0.39	0.18	0.79
5	34.0	2.25	0.00	2.43
6	28.5	1.64	0.04	2.04
Mean	23.2	1.27	0.06	1.65
SE	4.1	0.25	0.03	0.29

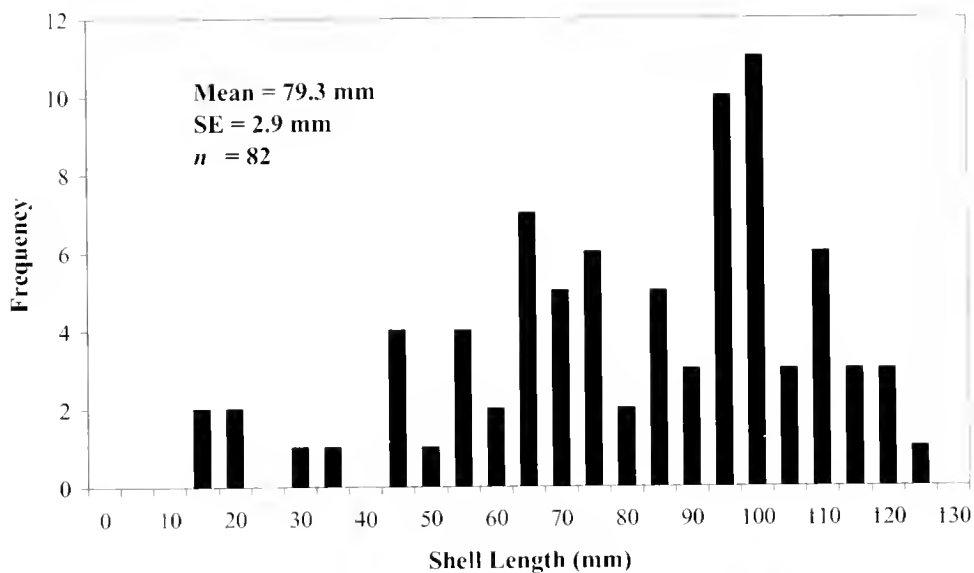Standard errors shown are for site groups ($n = 12$).

Figure 4. Size-frequency distribution of abalone measured within natural habitat samples during May and July 2002.

both artificial and natural habitat samples were conducted within an area greater than 10,000 m², juvenile abalone densities measured within artificial habitats were significantly greater than those within natural habitat samples (t-test, t = 3.049, d.f. = 14, P = 0.009). When all locations were included in the comparison, juvenile abalone densities measured within artificial habitats remained significantly greater than those within natural habitat samples (t-test, t = 5.597, d.f. = 26, P < 0.001). There was no significant difference in juvenile abalone densities measured in natural habitats at sites 1 to 4 when compared with sites 7 to 10, indicating that the presence of artificial structures at sites 1 to 4 did not influence juvenile abalone abundance in surrounding natural habitats.

DISCUSSION

The artificial habitat design tested in this study provided surrogate habitat for both juvenile and mature northern abalone. Within 10 months of installation, native abalone had discovered and occupied each of the 24 artificial habitats. The similar number

TABLE 3.

Mean number and density (#/m²) of abalone in 10 natural habitat samples at each of 8 sites surveyed in May and July 2002.

Site	No. of Abalone	Abalone Density (#/m²)		
		≤50 mm SL	>70 mm SL	All Sizes
1	8.0	0.15	0.45	0.80
2	1.0	0.05	0.45	0.65
3	6.5	0.05	0.10	0.30
4	3.0	0.00	0.25	0.35
7	3.5	0.05	0.55	0.60
8	6.0	0.05	0.40	0.60
9	6.0	0.20	0.35	0.70
10	7.0	0.00	0.10	0.10
Mean	5.1	0.07	0.33	0.51
SE	2.99	0.09	0.07	0.10

Standard errors shown are for site groups (n = 16).

of abalone occupying artificial habitats in July suggested the concrete blocks continued to provide preferred shelter throughout the summer months when surrounding food abundance is high and good quality alternative natural habitats were available. The specific length of time required for artificial materials to condition and attract abalone was difficult to determine due to the limited number of sample periods. The concrete materials appeared to be suitable for northern abalone within 7 months based on observations of abalone occupying most artificial habitats during structural inspections in February 2002. The conditioning time of this material was consistent with Davis (1995) who found "juvenile native *H. rufescens* and *H. corrugata* inhabited artificial habitats within 4 months of deployment" in California.

As indicated in Figure 3, juvenile abalone were the most abundant size class occupying artificial habitats. Both the small mesh size and high substrate complexity may have contributed to the size selectivity by limiting access and suitable shelter for abalone greater than 70 mm SL. Juvenile abalone densities measured within artificial habitats were significantly different between sites but similar within sites. This apparent ability of artificial structures to quantify juvenile abalone abundance within standardized sample areas at different locations may provide the feedback required to gauge the success of future stock restoration experiments. Benefits of the modular artificial habitat design tested here included the low cost of construction, ease of deployment, durability within high energy subtidal environments, and most importantly, their ease of being dismantled and reconstructed by divers *in situ*, without the destruction of natural habitat.

In this study, juvenile abalone recruitment measured within artificial habitats was not representative of recruitment measured within nearby natural habitat samples. At sites 1 to 4, juvenile abalone abundance measured within artificial habitats was significantly greater than natural habitat samples, ranging in magnitude from 4.3 times greater at site 3 to 42.9 times greater at site 2. Although each natural habitat sample was randomly located within good quality juvenile abalone habitat and the mean juvenile abalone density found within natural habitats was similar to Campbell et al. (2000), natural habitats provided little consistency with sub-

strate composition and hence, the lower abundance of sheltered habitat. Specific factors that made the artificial structures attractive to abalone were not investigated experimentally but were likely due to the consistent and availability of good quality sheltered habitat provided by the concrete blocks. Based on observations, additional factors that may have influenced the abundance of abalone in artificial habitats included easily accessed algal food growing on concrete bricks and a mesh frame that may have excluded large predators such as Sunflower seastars (*Pycnopodia helianthoides*).

The measured abundance of juvenile abalone within artificial habitats may have been at their annual spring peak, as surveys were only conducted during May and July, a similar time of year that Davis (1995) measured a peak in abalone recruitment. To calibrate artificial habitats into better juvenile abalone abundance instruments, it will be necessary increase the number of surveys and monitor fluctuations in abalone abundance throughout the year. Only by comparing the changes in abalone abundance from winter to summer can the magnitude of localized recruitment events be determined.

The use of artificial habitats as a standardized sampling instrument to estimate the abundance of cryptic juvenile abalone was supported by this research. The haphazardly oriented concrete blocks provided preferred habitat for juvenile abalone and the metal frame covered with wire mesh provides structural integrity and allowed each sampling unit to be quickly deployed or repositioned. A pair of divers could easily sample units *in situ*, with no destruction to either natural habitat or abalone adhering to concrete bricks. For proposed abalone rebuilding experiments, artificial habitats of this design can be used as an initial release site for cultured juveniles, as an affordable method of determining baseline juvenile abundance along coastlines of interest, and as a means to quantify changes in juvenile recruitment that may be due to experimental stock enhancement.

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EARLY REPRODUCTION IN HATCHERY-RAISED WHITE ABALONE, *HALIOTIS SORENSENI*, BARTSCH, 1940

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ABSTRACT White abalone, *Haliotis sorenseni*, is the first marine invertebrate species to be placed on the endangered species list in the United States. A controlled breeding program is underway to provide stocks to reestablish wild populations of this species. There is no knowledge of the reproductive potential of early adults. This study examines the onset of gonad maturation and spawning capability of one-year-old abalone. Both hydrogen peroxide and ultra-violet irradiated seawater induced spawning in males and females as small as 25 to 35 mm in shell length. More males spawned in each treatment than females ($P < 0.01$). The onset of gonad maturation is much smaller for white abalone than for other sympatric abalone in California. The implications for restocking strategies are discussed.

KEY WORDS: abalone, endangered species, *Haliotis sorenseni*, reproduction, maturation, hatchery

INTRODUCTION

The white abalone (*Haliotis sorenseni*) is one of seven species of a large marine gastropod inhabiting the waters off the west coast of North America. The deepest living of these species, the white abalone was historically found between depths of 20 to 60 m and was most abundant between 25 to 30 m (Cox 1960, Tutschulte 1976). The range of the white abalone extended from Point Conception in California to Punta Abreojos, Baja California, Mexico, with an historical center of abundance in southern California around the Channel Islands (Cox 1960). Like other abalone, this species was targeted by sport and commercial fishermen; 95% of white abalone landings occurred between 1969 and 1977 yielding 268 metric tons. Such exploitation was not sustainable and resulted in the dramatic decline of this species (Haaker 1994, Davis et al. 1996, Tegner et al. 1996, and Davis et al. 1998). With a life span of approximately 30 to 40 y, it is estimated that the last successful recruitment of the species was in 1966 (Hobday et al. 2000). Hobday and Tegner (2000) concluded that the population density of the surviving animals was too low to permit successful recovery and, without intervention the species may become extinct by 2010. In 2001 the white abalone was listed as endangered under Endangered Species Act (Anon 2001). For the first time in the United States, over-exploitation had pushed a marine invertebrate to the brink of extinction.

Artificial propagation is one method that has been explored to restore or re-establish species. Methods for the large-scale cultivation of abalone were first developed in Japan and have been adapted for a variety of species within this genus (McCormick 2000) with the intent of enhancing natural productivity. Hatchery-raised abalone from larvae to adults 80 mm in shell length (SL) adults have been out-planted with varying success (McCormick et al. 1994). To avoid costs associated with long-term hatchery cultivation of juvenile and adult abalone, the release of larvae has also been undertaken for a number of species. This approach has been shown to increase the number of newly recruited juveniles (Tong et al. 1987), however, the long-term impact of this method is difficult to assess. Schiel (1992) compared the costs of releasing larval and juvenile abalone, and concluded that the higher survival

of juvenile abalone more than offset the additional cultivation costs. Up to a point, increased survival has been correlated with increased size for some species of abalone. Inoue (1976) found that survival for *H. gigantea* increased from 10–70% after 1 y when the size of the abalone planted was increased from 10 mm to 30 mm SL. Saito (1979) found that survivorship of transplanted *H. discus hannai* reached a maximum when animals were 34 to 36 mm SL. Survival rates declined for both smaller and larger animals.

Successful enhancement programs require not only that the hatchery-reared animals survive in the wild, but reproduce as well. Rearing the abalone in a protected laboratory setting allows for the reintroduction of larger specimens, thus reducing losses resulting from natural mortality prior to sexual maturity. The age at which male and female abalone first reproduce and their fecundity will partially determine their contribution to the population after out-planting. Studies of several abalone species revealed that the minimum age of sexual maturity was found to be between 3 and 5 y, ranging from 29 mm SL for female *H. coccinea canariensis* (Pena 1986), and 40–120 mm SL for 14 other species. Field studies of abalone in southern California indicate that the age of sexual maturity for pink abalone (*Haliotis corrugata*) was 3 to 4 y (39 to 44 mm SL), for green abalone (*Haliotis fulgens*) was 5 to 7 y (61 to 89 mm SL), and for white abalone was 4 to 6 y (93 to 88 mm SL) (Tutschulte 1976). Ault (1985) found that wild red abalone (*Haliotis rufescens*) could be induced to spawn at sizes of 65 mm SL for males and 110 mm SL for females. Observations of a crop of white abalone grown in our hatchery indicated that these animals exhibited signs of sexual maturity at an age significantly younger than previously reported. Sexual maturation was quantified and several experiments were conducted to measure maturation, spawning response, and fecundity.

MATERIALS AND METHODS

Abalone Cultivation

In November 2000, wild adult white abalone were collected by the California Department of Fish and Game and transported to the Channel Islands Marine Resource Institute (CIMRI) in Port Hueneme for culture. In April 2001, two females and one male were successfully spawned using methods described by Morse et al. (1977). Sperm from the male was used to fertilize eggs from both

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females to create two half-sib families. Larvae were raised in flow-through systems (Tong & Moss 1992) and settled on plastic plates covered with cultures of micro-algae, diatoms and bacteria (Seki & Kanno 1980). Outdoor tanks received filtered sunlight and sand filtered UV sterilized seawater at ambient temperatures. Minimum and maximum temperatures ranged from 12°C to 20°C during the first 15 mo of culture. After 6 mo, cultivated Pacific dulse (*Palmaria mollis*) (Levin 1991, Evans & Langdon 2000) and wild giant kelp (*Macrocystis pyriferia*) were added to the culture tanks to supplement the micro-algae feeds. Tanks were periodically cleaned by siphoning detritus from the bottom.

Abalone Growth

Starting 79 days after spawning, the shell length of 50 abalone from each of 11 tanks was measured to the nearest 0.1 mm. Abalone were also periodically weighed to determine weight–length relationships. Normality of the lengths of the sampled individuals was first determined using the Kolmogorov–Smirnov test, and then data was subjected to ANOVA, which revealed no significant difference in growth between the two half-sib families or among the 11 cultivation tanks. A growth curve was then constructed using the von Bertalanffy growth function (Bertalanffy 1960) based on the mean lengths of the abalone within the 11 tanks.

Sex Determination

During routine measurement of shell length, abalone were inspected externally for presence of gonads. Histologic examinations could not be performed since such studies would require sacrifice of the animals. We used a non-lethal method developed by Uki and Kikuchi (1982). This method is used in many hatcheries to assess the spawning readiness of the broodstock. The Gonad Index (GI) ranking is as follows:

Gonad Index	Description of Gonad and Spawning Activity
0	No gonad observed. Not possible to determine sex. Abalone will not spawn.
1	Small volume of gonad observed. Possible to determine sex of abalone by gonad color. Abalone will not spawn.
2	Larger volume of gonad covers the conical appendage of the digestive gland. Easy to determine sexes. Gonad bulk visible. Abalone may spawn.
3	Volume of gonad quite large, may extend below the plane of the shell opening. Abalone will usually spawn.

Pluses and minuses attached to these values were sometimes used to designate a gonad index that fell between the index numbers. Sex was distinguished by color: the gonad of the males was a cream color while that of the females was green-grey.

All of the abalone examined were from the same family but were reared in two separate tanks. The GI and shell length of each animal were determined and recorded. From one tank, 146 animals were examined, from another, 255 for a total of 401 abalone (Table 1). From the two tanks examined, 115 abalone with a GI of "2" or higher were sequestered for the spawning study.

Spawning Methods

Two spawn inducing treatments, ultraviolet (UV) irradiated seawater (Kikuchi & Uki 1974) and hydrogen peroxide, H_2O_2, (Morse et al. 1977), were tested to determine if these methods

resulted in differences in the number of animals that spawned or in the quantity of gametes released. In both treatments, individual abalone were placed in 260-mL plastic containers. This prevented early spawners from stimulating neighboring animals and enabled us to isolate and count gametes from each animal. For the UV spawning method, 4.8 L/min seawater was filtered to 5 μm and passed through four 30-watt UV sterilizers. Two of the units failed during the experiment, resulting in a dosage of approximately 200 milli-Watt h/L. This is lower than the 800 milli-Watt h/L optimal dosage suggested by Kikuchi and Uki (1974), but still within the effective range. Abalone subjected to the UV irradiated water were also given a thermal shock by rapidly increased water temperature from 15°C to 21°C followed by gradual cooling back to 15°C (Ino 1952).

Hydrogen peroxide is commonly used in hatcheries to induce spawning in abalone. To test this method on white abalone, hydrogen peroxide and tris-(hydroxymethylamino) methane solutions were added to filtered, UV irradiated seawater at 15°C. Each spawning container received 200 mL each of hydrogen peroxide and tris solutions. When the abalone began to release gametes, or if the suggested 2.5 h had elapsed since initial exposure (Morse et al. 1978), the solutions were decanted and replaced with filtered, UV-irradiated seawater.

Both UV and H_2O_2 treatments were tested with a minimum of 25 females and 24 males. Table 1 shows the sex, GI, and, number of abalone used in this study. More males with a GI of 3 were used in the H_2O_2 treatments to determine the frequency of males able to spawn, and the correlation between the amount of gametes produced and shell length. In cases where there were an uneven number of individuals with a particular GI (for example, there were nine females with a GI of 3), the extra individual was randomly assigned to either the H_2O_2 or the ultraviolet (UV) spawning treatment. Because of a shortage of ripe females, five additional females were taken from the second tank used in the sex determination study previously mentioned. After both treatments had been completed, gametes were examined under ×400 magnification for visual identification of any defects in the gametes. None of the eggs were fertilized since our permit under the Endangered Species Act of 1976 was pending. Subsequently all gametes were destroyed and abalone were returned to their respective tanks, unharmed.

RESULTS

Abalone Growth

Growth of individuals within different tanks were compared, and found to be statistically similar (Anova, $P = 0.76$). Mean lengths were calculated for all 11 tanks, and then compared with lengths predicted by the von Bertalanffy growth function ($L_t = L_\infty(1-e^{-kt})$; $L_\infty = 231.70$, $k = 0.065$, $t =$ age of abalone. Total body weight was also measured for individuals of different lengths ($n = 317$) and fitted to a non-linear least squares regression, which denotes weight (W in g) as a function of shell length (L in mm) ($R^2 = 0.9957$), $W = 0.0001 L^{2.9695}$ (Fig. 1).

Sex Determination

Of the 401 individuals surveyed for sex determination, the lower limit on males exhibiting gonads was 15.9 mm SL, but this was an outlier. The next lowest was 20.8 mm SL. For females, the lower limit was 20.9 mm SL. A Kruskal Wallace test showed that

TABLE 1.

Percentage of abalone spawned and gamete production using ultraviolet and hydrogen peroxide.

		Ultraviolet + Temp. Shock			Hydrogen Peroxide		
	Gl	Number Tested	Percent Spawned	Number of Gametes $\times 10^3$	Number Tested	Percent Spawned	Number of Gametes $\times 10^3$
Female							
	2	9	11	1.3	10	70	0.07–1.1
	2+	11	46	0.2–10.2	11	36	0.39–1.3
	3	5	70	1.3	4	50	0.33–0.90
Male							
	2	8	63	76–3,210	9	67	–
	2+	11	100	163–1,686	11	64	–
	3	5	80	924–7,018	20	95	–

no significant difference existed in the SL of the males and females ($P = 0.43$). Also, Pearson's χ^2 tests revealed that there was no significant difference in the number of males in the population compared with the number of females ($P = 0.09$).

Spawning

The smallest female that spawned measured 25.6 mm SL, whereas the smallest male was 23.1 mm SL. There was no difference among different size classes of individuals with regard to the occurrence of spawning (Pearson's χ^2 test, $P = 0.42$). Greater than 95% of the eggs and sperm examined appeared normal, with no obvious defects. Significantly more males than females spawned in both treatments (Pearson's χ^2 test, $P < 0.001$ under UV treatment, $P = 0.009$ under H_2O_2 treatment) (See Table 1). However, there was no significant difference in the number of males that spawned in either UV or H_2O_2 ($P = 0.07$), nor was there a difference between the number of females that spawned in either of these treatments (Pearson's χ^2 test, $P = 0.08$). During the course of this study, males began releasing sperm early in the hydrogen peroxide treatment. The seawater solution was immediately decanted to minimize exposure to the hydrogen peroxide. In doing so, some sperm were lost and thus it was impossible to obtain accurate gamete counts.

For males in the UV treatment, no correlation existed between size and number of sperm released (Pearson correlation coefficient, r = 0.306). The largest amount of sperm (5.6 and 7×10^6) were released by abalone that measured 27.8 and 31.2 mm SL, respectively, while an intermediately sized male released only 7.6 $\times 10^3$ gametes. An average number was 1.5×10^6 gametes released

per male abalone. Similarly, for females no correlation existed between shell length and the number of eggs released, which ranged between 65 and 10,237 eggs per individual (Pearson correlation coefficient, r = 0.079). Because of large variation within treatments, a Mann–Whitney U-test indicated that there was no difference in the amount of eggs released between treatments ($P = 0.09$), although the average number of eggs released in the UV and H_2O_2 treatments was 2,880 and 673, respectively.

DISCUSSION

Growth of white abalone for the first year in the hatchery averaged 30.0 to 43.4 μm/day, yielding an average 16 mm SL, (minimum 7.0 mm SL to maximum 30.3 mm SL). This was somewhat less than hatchery growth rates expected for red and green abalone but was similar to that of pink abalone (McCormick, pers. obse.). No significant difference was observed in the frequency of males and females in the population of 1-year-old abalone examined (Pearson's χ^2 test, $P = 0.502$). This trend is considered common in organisms of many phyla, including invertebrates, where males and females reach maturation at approximately the same size and age. Analysis of 600 legal adult white abalone from the Channel Islands yielded a sex ration of 1:1 over all size classes (Tutschulte 1976, Tutschulte & Connell 1981).

The present work shows the onset of sexual maturation is significantly earlier than previously suggested, and hatchery-raised abalone, as young as 1 y in age (23–25 mm SL), are capable of spawning. Tutschulte and Connell (1981) proposed that the minimum age required for white abalone to reproduce is 4 y. However, the estimate was based on a sample of only three individuals smaller than 130 mm SL (Tutschulte 1976). The red abalone is a close relative to white abalone (Yang et al. 2000). Studies of sexual maturation of red abalone in northern California, (Giorgi & DeMartini 1977) have shown that wild red females matured at a minimum of 39.5 mm SL, whereas males were at least 84 mm SL before reaching sexual maturity. They also found that the onset of sexual maturation precedes that indicated by visual observation of the gonad. When examined histologically, animals as small as 25–50 mm SL were found to contain spermatozoa or oocytes. A similar phenomenon was found in the ormer, (H. tuberculata) where spermatozoa were present in animals as small as 28 mm SL but were not observable until the animals reached a size of 40 mm SL (Pena 1986). The smallest red abalone spawned by Ault (1985) were 65 mm SL for males and 110 mm SL for females.
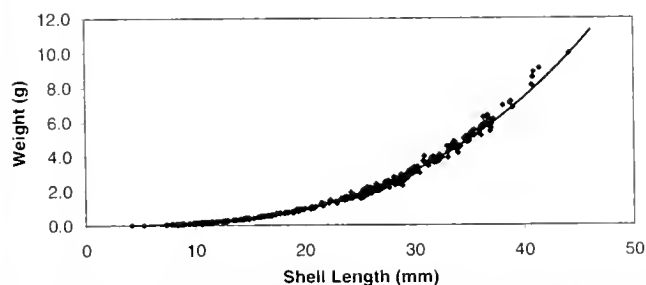

Figure 1. Shell length—whole wet live weight relation for white abalone. Equation for power curve: Weight = 0.0001 * Shell Length$^{2.9695}$ ($R^2 = 0.9957$, $n = 317$).

Hatchery-raised white abalone mature at sizes smaller than their wild counterparts. Hatchery conditions, most notably the continuous presence of abundant food sources, no doubt promote the early sexual maturation of white and some other species of abalone. Ault (1985) demonstrated this effect with red abalone and after conditioning them in the laboratory for 90 days. Conditioned animals spawned at minimum sizes of 55 mm and 60 mm SL for males and females, respectively. These sizes were 85% and 55% the length of the smallest wild spawners (see earlier). Ault (1985) also found that the improved diet of laboratory conditioned abalone increased fecundity. Laboratory conditioned red abalone less than 100 mm SL were as fecund as wild animals up to 140 mm SL.

Over the course of the last 2 decades, we have noted that crops of hatchery-raised red, green, and pink abalone start to mature at about 50 mm SL. This was less than wild animals but still twice the size of spawning white abalone in the present study (T. McCormick, pers. obse.). Tutschulte (1976) found that large (88 to 159 mm SL) wild white abalone were more fecund than either pink or green abalone. We now know that white abalone in the hatchery also mature at a smaller size. Mature gonads were observed in laboratory populations in animals as young as 10 months in age. Although the gametes were not tested for viability (due to permit constraints) no obvious defects were observed and we expected that the eggs and sperm would be as viable as those of older animals.

The implied higher fecundity of hatchery-raised white abalone may have an impact on enhancement efforts for this species. When hatchery-raised white abalone are used for enhancement efforts the number of animals that ultimately make a reproductive contribution depends upon the natural annual mortality rate (M) and the time required to reach sexual maturity. Even in natural communities, M is high for newly recruited abalone and may vary from 1–10 between populations (Schiel 1992, Shepherd & Breen 1992). For hatchery-raised abalone M is often higher than that of wild populations (Rogers-Bennett & Pearse 1998). Survival and consequent reproductive contribution may be improved by increasing the size of abalone at release. To date, many enhancement efforts utilizing hatchery-raised abalone have focused on abalone averaging 20–30 mm SL, with larger animals having greater survival (see summary in McCormick et al. 1994). Production of larger animals requires longer growout times and increased cultivation expense. Earlier sexual maturity and higher fecundity rates of hatchery-raised abalone may be another way of increasing reproductive contribution. We have shown that hatchery-reared white abalone mature at a much younger age and smaller size than anticipated. Providing white abalone with abundant food sources in the hatchery could make their initial reproductive contribution equivalent to that of animals much larger, as Ault (1985) observed.

Observations in our laboratory indicated that, unlike their wild counterparts that have highly synchronized maturation and spawning cycles, small hatchery-raised adults were apparently capable of spawning for much of the year. After first maturing in the late winter and early spring, the present crop of young adults remained gravid for over a year. Large wild adult abalone synchronize gonad maturation, culminating in a short spawning period in late winter (Tutschulte 1976, Tutschulte & Connell 1981). As with some other abalone species (Tutschulte & Connell 1981, Newman 1967, Poore 1974, and Shepherd & Laws 1974), food availability may regulate periodicity of the reproductive cycle.

CONCLUSIONS

This work defines the lower limit of sexual maturation in cultured white abalone. Field studies suggested that wild white abalone matured at 4 to 6 years in age at a size 80 mm SL or greater (Tuschulte 1976). At 25 mm SL, white abalone in our hatchery were sexually mature at a much smaller size than other species reared in North American hatcheries. Ault (1985) noted the same phenomena for red abalone in which wild abalone were approximately twice as large when sexually mature than hatchery conditioned animals. If the same relationship holds true for white abalone, we expect that wild white abalone could begin spawning at 50 mm SL. Tutschulte (1976) noted that white abalone have greater variation in reproductive success than do pink or green abalone and would benefit from an early age at sexual maturity and long life span. This would give them more opportunities for successful spawning. The present data seems to support this argument.

Studies quantifying survivorship of a range (25–100 mm SL) of young adult white abalone after outplanting into the marine habitat are necessary. Field research will be needed to document survival and changes in gonad maturation as young hatchery-raised adult white abalone acclimate to seasonal cycles of temperature and food abundance in their natural environment. Recruitment events and seasonal changes in gonad bulk will provide additional information on long-term impact of food resources on the abalone.

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DISTRIBUTION AND ABUNDANCE OF *HALIOTIS KAMTSCHATKANA* IN RELATION TO HABITAT, COMPETITORS AND PREDATORS IN THE BROKEN GROUP ISLANDS, PACIFIC RIM NATIONAL PARK RESERVE OF CANADA

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ABSTRACT Baseline information on the distribution and abundance of *Haliotis kamtschatkana* was obtained throughout the Broken Group Islands (BGI) in shallow- (2–5 m) and deep-water (6–9 m) habitats. The study demonstrates that abundance of northern (pinto) abalone varied spatially throughout the area and with depth. The shallow habitats in the study area supported significantly higher densities (0.18 abalone/m^2 ± 0.02 *SE*) of northern abalone when compared with deep habitats (0.10 abalone/m^2 ± 0.02 *SE*). Maximum and minimum sizes of northern abalone measured in BGI were 132 and 4 mm shell length (SL), respectively. There were significant differences in abalone SL among the 5 island groups and the 2 depth zones. Juvenile abalones were more abundant in the deep habitat than in the shallow habitat. A significant correlation was detected between abalone densities and the relative index of exposure. There was a positive correlation between abalone size and the abundance of benthic macroalgae and an inverse relationship between abalone size and the abundance of red sea urchins (*Strongylocentrotus franciscanus*). A positive correlation between abalone and red sea urchin densities was observed. Seven percent of juvenile abalone (≤45 mm SL) was found under the red sea urchins' spine canopy. Distribution and abundance of selected invertebrate species associated with northern abalone including its known predators (ie, sea stars, crabs, octopuses) were assessed. The abundance of northern abalone was inversely correlated with predator abundance and density of benthic macroalgae. Detailed surveys of associated organisms and substrate types suggest that the distribution and abundance of northern abalone is a complex function of community interactions and substrate habitat characteristics.

KEY WORDS: Northern (Pinto) abalone, *Haliotis kamtschatkana*, red sea urchins, competitors, predators, habitat, distribution

INTRODUCTION

Large, mobile invertebrates, such as abalone and sea urchins, are an important component on subtidal rocky reefs in the coastal waters of British Columbia. The northern (or pinto) abalone (*Haliotis kamtschatkana* Jonas, 1845) is found distributed from Alaska (Paul & Paul 1981) to California (Sloan & Breen 1988) along the west coast of North America. Historically, *H. kamtschatkana* was widely distributed in British Columbia with preference to semi-exposed to exposed coastal habitats where they graze mainly on attached or drift macroalgae and diatoms. Abalone are slow growing and long-lived gastropods, characterized by patchy distribution, sporadic recruitment, density dependent reproduction and short larval period (Hobday et al. 2001). They are dioecious broadcast spawners with peak reproductive activity during the summer (Breen & Adkins 1980). During spawning events abalone aggregate in shallow subtidal areas to maximize fertilization success, which depends on their aggregation density (Babcock & Keesing 1999). It is now recognized that northern abalone is particularly vulnerable to overexploitation because of this life history strategy.

The coastal First Nations of British Columbia have a long history of harvesting northern abalone for a wide range of uses, ranging from subsistence harvests to use in native art and cultural activities (Stewart 1977). The first record of modern commercial abalone fishery in British Columbia dates to the early 1900s (Sloan & Breen 1988). Prior to the invention of SCUBA, the abalone fishery targeted mainly the intertidal populations; thus subtidal areas were in effect natural refugia. The use of SCUBA to harvest abalone started in the 1950s, but was generally restricted to few operators. Abalone commercial landings in British Columbia peaked in 1977 to 1978 (428–433 tons, respectively) and then continued to decline. Northern abalone was targeted by recreational and commercial dive fisheries until 1990, when the fishery was closed due to major stock declines (Campbell et al. 2000). The purpose of the 1990 commercial fishery closure in British Columbia was to allow the abalone populations to rebuild. However,

numerous stock assessment surveys by Department of Fisheries and Oceans Canada (DFO) during the 1990s have shown no evidence of recovery (Campbell 2000). As a result, *H. kamtschatkana* was designated as threatened in 1999 by the Committee on the Status of Endangered Wildlife in Canada (COSEWIC). Recovery strategy and action plans are now in place to assist in rebuilding the northern abalone population to sustainable levels. This study is part of that strategy.

The present study was conducted in the Broken Group Islands (BGI), which are part of the Pacific Rim National Park Reserve of Canada (PRNPR). A recent DFO survey of abalone populations in southeast Barkley Sound, adjacent to BGI, provided no evidence of recovery of abalone populations since the province-wide closure in 1990 (Lucas et al. 2002). The mean reported density of 0.1 abalone/m^2 is significantly lower than the mean density of 0.52 abalone/m^2 reported by Emmett and Jamieson (1988) from the same area prior to the 1990 closure. The objective of the present study is to provide baseline information for the managers of PRNPR on the distribution and abundance of northern abalone throughout the BGI at two depth zones (shallow: 2–5 m below chart datum; and deep: 6–9 m below chart datum). The study was designed to explore the association of abalone with other components of their subtidal habitats, by providing key information on the distribution and abundance of organisms associated with the species, including its major known predators. The study forms the baseline against which to compare future response of abalone populations to sea otter (*Enhydra lutris* Linnaeus, 1758), recolonization of BGI, and to the expected increase in climate variability associated with climate change.

MATERIALS AND METHODS

Description of Study Sites

The BGI Archipelago is located on the Pacific coast of Vancouver Island in Barkley Sound, roughly between latitudes

48°57.683′N and 48°50.233′N, and longitudes 125°12.700′W and 125°24.700′W (Fig. 1). Based on geographic and oceanographic features, the BGI were sub-divided into 5 island groups and stratified in two depths. The 5 island groups were: Group 1, Hand; Group 2, Dodd; Group 3, Clark; Group 4, Wouwer; and Group 5, Gibraltar (see Fig. 1). The tidal range within the BGI is approximately 3.8 m. Based on past studies by DFO, each of these island-groups was stratified into shallow (2–5 m below chart datum) and deep (6–9 m below chart datum) zones reflecting the distribution of northern abalone populations (eg. McShane & Naylor 1995, Sloan & Breen 1988, Campbell et al. 1998, Campbell et al. 2000, Lucas et al. 2002).

SAMPLING PROTOCOL

A 200 × 200 m geo-referenced grid was laid over each of the 5 island groups using *ArcGIS* 8.x software. All 200 × 200 m blocks that intersected a shoreline or offshore reefs within each island group were sequentially numbered. The number of blocks that intersected a shoreline or an offshore reef ranged from 58 in Group 3 to 295 in Group 5. Random selection without replacement was used to select 4 sampling locations (ie, blocks) in Group 1, 5 locations in Group 2, 4 locations in Group 3, 4 locations in Group 4, and 5 locations in Group 5, for a total of 22 sampling locations (see Fig. 1). A relative index of exposure was computed for each site following procedures described by Ekebom et al. (2002). At each location, 2 sites approximately 30 m apart were sampled. Sampling was conducted by 2 dive teams.

Sampling at each site was conducted by randomly placing 1 m^2 quadrats along 25 m virtual transects that were laid randomly parallel to the depth contour. Two transects were sampled within each depth zone at each site. The position of transects within each depth zone was determined by randomly selecting two specific
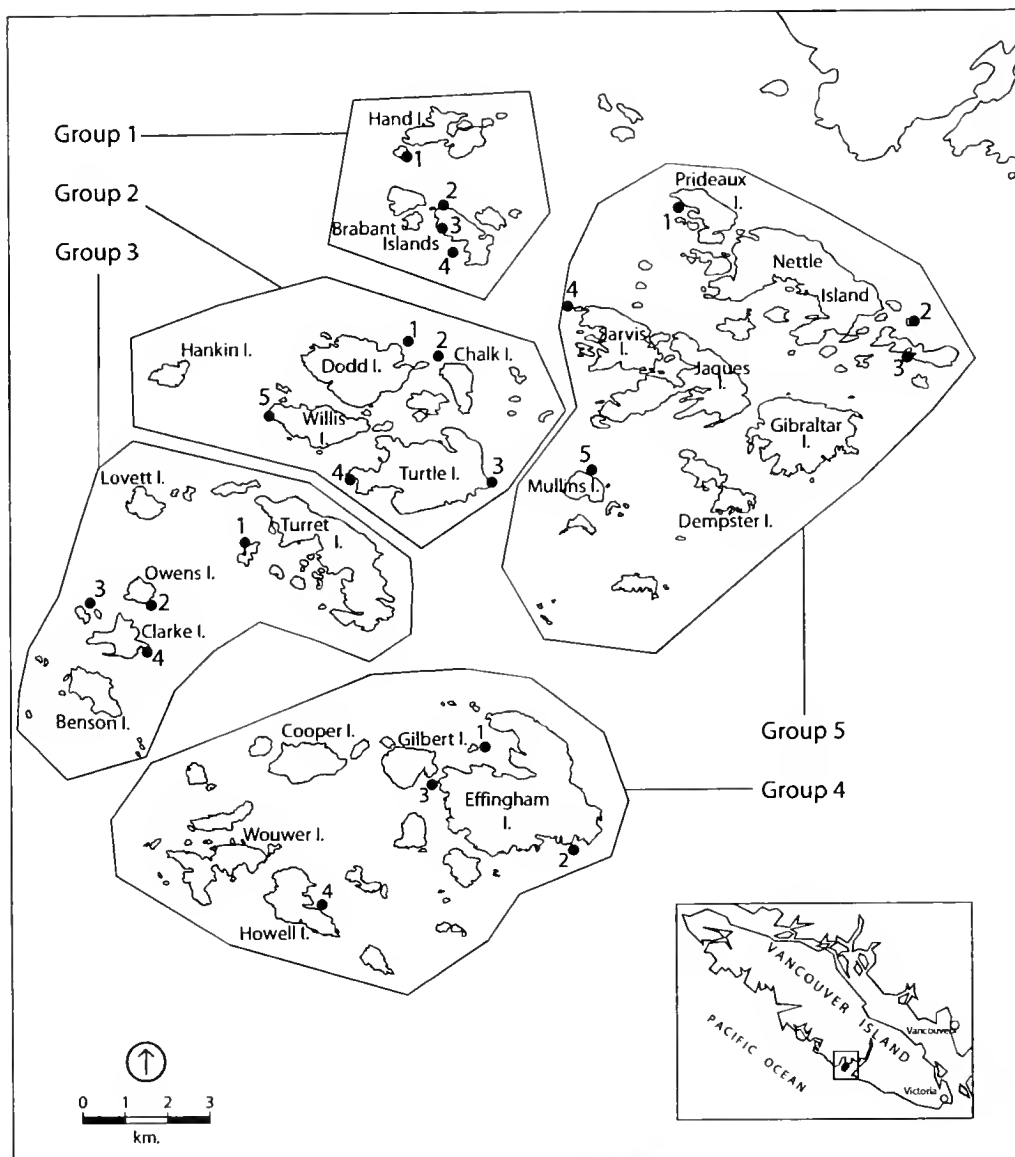

Figure 1. Map of the Broken Group Islands within the Pacific Rim National Park Reserve located on the west coast of Vancouver Island, British Columbia, Canada. Dark lines represent the rough boundaries of the 5 groups in which randomly chosen study locations were set up. Black dots and associated numbers indicate the number and position of each study location in the survey. The 5 geographic groups were: Group 1, Hand; Group 2, Dodd; Group 3, Clark; Group 4, Wouwer; and Group 5, Gibraltar.

depths within each zone (shallow zone: 2, 3, 4, and 5 m; deep zone: 6, 7, 8, and 9 m). Ten random 1 m² quadrats were sampled along each 25 m virtual transect. The positions of the 10 random quadrats along each transect were determined by randomly selecting 10 numbers between 1 and 25. The random quadrat selection was conducted prior to the survey and marked on underwater recording sheets that were specific for each sampling transect. The starting point of each transect was selected haphazardly by swimming along the depth contour and dropping the quadrat after about a 1 to 2 min swim. Once the starting point was determined the divers proceeded to flip the 1 m² quadrat along the virtual transect until they reached the first predetermined randomly selected position. Quadrat sampling included divers carefully lifting up (but not removing) all large macrophytes from the quadrat area to facilitate the systematic search for both emergent and cryptic specimens. Rocks were not removed or turned over in this survey. Once sampling of the quadrat was completed divers proceeded to flip the quadrat to the next randomly selected position along the virtual transect. This procedure was repeated until all 10 quadrats were sampled, or divers were forced to surface due to safety considerations.

Abalone and red sea urchin (*Strongylocentrotus franciscanus* Agassiz, 1863) counts, including maximum shell length (SL) and test diameter (TD) measurements in mm, were recorded in all 10 quadrats in each transect. The green sea urchin, *Strongylocentrotus droebachiensis* (O.F. Müller, 1776) and the purple sea urchin, *Strongylocentrotus purpuratus* (Stimpson, 1857) were also recorded. The number and size of juvenile abalone found under the red sea urchin spine canopy were also recorded in each quadrat. Predator densities, including dungeness crab (*Cancer magister* Dana, 1852), red rock crab (*Cancer productus* Randall, 1839), octopus (*Enteroctopus dofleini* Wülker, 1910), and sunflower sea star (*Pycnopodia helianthoides* (Brandt, 1835)), were recorded in all quadrats along each transect. For octopuses, either individuals or inhabited dens were counted. Sea otters were not observed in the study area.

Densities of benthic macroalgae were estimated in 5 randomly selected 1-m² quadrats (from the original 10 quadrats) along each transect. Because of time constraint, ease of taxonomic identification and reporting efficiency, the following macroalgae were included: (1) *Macrocystis integrifolia* Bory, 1826; (2) *Nereocystis luetkeana* (Mertens) Postels et Ruprecht, 1840; (3) *Fucus* spp.; (4) *Eisenia arborea* Areschoug, 1876; (5) *Hedophyllum sessile* (C. Agardh) Setchell, 1901; (6) *Agarum clathratum* Dumortier, 1822; (7) *Pterygophora californica* (Ruprecht, 1852); (8) other browns; and (9) green algae. In each quadrat, the macroalgae were identified and counted. Algal holdfasts were counted for all species except *M. integrifolia* for which the number of stipes was used.

The following substrate cover types were defined in the present study: (1) encrusting coralline algae, (2) articulated coralline algae, (3) brown algae, (4) green algae, (5) bryozoans, (6) sponges, (7) other invertebrates, and (8) sand. The percentage cover of each substrate type was quantified in 3 randomly selected 1 m² quadrats (from the original 10 quadrats) in each transect using a point-intersect method. This method involved the use of a quadrat, which was permanently marked along one side with 20 points (5 cm apart), and a 1 m PVC bar that was permanently marked with 5 random points. Sampling involved placing the 1 m PVC bar across the quadrat from 3 randomly chosen points along the side of the quadrat and recording the substrate type that was found under each of the 5 points on the PVC bar. The three random points along the

side of the quadrat were chosen earlier and were marked on recording sheets. Each quadrat was sampled with 15 points (45 points per transect).

STATISTICAL ANALYSES

All data analyses were conducted using the NCSS statistical package (Hintze 2001). Tests of normality and homogeneity of variance were performed on all data sets using normal probability plots and modified Levene equal-variance test, respectively. Square root (SQRT) and ARCSINE(SQRT(X)) transformations were performed as appropriate (Zar 1996) and the assumptions were tested again on the transformed data sets to verify the success of the transformations.

A nonparametric Kruskal–Wallis one-way ANOVA on ranks was used to compare abalone and red sea urchin densities, as well as red sea urchin test diameters, among the island groups and depth zones, since no transformation was able to normalize the data. This non-parametric procedure tests the null hypothesis that all medians are equal and is an accepted substitute for one-way ANOVA. Where significant ($P < 0.05$) among group differences were indicated, we used the Kruskal–Wallis multiple-comparison Z-value test to find specific among group differences. The Z-values are appropriate for testing whether the medians of any two groups are significantly different.

One-way ANOVA (fixed model) was used to compare abalone shell lengths (untransformed) among the 5 island groups and between the two depth zones. To identify specific among group difference we used the Tukey–Kramer multiple-comparison test, which examines all pairs of group means. The Kolmogorov–Smirnov goodness of fit test was used to compare abalone and red sea urchin size frequency distributions between the shallow and deep zones. The nonparametric Spearman Rank Correlation was used to assess the relationship between the relative exposure index and abalone densities, since transformations failed to normalize the data. The test produces a correlation coefficient (r_s), which may range from −1 to 1, and it has no units (Zar 1996). The parametric Pearson Product-moment Correlation analysis was used to identify significant relationships between the abundance of northern abalone (SQRT transformed) and the various substrate cover types (ARCSINE(SQRT(X)) transformed). This procedure was also used to examine relationships among abalone, red sea urchin, predator, and macroalgae densities (SQRT transformed). The test produces a simple correlation coefficient (r), which is unitless and ranges from −1 to 1. Simple linear regression analyses were used to assess the relationships between abalone size (untransformed), red sea urchin densities (SQRT transformed), and benthic macroalgae densities (untransformed). One location (group 1, location 2) was excluded from these regression analyses because no abalone were found at this location.

RESULTS

Distribution and Abundance of Abalone

Northern abalone were present at all island groups surveyed in this study, although in varying densities (Table 1). The result of the Kruskal–Wallis one-way ANOVA on ranks indicated significant differences ($P < 0.05$) in abalone densities among the 5 island groups. The Kruskal–Wallis multiple-comparison Z-value test revealed that abalone densities in Group 3 were significantly higher ($P < 0.05$) than at all other groups. There were no significant

TABLE 1.

Northern abalone, red sea urchin, predator and macroalgae densities (mean ± SE) at the 5 island groups and 2 depth zones in the study. The numbers in parentheses are sample size - *n* (ie, number of quadrats). *n* for red sea urchins and predators is same as for abalone.

Island Group	Abalone (Number/m²)	Red Sea Urchin (Number/m²)	Predators (Number/m²)	Macroalgae, (Number/m²)
Group 1	0.143 ± 0.03 (280)	0.596 ± 0.06	0.200 ± 0.03	0.943 ± 0.17 (140)
Shallow	0.163 ± 0.05 (160)	0.700 ± 0.12	0.225 ± 0.04	0.725 ± 0.21 (80)
Deep	0.117 ± 0.04 (120)	0.458 ± 0.08	0.167 ± 0.04	1.233 ± 0.29 (60)
Group 2	0.131 ± 0.03 (320)	0.456 ± 0.07	0.228 ± 0.03	4.176 ± 0.58 (165)
Shallow	0.165 ± 0.03 (230)	0.370 ± 0.07	0.261 ± 0.03	5.042 ± 0.76 (120)
Deep	0.044 ± 0.03 (90)	0.678 ± 0.17	0.144 ± 0.04	1.867 ± 0.51 (45)
Group 3	0.266 ± 0.04 (320)	2.538 ± 0.13	0.166 ± 0.03	0 ± 0 (160)
Shallow	0.288 ± 0.06 (170)	3.059 ± 0.19	0.141 ± 0.03	0 ± 0 (85)
Deep	0.240 ± 0.05 (150)	1.947 ± 0.16	0.193 ± 0.04	0 ± 0 (75)
Group 4	0.104 ± 0.02 (240)	1.242 ± 0.12	0.208 ± 0.04	2.565 ± 0.43 (124)
Shallow	0.154 ± 0.03 (130)	0.915 ± 0.14	0.208 ± 0.05	3.072 ± 0.62 (69)
Deep	0.045 ± 0.02 (110)	1.627 ± 0.20	0.209 ± 0.05	1.927 ± 0.58 (55)
Group 5	0.085 ± 0.02 (353)	0.244 ± 0.06	0.241 ± 0.03	4.486 ± 0.42 (181)
Shallow	0.135 ± 0.03 (193)	0.264 ± 0.10	0.306 ± 0.04	4.367 ± 0.55 (98)
Deep	0.025 ± 0.01 (160)	0.219 ± 0.06	0.163 ± 0.04	4.627 ± 0.66 (83)
Total	0.147 ± 0.01 (1513)	0.997 ± 0.05	0.210 ± 0.01	2.534 ± 0.19 (770)
Shallow	0.180 ± 0.02 (883)	1.005 ± 0.07	0.233 ± 0.02	2.883 ± 0.27 (452)
Deep	0.100 ± 0.02 (630)	0.987 ± 0.07	0.176 ± 0.02	2.038 ± 0.24 (318)

differences in abalone abundance among the other 4 groups ($P > 0.05$). Mean abalone densities in the shallow zone (0.18 ± 0.02 SE) were almost twice as high than in the deep zone (0.10 ± 0.02 SE). The Kruskal–Wallis Z-value test revealed significant differences ($P < 0.05$) in northern abalone densities between the shallow and deep zones.

The mean SL of *H. kamtschatkana* measured in this study was 59.4 mm ($\pm$ 2.0 SE; $n = 222$). The results of one-way ANOVA revealed significant differences in the mean SL of northern abalone among the 5 island groups. The mean SL of abalone in Group 3 was significantly smaller ($P < 0.05$) than those of Groups 2, 4, and 5 (Table 2 and Table 3). Largest abalone were found in Group 2 followed by Group 4. No differences ($P > 0.05$) in abalone size were found between groups 1 & 3, 1 & 4, 2 & 5, and 4 & 5. The area with the highest densities of abalone (i.e., Group 3) was also the area with the smallest abalone. In general, the mean SL of abalone in the shallow depth zone ($0 = 64.7$ mm $\pm$ 2.4 SE) was significantly larger than in the deep-water habitat ($0 = 46.3$ mm $\pm$ 3.2 SE) (one-way ANOVA; $F = 18.1$; $P < 0.001$). The results of the Kolmogorov–Smirnov test indicated that differences in abalone size frequency distributions between the shallow and deep zones were statistically significant ($P < 0.001$) (Fig. 2).

Distribution and Abundance of Red Sea Urchins

The red sea urchin (*S. franciscanus*) was the most abundant echinoid in the study area. The abundance of both green (*S. droebachiensis*) and purple (*S. purpuratus*) sea urchins was so low (i.e., 17 and 5 individuals, respectively) that they were left out of the analysis. Red sea urchins were found in all island groups (Table 1). Significantly higher mean red sea urchin densities (urchins/m²) were found in Group 3 than anywhere else in the study area (Table 4). No significant differences in red urchin densities were observed between groups 1 & 2 and groups 2 & 5. For all areas combined, red sea urchin mean densities were not different between the shallow-water zone (1.01 ± 0.07 SE, $n = 833$) and the deep-water zone

(0.99 ± 0.07 SE, $n = 630$) (Kruskal–Wallis Z-test; $z = 1.936$; $P > 0.05$).

The results of the Kruskal–Wallis Z-value test revealed significant differences ($P < 0.05$) in red sea urchin TD among the 5 island groups (see Table 2; Table 5). The mean TD of red sea urchins in Group 3 was significantly smaller (80.4 mm $\pm$ 1.0 SE) when compared with other groups, with the exception of Group 5. Red sea urchins in Group 4 had largest mean TD (91.3 mm $\pm$ 1.1 SE). No

TABLE 2.

Summary statistics (mean ± SE) for maximum shell length (mm) of northern abalone and maximum test diameter (mm) of red sea urchin at the 5 island groups and 2 depth zones in the study. Sample size (ie, number of individuals measured) in parentheses.

Island Group	Abalone Shell Length (mm)	Red Sea Urchin Test Diameter (mm)
Group 1	51.2 ± 4.3 (38)	87.5 ± 4.0 (136)
Shallow	57.5 ± 6.1 (24)	82.0 ± 5.5 (82)
Deep	40.4 ± 3.6 (14)	95.8 ± 5.3 (54)
Group 2	83.0 ± 4.7 (42)	88.9 ± 2.7 (143)
Shallow	84.7 ± 4.6 (38)	92.4 ± 3.4 (84)
Deep	66.3 ± 23.5 (4)	83.9 ± 4.3 (59)
Group 3	46.1 ± 2.5 (87)	80.4 ± 1.0 (811)
Shallow	49.9 ± 3.2 (50)	82.8 ± 1.2 (517)
Deep	41.0 ± 3.8 (37)	76.0 ± 1.5 (294)
Group 4	62.7 ± 6.1 (25)	91.3 ± 1.1 (301)
Shallow	61.3 ± 7.0 (20)	91.8 ± 1.6 (179)
Deep	68.4 ± 13.4 (5)	90.4 ± 1.4 (122)
Group 5	72.4 ± 4.4 (30)	83.6 ± 6.0 (75)
Shallow	73.0 ± 4.9 (26)	96.0 ± 8.5 (35)
Deep	68.5 ± 10.0 (4)	72.8 ± 8.3 (40)
Total	59.4 ± 2.0 (222)	84.2 ± 0.8 (1466)
Shallow	64.7 ± 2.4 (158)	84.3 ± 1.1 (845)
Deep	46.3 ± 3.2 (64)	84.1 ± 1.2 (621)

TABLE 3.

Results of one-way ANOVA and the Tukey–Kramer multiple-comparison test to discern statistically significant differences in the shell length (mm) of northern abalone among the 5 island groups in the study. Group designation as in Figure 1.

Source Term	DF	Sum of Squares	Mean Square	F-Ratio	Prob Level
A: Group	4	46536.75	11634.19	16.96	0.000*
S(A)	217	148823.5	685.8224		
Total (Adjusted)	221	195360.2			
Total	222				

* Term significant at alpha = 0.05

	Tukey-Kramer Multiple Comparison Test				
Group	1	2	3	4	5
1	—				
2	*	—			
3	NS	*	—		
4	NS	*	*	—	
5	*	NS	*	NS	—

Represents significant difference at least at *P* < 0.05 level. NS indicates no significant difference between groups. This report provides multiple comparison tests for all pairwise differences between the means.

significant differences in the TD of red urchins were found between groups 1 & 2, 1 & 4, 1 & 5, 2 & 4, and 3 & 5. There were no differences in red sea urchin TD between the two depth zones (Kruskal–Wallis Z-test; $z = 0.443$; $P > 0.05$). The size frequency distribution of red sea urchins in BGI for both depth zones were combined (Fig. 3), since the Kolmogorov–Smirnov goodness of fit test indicated no differences ($Dmn = 0.06$, $P > 0.05$) in size frequency distribution between the two depth zones.

Habitat Relationships

The relative index of exposure was positively correlated with abalone densities ($r_s = 0.61$, $P < 0.003$; $n = 22$), red sea urchin densities ($r_s = 0.54$, $P < 0.01$; $n = 22$) and with encrusting coralline algae ($r_s = 0.44$, $P < 0.05$; $n = 22$), but was inversely related to predator abundance ($r_s = -0.45$; $P < 0.05$; $n = 22$). The

TABLE 4.

Kruskal–Wallis multiple comparisons Z-value test to discern statistically significant differences of red sea urchin (*S. franciscanus*), densities (# individuals/m²) among 5 island groups. Numbers represent *Z-values* for the Bonferroni Test (Hintze, 2001). Bold numbers indicate significant differences among groups at *P* < 0.05. Group designation as in Figure 1.

Group	1	2	3	4	5
1	—				
2	2.29	—			
3	13.80	16.65	—		
4	7.78	6.09	9.33	—	
5	3.90	1.62	18.67	7.71	—

Bonferroni Test: Medians significantly (*P* < 0.05) different if *Z-value* > 2.81

relative index of exposure was not correlated with macroalgae densities and other substrate cover types. Encrusting coralline algae were a dominant substrate cover type in all groups, ranging from 50% to 86% (Table 6). In Groups 1, 3, and 5 encrusting coralline algae occupied more than 70% of the available substrate. The highest percent cover by encrusting coralline algae was measured in Group 3, where they covered 85.9% of the substrate. The percent cover of encrusting coralline algae in Group 3 was significantly higher that in any other group (Kruskal–Wallis multiple comparison Z-value test; $P < 0.05$). Articulated coralline algae represented relatively low percentage of substrate throughout the study area, ranging between 2% to 6%.
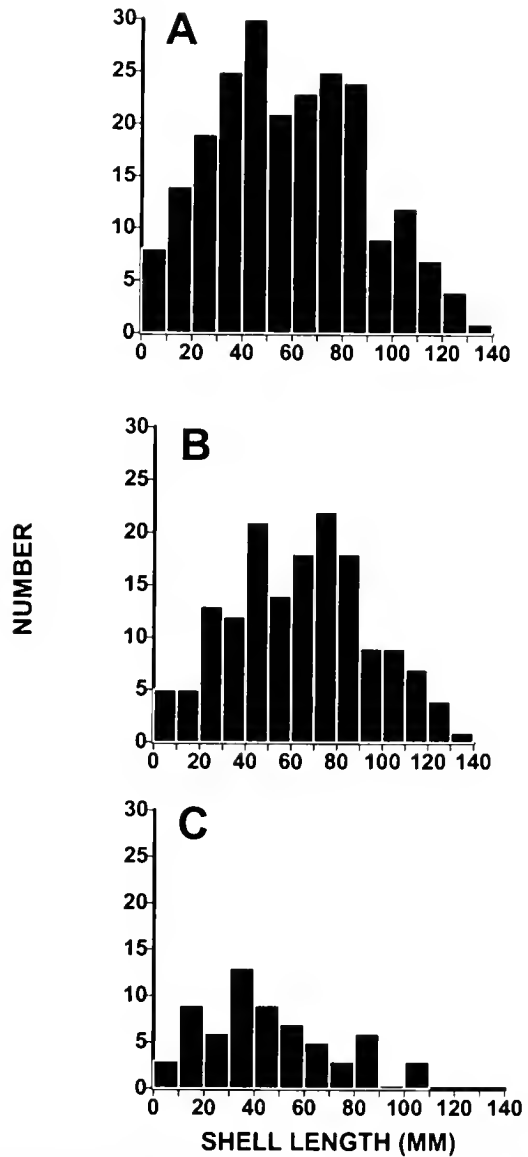

Figure 2. Size frequency distributions of northern abalone (*H. kamtschatkana*) from BGI measured during the study. (A) All abalone measured during the study in BGI at both shallow and deep zones, (B) abalone measured in shallow zones, (C) abalone measured in deep zones. The size frequency distributions at the shallow (B) and deep (C) zones were significantly different (Kolmogorov–Smirnov goodness of fit test *Dmn* = 0.32; *P* < 0.001). The vertical axes represent number of abalone per size class.

Kruskal–Wallis multiple comparisons Z-*value* test to discern statistically significant differences in the test diameter (mm) of red sea urchin (*S. franciscanus*) among 5 island groups. Numbers represent Z-*values* for the Bonferroni Test (Hintze, 2001). Bold numbers indicate significant differences among groups at $P < 0.05$. Group designation as in Figure 1.

Group	1	2	3	4	5
1	—				
2	0.61	—			
3	**3.83**	**4.73**	—		
4	0.32	0.40	**5.75**	—	
5	2.68	**3.22**	0.25	**3.24**	—

Bonferroni Test: Medians significantly different if Z-*value* > 2.8070

Community Relationships

The results of simple linear correlation analysis revealed a significant positive relationship between abalone and red sea urchin densities ($r = 0.48$, $P < 0.05$; $n = 22$). A significant inverse relationship was found between abalone and predator densities ($r = -0.61$; $P < 0.01$; $n = 20$), as well as between abalone and benthic macroalgae densities ($r = -0.43$; $P < 0.05$; $n = 22$). A significant negative correlation was also found between red sea urchin and benthic macroalgae densities ($r = -0.69$; $P < 0.001$; $n = 22$). While encrusting coralline algae showed a strong positive correlation with red sea urchin densities ($r = 0.75$; $P < 0.001$; $n = 22$), they showed no correlation with abalone densities ($P > 0.05$). The results of simple linear regression analysis revealed a significant inverse relationship between abalone size and red sea urchin densities ($r^2 = 0.33$, $P < 0.001$; $n = 21$). Furthermore, simple linear regression found a significant positive relationship between abalone size and abundance of benthic macroalgae ($r^2 = 0.54$, $P < 0.001$; $n = 21$).

DISCUSSION

The results of this study concur with recent surveys by DFO (Lucas et al. 2002). The estimated mean density of abalone in this study ($0.15/m^2$) is similar to the mean abalone density ($0.10/m^2$)
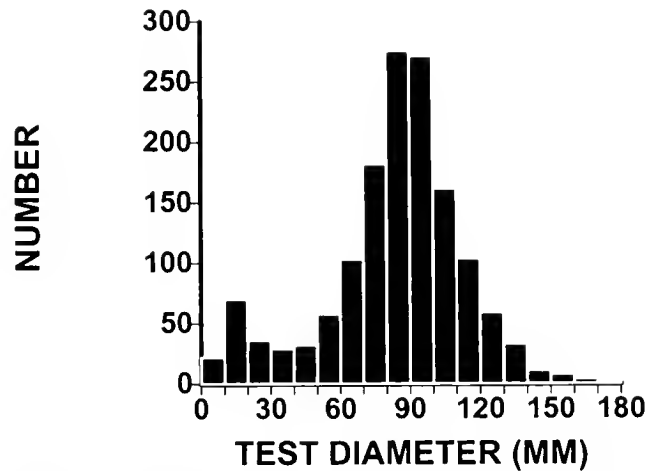

Figure 3. Size frequency distribution of red sea urchins (*S. franciscanus*) from BGI measured during the study. Test diameter frequencies from shallow and deep zones were combined. The vertical axis represents number of red sea urchins per size class.

reported by Lucas et al. (2002) from an adjacent area only a few kilometers away. These values are in sharp contrast to mean density values reported from the north coast of British Columbia between 1978 and 1984 (0.65 to 2.86 abalone/m^2, Sloan & Breen 1988). Surveys conducted in the Queen Charlotte Islands in 1978 reported densities of up to 28 abalone/m^2 (Breen & Adkins 1979). The size range of abalone in Barkley Sound changed from 51 to 146 mm SL in 1964 (Quayle 1971) to 38 to 119 mm SL in 2000 (Lucas et al. 2002). The size range recorded in this study was 4 to 132 mm SL, with a mean of 59.4 mm SL. Roughly 10% of the abalone population measured in this study was more than 100 mm SL, while 58% of the sampled abalone population was more than 50 mm SL (Fig. 2A). Although northern abalone reach sexual maturity between 50 to 55 mm SL (Sloan & Breen 1988), juvenile abalone represented 42% of the sampled population. This suggested that abalone recruitment was occurring, albeit at relatively low numbers.

The low densities of abalone, as well as low abundance of large size individuals recorded in this study may be related to several

TABLE 6.

Summary statistics for percentage cover of eight (8) substrate cover types measured during the study.

Island Group	Substrate Types								n
	EC	AC	BA	GA	BR	S	SP	O	
1	75.7	4.7	0.4	0	0.6	11.0	0.2	5.8	252
	(±1.9)	(±1.0)	(±3.8)	(±0)	(±0.4)	(±1.4)	(±0.1)	(±1.1)	
2	53.3	4.9	2.2	0	0.7	22.5	0.1	15.6	279
	(±2.2)	(±0.9)	(±0.6)	(±0)	(±0.3)	(±1.9)	(±0.1)	(±1.8)	
3	85.9	2.9	0	0.1	0.2	6.3	0	3.7	288
	(±1.3)	(±0.7)	(±0)	(±0.1)	(±0.1)	(±0.9)	(±0)	(±0.7)	
4	50.2	5.2	10.0	0.1	4.2	9.0	0	13.7	219
	(±2.8)	(±1.2)	(±1.6)	(±0.1)	(±1.1)	(±1.5)	(±0)	(±1.9)	
5	70.2	6.2	2.8	0	1.8	12.0	0.3	5.8	309
	(±1.9)	(±0.9)	(±0.5)	(±0)	(±0.4)	(±1.4)	(±0.2)	(±0.9)	

First number is the mean % cover; second number in brackets is ± standard error (*SE*). Acronyms: EC, encrusting coralline algae; AC, articulated coralline algae; BA, brown macroalgae; GA, green macroalgae; BR, bryozoans; S, sand; SP, sponges (Porifera); O, other invertebrates. *n*, sample size (# of quadrats). Island Group designation as shown in Figure 1.

factors, such as human exploitation (i.e., poaching), competition, predation, starvation, disease, differential mortality, or environmental factors. Ocean-climate variability may also play a role in keeping abalone populations at their current low levels. Tegner et al. (2001) demonstrated a strong link between declines in landing of red abalone (*Haliotis rufescens* Swainson, 1822) in southern California and increased variability in sea surface temperatures (SSTs) associated with El Niño events that affect kelp abundance. However, the inverse relationship between abalone and predator densities found in this study suggests that predation may be an important factor contributing to present day structure of abalone populations. The low abundance of large abalone (>110 mm SL) suggests that large abalone may be more susceptible to predation than small individuals (<70 mm SL). Predators may be preferentially selecting larger individuals, or as abalone reach larger size they may loose the ability to either outrun or hide from the predators. In contrast, Watson (2000), citing Sloan & Breen (1988), suggested that only sea otters and human exploitation seem to have a significant impact on the abundance and size of abalone populations.

There was a significant positive correlation between abalone and red sea urchin densities. This is in contrast to studies conducted in California, where consistent negative correlations between *H. rufescens* and red sea urchins were found, suggesting spatial inter-specific exclusion between these two species (Karpov et al. 2001). The red sea urchin is viewed as perhaps abalone's most important competitor for space and food, since both species are grazers competing for the same food resource and space. The positive relationship therefore suggests that competition for food and space may not be direct, and that abalone may be in some way benefiting from their association with the red sea urchin. The positive relationship between abalone and red sea urchins in this study may be partly a function of lower population densities than those reported previously. For example, Watson (1993) reported that mean red sea urchin densities in Barkley Sound between 1988 to 1989 were about 6.9 urchins/m^2, which is about 7 fold higher than at present. Current abalone densities in the BGI are about 4 times lower that pre-closure (Emmett & Jamieson 1988). At these low densities direct competition between abalone and red sea urchins may not be apparent. However, we also found a significant negative correlation between abalone size and red sea urchin abundance. This negative relationship suggests that the urchins may be exerting some degree of influence on abalone populations through their effect on either encrusting coralline algae or benthic macroalgae. Densities of both species exhibited a significant negative correlation with macroalgae abundance. Although abalone size also showed a strong positive relationship with benthic algal abundance, food availability may have played a key role in this interrelationship.

The significant positive relationship between red urchins and encrusting coralline algae and the negative relationship with benthic macrophytes suggests that the presence of sea urchins may

in some way benefit abalone through their maintenance of a habitat that is preferred by juvenile abalone. Sloan & Breen (1988) suggested that abalone settlement occurs on encrusting corallines in deeper water and that juveniles and adults migrate upwards as they grow. Sasaki & Shepherd (2001) showed that ezo abalone (*Haliotis discus hannai* Ino, 1952) settled on encrusting coralline algae and moved into shallow *Eisenia* forest as they aged. Several other studies have also suggested that abalone prefer to settle on substrates dominated by encrusting coralline algae (Shepherd & Turner 1985, McShane 1995). However, the primary food source for abalone, essential for rapid growth of post-larval abalone, may be the associated diatoms rather than the encrusting coralline algae themselves (Takami et al. 1997). Encrusting corallines were found to occupy about 71% of the substrate in the deep zone (6–9 m), which was statistically higher than the 66% cover in the shallow zone (2–5 m).

Vance (1979) suggested that echinoids play a key role in structuring algal turf communities by removing encrusting invertebrates, thus promoting the growth of encrusting coralline algae. This study found a strong negative correlation between encrusting coralline algae and "other invertebrates". By maintaining encrusting coralline algae free of other invertebrates, the red sea urchin may indirectly influence abalone settlement rates and perhaps post settlement survivorship. However, the present study supports earlier studies in British Columbia that found no significant association of juvenile abalone with red sea urchin spine canopy (Sloan & Breen 1988), even though there was a positive correlation in the densities of these two species. In contrast, several recent studies around the world have shown that juveniles of some *Haliotis* species have a strong association with the sea urchin spine canopy (eg, Day & Branch 2002). We found only six juvenile abalones (≤45 mm SL) under the red sea urchin spine canopy, which represents only 7% of all juvenile abalones recorded in this study. Rogers–Bennett & Pearse (2001) reported that one third of juvenile abalone inside a marine protected area was found under the spine canopy of sea urchins.

ACKNOWLEDGMENTS

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IMPLICATIONS OF HIGH LEVELS OF GENETIC DIVERSITY AND WEAK POPULATION STRUCTURE FOR THE REBUILDING OF NORTHERN ABALONE IN BRITISH COLUMBIA, CANADA

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ABSTRACT   In the past 25 years, the abundance of northern abalone (*Haliotis kamtschatkana*) has declined by 80% in British Columbia (BC), leading to concern over a possible loss of genetic diversity and fragmentation of breeding aggregates in the species. Abalone from 31 sites in BC and one site in southeastern Alaska were surveyed for variation at eight polymorphic microsatellite loci. The high level of H_E characterizing all samples resulted in a large estimated effective population size for northern abalone (>350,000), consistent with high estimates for the historical average number of migrants entering abalone aggregations each generation (~20–125). Hierarchical analysis of gene diversity revealed that 99.6% of genetic variation was contained within abalone samples and only 0.4% partitioned among samples. Approximately half of the variation was accounted for by differences between abalone of the Queen Charlotte Islands, Alaska and those from central, southern British Columbia while the other half was caused by differences among samples within the two regions. Little allele frequency variation was observed among size classes or between repeat samples from sites sampled in more than 1 year. The results indicated that, historically, northern abalone aggregations did not represent isolated breeding units and any disruption of gene flow that may have been caused by recent low abundance levels cannot yet be detected. These results are discussed with respect to rebuilding efforts to be undertaken for northern abalone within BC.

KEY WORDS: *Haliotis kamtschatkana*, genetic variation, microsatellite, gene flow, inbreeding, population structure

INTRODUCTION

Exploited species of abalone throughout the world have suffered severe declines in abundance (Davis et al. 1992, Prince & Shepherd 1992) and some are close to extinction (Davis et al. 1998). The northern or pinto abalone (*Haliotis kamtschatkana*), which inhabits shallow coastal waters of the northeastern Pacific Ocean from southern California to Alaska, declined in abundance by 75% to 80% in British Columbia (BC) between 1978 and 1990 (Campbell 2000). Abalone abundance did not increase with implementation of a complete harvest closure in 1990 and northern abalone was listed as a "threatened" species (i.e., one likely to become in imminent danger of extinction or extirpation if limiting factors are not reversed) by the Committee on the Status of Endangered Wildlife in Canada in 1999 (COSEWIC 2000). Two factors identified as major threats to northern abalone recovery were low recruitment levels and continued (illegal) harvest. In this study, we undertake a genetic assessment of population structure in northern abalone as an element of a comprehensive recovery plan for the species in BC.

Northern abalone are distributed in patches on exposed and semi-exposed rocky coastal areas in BC. Species at low abundance partitioned into isolated small populations are at risk for extirpation and extinction from stochastic demographic, environmental, and genetic factors. The biology of northern abalone makes it vulnerable to all three types of processes. Spawning is usually restricted to summer months (i.e., May to August), and the pelagic larval stage is of short duration, varying from 4 to 8 days with local factors such as temperature (14°C to 10°C) (Sloan & Breen 1988). The current low abundance and low densities of mature abalone (Campbell 2000) may reflect not only the historical commercial harvest but also adverse environmental conditions likely to have hindered successful recruitment over the period 1975 to 1983 (Breen 1986). In turn, low abalone abundance hinders successful spawning because external fertilization requires high-density aggregations of mature individuals (Babcock & Keesing 1999). Fi-

nally, reduced spawning success may lead to disruption of the larval-mediated gene flow among spawning aggregates that typically offsets a loss of diversity within local populations. Expected results would include increased inbreeding within, and genetic drift among, local populations.

The level and distance of larval dispersal mediate both demographic and genetic processes in sedentary marine organisms. Larval dispersal levels for abalone species are generally not known but are apparently sufficiently low to ensure that demographic processes occur on a local scale (i.e., recruitment is primarily local, ranging from a few meters or kilometers) and sufficiently high enough to prevent strong genetic differentiation over large geographic ranges (Brown 1991, Hamm & Burton 2000, Huang et al. 2000). Nevertheless, the genetic studies have provided evidence for different scales of population structure in abalone species, even those that are sympatric. This indicates factors such as habitat utilization, spawning season, and larval duration may also influence abalone population structure.

For blacklip abalone, *H. rubra*, sampled along the southern coastline of Australia, genetic data indicated there was "isolation by distance", but even the most geographically distant (>1000 km) populations were genetically similar. The F_{ST} value for this species estimated from allozymes was 0.022 (Brown 1991) and from microsatellites was 0.077 (Huang et al. 2000). Greater microspatial genetic heterogeneity was observed in Australian greenlip abalone, *H. laevigata*, a species with a more patchy distribution than *H. rubra*, but the estimated F_{ST} value (0.014) was not greater than that of the blacklip abalone (Brown & Murray 1992, Shepherd & Brown 1993). Microspatial variability contrasting with genetic homogeneity was also evident in the sympatric Roe's abalone, *H. roei*, for which an F_{ST} value of 0.009 was estimated from samples collected over almost 3000 km of coastline (Hancock 2000).

Differences in population structure have also been observed in two sympatric abalone species of California. Red abalone, *H. rufescens*, from northern and southern California were little differen-

tiated at allozyme loci, in mitochondrial DNA sequence, or at a single microsatellite locus, with the F_{ST} value of 0.012 estimated from allozyme data not significantly different from zero (Gaffney et al. 1996, Kirby et al. 1998, Burton & Tegner 2000). In contrast, significant genetic differentiation among samples of black abalone, *H. cracherodii*, in central California ($F_{ST} = 0.039$) was attributed to a restricted spawning season that limits larval dispersal (Hamm & Burton 2000). These results indicated that immigration from distant sources was unlikely to be sufficiently great to accelerate recovery in the depleted black abalone populations of southern California, estimated to have declined in abundance by as much as 97% (Altstatt et al. 1996).

Low levels of intraspecific variation may make the partitioning of genetic diversity within abalone species most amenable to examination with highly polymorphic, rapidly evolving microsatellite loci (Huang et al. 2000, Withler 2000). In the present study, we survey variation at eight polymorphic microsatellite loci in northern abalone collected from 31 sites in BC and one site in southeast Alaska. The objectives of this study are to determine levels of genetic variation within and among aggregations of northern aba-

lone in BC, and to estimate effective population sizes and inbreeding levels for the species. We examine the genetic data for evidence of recent bottlenecks in population abundance that might have reduced genetic variation within, or increased variation among, extant abalone aggregations and incorporate the genetic data into recommendations for conservation efforts likely to benefit the northern abalone of BC.

MATERIALS AND METHODS

Epipodial tissue samples from adult abalone were collected from 31 sites within BC and one site in southeast Alaska between 1998 and 2002 (Table 1, Fig. 1). Abalone were collected in 2 different years at six sites. SCUBA dive teams searched for emergent or exposed (visible on rocks) individuals because most are easily found, whereas immature abalone tend to be cryptic (Campbell 1996). Samples from abalone within 10 to 200 m were used to represent each collection area. The small epipodial tissue sample removal from each abalone was considered non-destructive, causing no mortality to the abalone (A. Campbell unpublished data on

TABLE 1.

Locations, years and sample sizes of *Haliotis kamtschatkana* collections for microsatellite DNA analysis.

| Site	Latitude	Longitude	Year	N
West coast Vancouver Island				
Elbow Island	48 54.060	125 16.556	2000	45
Vargas Island	49 09.429	125 57.729	2000	70
Dempster Island	48 54.000	125 16.000	2002	170
Hankin Island	48 55.000	125 22.000	2002	170
Turret Island	48 54.000	125 20.000	2002	180
Austin Island	48 51.370	125 19.100	2002	180
Deer Group Islands	48 53.000	125 08.000	2002	30
Bamfield Inlet	48 49.000	125 08.000	2001	90
Georgia Strait				
Denman Island	49 28.883	124 41.209	1999, 2000	45, 85
Queen Charlotte Strait				
Alert Bay	50 35.000	126 55.000	2000	40
BC central coast				
Cranstown Point	51 22.500	127 46.500	1999	110
Nalau Passage	51 47.000	128 06.500	1999	115
Simonds Group	51 57.800	128 16.700	1999, 2001	80, 70
Iroquois Island	52 02.895	128 19.445	1999	110
Stryker Island	52 05.990	123 23.207	1999, 2001	90, 20
Nowish Islands	52 31.000	128 26.000	1999	112
Higgins Passage	52 28.500	128 45.500	1999	90
Lotbiniere Bay	53 01.372	129 31.770	2000, 2001	28, 90
Hankin Point	53 42.400	130 24.610	2000, 2001	55, 25
Freeman Passage	53 49.300	130 31.600	2000, 2001	75, 85
Kitasu Bay	52 32.500	128 49.300	2001	35
Mosquito Island	51 50.195	128 09.900	2001	110
Rennison Island	52 51.308	129 20.540	2001	95
Kingkown Inlet	53 29.685	130 27.559	2001	85
Queen Charlotte Islands				
Louscoone Inlet	52 07.692	131 14.127	1999	130
Montserrat Bay	52 06.227	130 59.170	1998	70
Skincuttle Inlet	52 20.780	131 14.260	1998	73
Faraday Island	52 36.770	131 27.800	1998	72
Virago Sound	54 04.000	132 31.000	1998	70
Bruin Bay	54 10.017	132 58.752	1999	90
Carpenter Bay	52 13.341	131 03.260	2002	90
Alaska				
Sitka Sound	57 03.100	135 20.500	1999	95
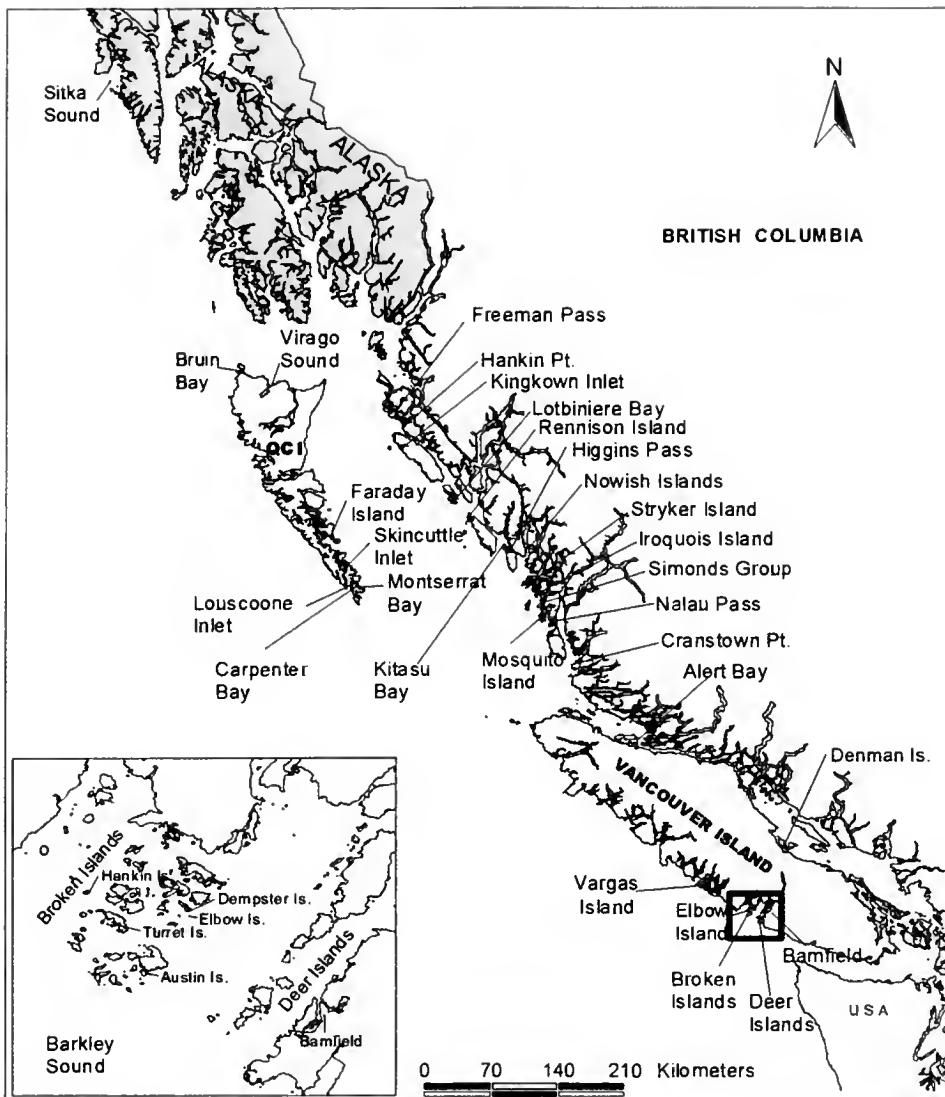
Figure 1. Map showing locations of *Haliotis kamtschatkana* sample collections made in British Columbia and southeast Alaska between 1998 and 2002.

a laboratory experiment). Samples were stored in 95% ethanol prior to DNA extraction using DNeasy kits (Qiagen, Valencia, CA).

Variation at eight microsatellite loci isolated from northern abalone (*Hka*12, *Hka*28, *Hka*40, *Hka*43, *Hka*48, *Hka*56, *Hka*65, *Hka*85) was surveyed using the primers and protocols outlined by Miller et al. (2001). The microsatellite loci consisted of di-, tri- and tetra-nucleotide repeat sequences (Table 2). Alleles at each locus were generally differentiated by the number of basepairs (bp) of the predominant repeat unit, but alleles differentiated by a single base pair were observed and scored without binning at two imperfect dinucleotide loci (*Hka*48 and *Hka*65). Allele frequencies for all samples surveyed in this study are available at <http://www.pac.dfo-mpo.gc.ca/sci/aqua/bgsid_e.htm>.

Analysis of the allelic and genotypic frequency data was carried out using the Genetic Data Analysis (GDA) program of Lewis & Zaykin (2000), GENEPOP version 3.1d (Raymond & Rousset 1995) and FSTAT version 2.9.3.2 (Goudet 2001). Genotypic frequencies at each locus in each sample were tested for conformance to Hardy Weinberg equilibrium (HWE) distributions in

GENEPOP. Weir & Cockerham's (1984) F_{ST} values were computed over all samples and on a pairwise basis between samples using FSTAT. The significance of the multilocus F_{ST} value over all samples was determined by jackknifing over loci. FSTAT was used to measure the "allelic richness" (allelic diversity standardized to a sample size of 15) for each sample and to perform Mantel's (1967) regression of the pairwise F_{ST} values on geographic distance to test for "isolation by distance" among abalone samples. Geographic distances were measured as the shortest direct distances between sites.

Pairwise F_{ST} values were clustered with the neighbor-joining algorithm to provide a dendrogram of the genetic relationships among abalone samples. The pairwise average number of migrants (N*m*) between samples was estimated by the private alleles method of Barton and Slatkin (1986) using GENEPOP and with the expression $F_{ST} = 1/(4Nm + 1)$, a relationship based on the assumption of island model of population structure (Whitlock & McCauley 1999). The effective population size (N$_e$) for northern abalone was calculated from expected heterozygosity (H$_E$) values for the eight microsatellite loci using the relationship $N_e = (1/[1-H_E]^2-1)/8\mu$,

TABLE 2.

Microsatellite loci examined in samples of *Haliotis kamtschatkana* from 32 locations in British Columbia and Alaska. The total number (A) and size range (in base pairs) of alleles, the expected (H_E) and observed (H_O) heterozygosity values, the F_{ST} value and the inbreeding coefficient (f_{is}) calculated over all samples for each locus are shown. The effective population size (N_e) estimated from H_E is also shown.

Locus	Repeat	A	Size Range (bp)	H_E	H_O	F_{ST}	f_{is}	N_e/1000
*Hka*12	di	82	171–377	0.92	0.89	0.000	0.03	190
*Hka*28	di	37	183–271	0.94	0.57	0.001	0.40	350
*Hka*40	di	37	112–210	0.91	0.85	0.001	0.07	150
*Hka*43	tetra	24	163–263	0.88	0.87	0.005*	0.01	90
*Hka*48	di	68	93–250	0.97	0.71	0.002*	0.27	1390
*Hka*56	di	35	93–164	0.92	0.86	0.001*	0.07	190
*Hka*65	di	58	115–250	0.95	0.87	0.001	0.09	500
*Hka*85	tri	49	122–390	0.89	0.49	0.000	0.45	100
Mean	–	49	–	0.92	0.76	0.002*	0.17	370

* $P < 0.05$.

where μ is the mutation rate for the microsatellite loci (Lehmann et al. 1998). Little is known of the mutation rate of microsatellite loci in invertebrate organisms except *Drosophila*, in which the observed rate ($\sim10^{-6}$) is much lower than in mammals ($\sim10^{-4}$). N_e for northern abalone was estimated in this study using the conservative assumption that $\mu = 10^{-4}$, with recognition that N_e values are 100 times greater if the true value is 10^{-6}.

Hierarchical analyses of allele frequency variation were carried out with nested ANOVA (random effects model) as described by Weir (1996) using GDA. The significance of differences in allele frequencies between pairs of samples collected in different years at each of six sites was examined. Similarly, the significance of allele frequency differences attributable to two geographic regions identified in the dendrogram based on genetic distances (the Queen Charlotte Island [QCI] and southeastern Alaskan sites versus remaining sites from coastal BC) was tested in a hierarchical model with sample sites nested within regions.

Heterogeneity among size classes within samples was investigated in abalone from 16 sites (within year samples). Abalone from each site were divided among 4 size classes based on shell length: up to 50 mm, immature; 51 to 69 mm, transition of immature to mature; 70 to 99 mm, mature; and more than 99 mm, fishery; defined by size at maturity estimates by Campbell et al. (1992). For each site, abalone from between 2 and 4 of the size classes were obtained. Each of the size groups contained a range of ages whose growth rates could have been influenced by local environmental conditions: less than or equal to 2 to less than or equal to 4 y ($\leq$50 mm SL), between 2 and 7 y (51–69 mm SL), between 3 and 14 y (70–99 mm SL) and more than 6 or more than 14 y (>99 mm SL) estimated from (Fig. 8 in Sloan & Breen 1988). The maximum age of *H. kamtschatkana* is not known, but individuals reach ages of 30 y and older (Breen 1980). Thus, the potential number of cohorts contained within each size class increases with size class. Allele frequencies in the two or three size classes containing the most abalone at each site were analyzed by ANOVA to examine the possibility that small numbers of adults contribute to recruitment in individual cohorts of northern abalone, leading to low genetic variability within cohorts and significant variation among cohorts within abalone aggregations. The allelic richness and inbreeding coefficient was estimated using FSTAT for each size group containing at least 20 abalone from each site.

RESULTS

Genetic Variation Within Populations

All microsatellite loci examined were highly polymorphic, exhibiting high numbers of alleles and high values of both observed (H_O) and expected (H_E) heterozygosities (Table 2). Genotypes at all eight loci showed an excess of homozygotes in comparison to those expected under HWE, but the level of heterozygote deficiency varied greatly among loci (Table 2). Estimates of f_{is} (the level of population subdivision and inbreeding if the excess of homozygotes was due entirely to assortative mating) ranged from 0.01 at *Hka*43 to 0.45 at *Hka*85.

Differences in allele frequencies between pairs of samples collected in 2 different years from each of six sites were not significant ($F_{5, 700} = 1.75$, $P > 0.10$). The F_{ST} values between the sample pairs ranged from 0 to 0.003, with an average value of 0.001. In each case, samples from the same site were combined for further analysis.

All 32 samples of northern abalone displayed high levels of allelic diversity (mean numbers of alleles observed over all loci) and the standardized number of alleles, termed allelic richness, averaged 14.4 over all samples and did not differ among samples ($P > 0.10$) (Table 3). Each locus was characterized by between two and seven common alleles, with frequencies of common alleles rarely exceeding 0.25 in a sample. None of the loci possessed a single allele that was present at the highest frequency in all samples. Although allelic diversity was high, private alleles (those observed in a single sample) were rare. Of the 390 alleles observed over all eight loci, only 30 were private and each was present at a frequency of less than 0.025 in the single sample in which it was observed. Average H_O by sample ranged from 0.73 to 0.79 (mean of 0.76), but in all cases was less than the H_E, which was essentially 0.92 for all samples (Table 3). Thus, the estimated f_{is} value varied much less among samples (from 0.14–0.21) than among loci. The great range of f_{is} values among loci and the consistency of the f_{is} values for a given locus among samples indicate that population structure was not the sole explanation for the large observed heterozygote deficits at *Hka*28, *Hka*48, and *Hka*85.

Using the mammalian microsatellite mutation rate (10^{-4}) and H_E values estimated for the abalone microsatellite loci of this study, we obtained locus-specific estimates of effective population

TABLE 3.

Genetic variation within samples of *Haliotis kamtschatkana* sampled from locations in British Columbia and southeast Alaska. The average number of alleles (A_P), standardized allelic richness (A_R), and expected (H_E) and observed (H_O) levels of heterozygosity are shown for each sample. The inbreeding coefficient calculated over all loci (f_{is}—all loci) and over the five loci at which there was no evidence of non-amplifying alleles (f_{is}—5 loci) are also shown.

Site	N	A_P	A_R	H_E	H_O	f_{is} (All loci)	f_{is} (5 loci)
West coast Vancouver Island							
Elbow Island	45	22.6	14.3	0.92	0.78	0.15	0.08
Vargas Island	70	25.9	14.1	0.91	0.74	0.19	0.09
Dempster Island	170	32.4	14.5	0.92	0.76	0.17	0.06
Hankin Island	170	32.0	14.3	0.92	0.76	0.18	0.07
Turret Island	180	31.9	14.4	0.92	0.73	0.21	0.08
Austin Island	180	31.5	14.3	0.92	0.74	0.19	0.06
Deer Group Islands	30	19.0	14.4	0.93	0.74	0.20	0.06
Bamfield	90	27.1	14.6	0.92	0.77	0.17	0.02
Georgia Strait							
Denman Island	130	29.9	14.4	0.92	0.77	0.17	0.03
Queen Charlotte Strait							
Alert Bay	40	21.5	14.3	0.92	0.78	0.16	0.06
BC central coast							
Cranstown Point	110	29.6	14.9	0.93	0.79	0.15	0.04
Nalau Passage	115	30.9	14.6	0.92	0.78	0.16	0.06
Simonds Group	150	31.9	14.5	0.92	0.74	0.20	0.08
Iroquois Island	110	31.6	14.8	0.92	0.78	0.16	0.04
Stryker Island	110	28.1	14.2	0.92	0.77	0.16	0.04
Nowish Islands	112	28.9	14.3	0.92	0.73	0.20	0.08
Higgins Passage	90	27.1	14.2	0.92	0.76	0.18	0.05
Lotbiniere Bay	118	29.9	14.3	0.92	0.76	0.17	0.07
Hankin Point	80	27.5	14.2	0.92	0.77	0.16	0.05
Freeman Passage	160	32.8	14.7	0.92	0.79	0.14	0.04
Kitasu Bay	35	20.0	14.2	0.92	0.76	0.17	0.01
Mosquito Island	110	30.5	14.6	0.92	0.77	0.17	0.06
Rennison Island	95	28.1	14.3	0.92	0.78	0.16	0.06
Kingkown Inlet	85	26.3	13.9	0.92	0.74	0.19	0.05
Queen Charlotte Islands							
Louscoone Inlet	130	32.4	14.8	0.92	0.77	0.17	0.05
Montserrat Bay	70	28.0	14.7	0.92	0.76	0.17	0.04
Skincuttle Inlet	73	27.6	14.3	0.92	0.75	0.18	0.02
Faraday Island	72	26.4	14.2	0.92	0.74	0.19	0.04
Virago Sound	70	26.4	14.2	0.92	0.79	0.14	0.06
Bruin Bay	90	29.6	14.6	0.92	0.78	0.14	0.02
Carpenter Bay	90	29.9	15.1	0.93	0.76	0.18	0.06
Alaska							
Sitka Sound	95	28.5	14.5	0.92	0.76	0.17	0.05
Total/Mean	3345	28.3	14.4	0.92	0.76	0.17	0.05

size (N_e) ranging from 90,000 to 1,390,000 and a mean value of 370,000 (Table 2). Use of the possibly more realistic mutation rate of 10^{-6} provides estimates 100 times larger.

Genetic Variation Among Size Groups Within Samples

Allele frequencies did not differ significantly among size classes within each site ($F_{29, 1200} = 1.39$, $P > 0.05$) and size accounted for none of the variation observed within and among the samples of abalone subdivided into size classes. The mean allelic richness of the individual size samples (14.3 alleles) was the same as that of the total samples indicating that there was not reduced diversity within cohorts. Fewer ages (year classes) contributed to the smaller (immature and transition) than the larger (mature and fishery) abalone size classes. However, neither allelic richness nor

the inbreeding coefficient varied among size classes (both $P > 0.05$), providing little evidence that individual cohorts were the products of small numbers of or highly related abalone parents. The lack of allele frequency variation among size groups also indicated that the number of abalone participating in individual spawning events was not extremely low.

Genetic Variation Among Samples

The F_{ST} value calculated over the eight loci among all samples was low but significantly greater than zero (0.002; SE 0.000). Examined on a single locus basis, F_{ST} values ranged from 0.000 to 0.005, and were significantly greater than 0 for three of the eight loci examined ($P < 0.05$) (Table 2). There was no strong geographic clustering of samples apparent in the dendrogram (Fig.

2). The seven QCI and single Alaskan samples clustered together, but the central coast, Georgia Strait and west coast Vancouver Island samples did not cluster geographically. The hierarchical analyses of gene diversity indicated that 99.6% of the observed genetic variation occurred within samples and only 0.4% was attributable to differentiation among samples. Of the differentiation among samples, approximately half (0.2%) was due to differences between the two regions (QCI/Alaska vs. coastal BC) and the other half to differences among samples within regions. The effect of region was not highly significant ($F_{1, 30} = 3.46$, $0.05 < P < 0.10$) and there was no significant differentiation among samples within region ($F_{3, 3300} = 1.39$, $P > 0.05$).

The regression of all pairwise F_{ST} values on geographic distance was significant ($P < 0.05$), but geographic distance accounted for very little of the observed variation in F_{ST} values ($r^2 = 0.11$) (Fig. 3A). The distinctiveness of the QCI and Alaskan samples and their relatively great geographic distance from many of the remaining samples accounted for the relationship between geographic and genetic differentiation. With the QCI and Alaskan samples removed from the data set, there was no relationship ($P = 0.67$) and distance accounted for less than 1% of the observed variation in F_{ST} values (Fig. 3B). For this set of coastal BC populations, pairwise F_{ST} values did not exceed 0.005 (note the change in the F_{ST} scale between Figs. 3A and 3B), and F_{ST} values of 0 were
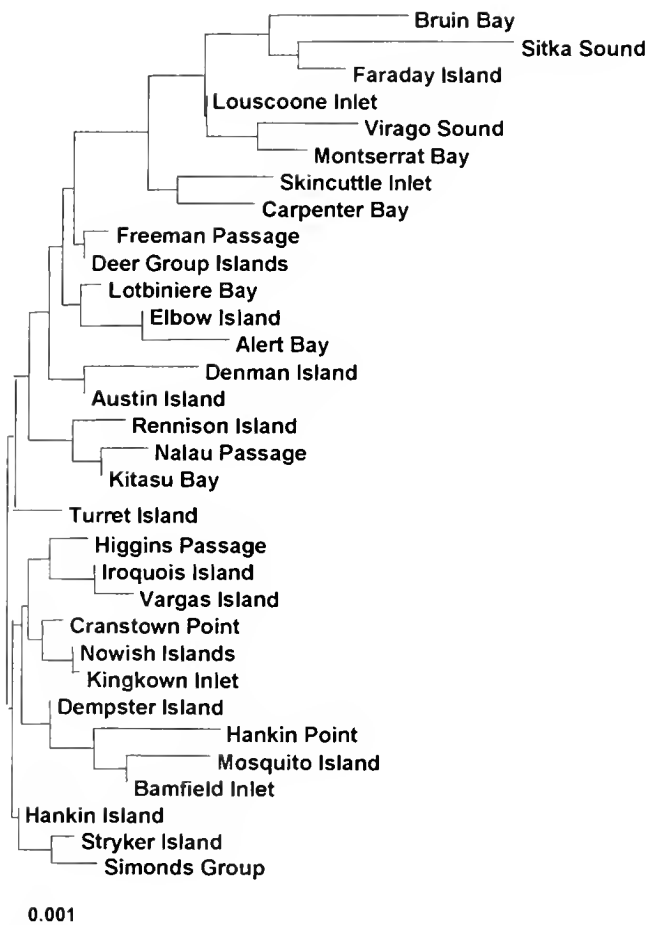
A

B
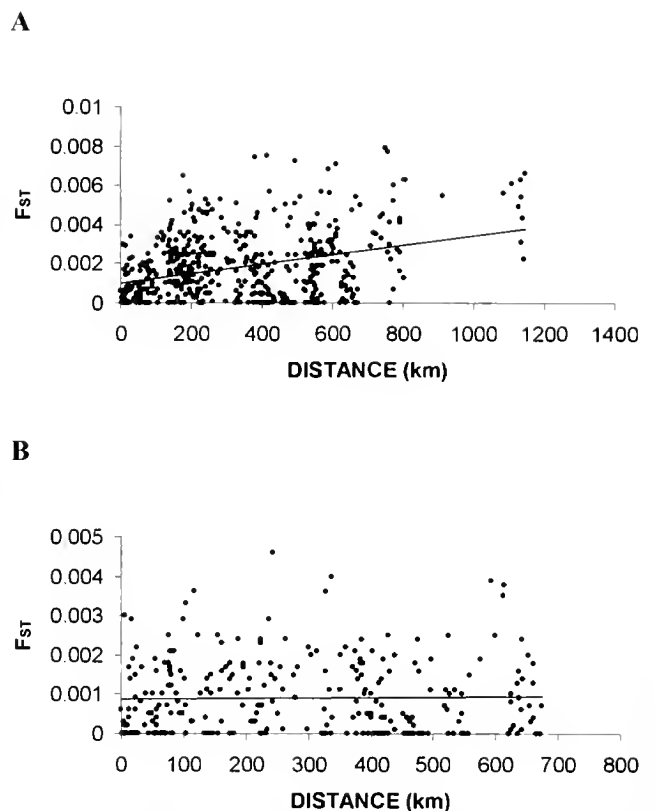
Figure 3. Regression of pairwise values of genetic differentiation (F_{ST}) on geographic distance for (A) all *Haliotis kamtschatkana* samples and (B) coastal British Columbia samples only (excluding samples from the Queen Charlotte Islands and southeast Alaska). Note the difference in y-axis scale between A and B.

observed between pairs of samples over the entire range of geographic separation from 1 to 700 km.

Using the entire data set, the regression of F_{ST} on geographic distance intersected the average F_{ST} value between repeat samples from the same geographic location at the y-axis intercept, a value of 0 km in geographic distance. This would suggest a neighborhood size of less than 1 km, the smallest distance by which samples in this study were separated. However, using the data set for coastal BC sites only, the regression of F_{ST} on distance was essentially a straight line (slope $= 1.2 \times 10^{-7}$), indicating an average pairwise F_{ST} of 0.0008 over the entire 700 km range. This line coincided with the F_{ST} value of 0.001 obtained between repeated samples from the same site and suggested that the entire coastal range of BC sampled in this study, exclusive of the QCI, constituted a single genetic neighborhood.

The average number of migrants per generation into the abalone aggregations represented by each sample was estimated by the private alleles method as 18.7, a number consistent with the observed lack of genetic differentiation among samples. This value changed little when only QCI/Alaskan samples (22.5) or only non-QCI samples (22.6) were considered. Calculating the average number of migrants using the standard expectation for the relationship between F_{ST} and Nm provided an estimated 125 migrants entering abalone aggregations each generation.

DISCUSSION

Northern abalone throughout BC and southeast Alaska were characterized by very high levels of microsatellite DNA variation

Figure 2. Neighbor-joining dendrogram of relationships among *Haliotis kamtschatkana* samples based on pairwise F_{ST} values. Samples from the Queen Charlotte Islands and southeast Alaska cluster independently from samples from coastal British Columbia locations.

within and very low levels of differentiation among spawning aggregates. Less than 1% of genetic variation was attributable to differences among samples and little geographic structure was observed. The lack of strong differentiation among sample locations, between repeated samples from single locations and among abalone of different size classes within samples suggested that gene flow among abalone breeding aggregations throughout BC has been extensive. If the recent low abundance of abalone has disrupted historical patterns of gene flow, it is not yet evident among abalone of the age groups encompassed in this study.

During the last Cordilleran glaciation of North America, which ended approximately 12,000 y ago, the QCI and Alaskan coastal regions may have provided refugial habitat for terrestrial and marine organisms (Warner et al. 1982). Thus, northern abalone throughout much of coastal BC and those of the QCI (and perhaps northern BC and southeast Alaska) may be descendants of different refugial populations. Two distinctive clades in mitochondrial DNA sequences of the littorinid snail *Littorina subrotundata* throughout BC and Washington have been attributed to dispersal from separate glacial refugia (Kyle & Boulding 1998, Kyle & Boulding 2000). The small differences in microsatellite allele frequencies between coastal and QCI samples of northern abalone may reflect either historical isolation in separate refugia or more recent restrictions of gene flow between coastal and QCI habitats. Even if extant abalone are descendants of different refugial populations, the high level of intraspecific variability and low level of intersample differentiation indicated that refugial population sizes were large and limited genetic divergence occurred during isolation, or that gene flow has occurred since the glacial period.

All of the microsatellite loci examined in this study exhibited an excess of homozygosity such as that observed in surveys of other mollusks, including abalone species (Brown 1991, Hara & Kikuchi 1992, Beaumont et al. 1993, Huang et al. 2000, Pérez–Losado et al. 2002). For abalone, the deficiencies generally have been attributed to inbreeding. In northern abalone, more variation was observed among loci than among samples in the level of heterozygote deficiency, indicating that locus-specific factors such as non-amplifying alleles were also involved. Thus, some level of inbreeding may have occurred in northern abalone, as in other abalone species, the level of which is best estimated by those loci showing the least evidence of non-amplifying alleles (i.e. those loci with genotypic frequencies closest to HWE). The average inbreeding coefficient over all samples for the five loci closest to HWE was 0.05. This may represent the typical level of inbreeding in northern abalone populations.

High levels of local larval recruitment or asynchronous spawning on a small geographic scale may have contributed to inbreeding in *H. kamtschatkana*, as suggested for blacklip abalone (Huang et al. 2000). However, the analysis of allelic differentiation among size classes of northern abalone within samples provided no evidence of the increased genetic differentiation among cohorts and the reduced genetic diversity within cohorts expected under "sweepstakes-style" recruitment success (Hedgecock 1994). According to this model, spatial and temporal variability in recruitment success may lead to detectable genetic drift among cohorts and to "chaotic genetic patchiness", in which samples in very close proximity are as genetically differentiated as ones very far apart (Larson & Julian 1999). Although proximal samples of northern abalone in coastal waters were as different as distal ones, all samples were highly polymorphic and little differentiated. Samples representing individual size and restricted age groups were as al-

lelically "rich" as samples containing all size classes from a single location. Moreover, the average F_{ST} value between size classes within sites (0.001) was the same as that between repeat samples from the same site and that between coastal sites. Thus there was no evidence that the successful spawners at any given time were sufficiently small in number or closely related to result in accelerated genetic drift. Instead, the coastal abalone populations in this study could be considered to form a single genetic neighborhood, with genotype distributions showing no departure from those expected under panmictic mating. The abalone of the QCI and southeastern Alaska may constitute a second, only slightly differentiated neighborhood.

The high abundance of rare alleles in all northern abalone samples (>80% of alleles were present at frequencies <0.1) suggested that populations have existed at long-term stable sizes (ie, not suffered recent bottlenecks) (Luikart et al. 1998). This observation and the high estimates of effective population size indicated that the small local aggregations of mature abalone observed in census studies (Wallace 1999, Campbell 2000) did not represent genetically isolated breeding units. "Cryptic" abalone, not recently included in census counts, possibly also contributed to reproduction in northern abalone. However, it is evident that local northern abalone aggregations have been connected by gene flow as the result of larval dispersal.

The strong genetic homogeneity of northern abalone, a seasonal spawner, contrasts with results obtained for the black abalone, in which higher levels of genetic differentiation were attributed at least in part to the limited spawning season and strong seasonal differences in oceanographic patterns in the coastal waters of California (Hamm & Burton 2000). The lack of genetic structure in northern abalone is more similar to the low level of genetic differentiation observed in the red abalone of California, which spawns throughout the year (Burton & Tegner 2000) and in three sympatric abalone species inhabiting the waters of southern Australia. The Australian blacklip, greenlip, and Roe's abalone all show low levels of genetic differentiation over spatial scales as large or larger than those encompassed in the present study (Brown 1991, Brown & Murray 1992, Hancock 2000).

Small-scale genetic heterogeneity coupled with large-scale homogeneity in Roe's abalone was attributed to predominantly local recruitment, with the high gene flow resulting more from large effective population sizes than from large migration rates (Hancock 2000). Hancock also suggested that rare cases of successful long-distance dispersal might play a role in maintaining the observed large-scale genetic homogeneity. Little small-scale heterogeneity was observed among samples of northern abalone. The lower F_{ST} values observed over short distances in northern abalone suggest that the N_e of this species is larger, or that larval dispersal is greater, than that observed for Roe's abalone. Given the high densities observed for Roe's abalone (Hancock 2000), it seems unlikely that the N_e for northern abalone exceeded that for Roe's abalone even before the recent decreases in abundance. The large estimated numbers of successful migrants among the samples in this study support the idea that dispersal may contribute more to the low observed levels of differentiation in northern abalone than in many other species. Whether successful larval dispersal in northern abalone occurs on a regular basis or is predominantly the result of rare, but highly effective, long distance dispersal events is not known.

Marine species with extended longevity possess a "storage capacity" for genetic variation in the face of fluctuating environments

in the large cohort of adults produced from each strong recruitment (Warner & Chesson 1985, Ellner & Hairston 1994, Ellner 1996, Gaggiotti & Vetter 1999). Each large cohort effectively "stores" many genotypes within the reproductive population over many spawning periods that are capable of contributing to both population size and genetic diversity when favorable spawning and recruitment conditions return. However, extended periods of low reproductive or recruitment success may be masked in genetic surveys heavily influenced by the genetic variability being stored in, but not transmitted from, the older age groups. The analysis of genetic variation in different size classes of abalone at several sites in this study provided no indication that younger abalone were less diverse than older ones, but sampling of the younger ages did not include newly recruited "cryptic" individuals. In the black abalone of southern California, recruitment failure was observed after abalone abundance dropped by approximately 50% (Richards & Davis 1993). Because of the longevity of northern abalone individuals, it is essential that recruitment be measured to determine current levels of reproductive success. Long-term genetic monitoring of newly recruited abalone would reveal the loss of genetic diversity and population fragmentation that might follow a disruption of gene flow at low abundances, but only some years after the fact.

Options for rebuilding abalone abundance in BC include maintaining fishery prohibitions, aggregation of reproductive adult abalone in the wild to increase density and improve reproductive success, and out-planting of hatchery-raised juvenile abalone to the wild to enhance recruitment. The possibility of disrupting natural population structure in northern abalone by aggregating adults or out-planting juveniles over geographic areas larger than the small aggregations monitored for stock assessment purposes appears unlikely given the low level of microsatellite differentiation observed in this study. However, studies on other marine molluscan organisms have provided indications that both adaptive genetic and non-genetic inducible phenotypic changes may be typical responses to different environments (Kim et al. 2003, Trussell & Smith 2000). Rearing abalone in "common-garden" conditions to assess differences in fitness-related traits may be required to determine at what geographic scale, if any, adaptive differences occur, but it seems likely that transplanting northern abalone will be limited more by disease transfer than by genetic concerns.

Two other concerns associated with the out-planting of hatchery produced organisms are the random loss of genetic diversity due to a limited number of spawners and, if the broodstock is maintained in the hatchery over generations, the development of a strain that is not well adapted to survival and reproduction in the wild. Hatchery strains that are intended for reseeding into natural populations should be carefully monitored to ensure that high levels of genetic variation are maintained, and should be open populations that incorporate naturally produced individuals on a regular basis. Genetic monitoring may also contribute to evaluation of the success of enhancement efforts (Burton & Tegner 2000). This study has indicated that, in a genetic sense, northern abalone in BC are poised for recovery under favorable environmental circumstances. Whether or not active intervention in abalone reproduction is undertaken, prudent management activities would include the identification, protection, and monitoring of spawning aggregates (and recruits) on a regional basis to examine both demographic and genetic parameters for signs of population recovery or decline.

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STATUS OF STEWARDSHIP PROJECTS

ABALONE STEWARDSHIP IN HAIDA GWAII: FORGING A LONG-TERM COMMITMENT. Russ Jones and **Bart De-Freitas**, Haida Fisheries Program, PO Box 98, Skidegate, Haida Gwaii, BC Canada V0T 1S0; **Norm Sloan**, Gwaii Haanas National Park Reserve / Haida Heritage Site, PO Box 37, Queen Charlotte City, Haida Gwaii, BC Canada V0T 1S0; **Lynn Lee**, World Wildlife Fund, PO Box 74, Tlell, Haida Gwaii, BC Canada V0T 1Y0; **Kimiko von Boetticher**, Haida Gwaii Marine Resource Group Association, P.O. Box 680, Massett, Haida Gwaii, BC Canada V0T 1M0; and **Greg Martin**, Laskeek Bay Conservation Society, PO Box 867; Queen Charlotte City, Haida Gwaii, BC Canada V0T 1S0.

Local stewardship is a possible solution to the vexing problem of rebuilding over fished northern abalone (*Haliotis kamtschatkana*) stocks. Northern abalone fisheries in British Columbia were closed coastwide in 1990 but stocks have failed to rebuild and the species became federally listed as "threatened" in 1999. We describe 3 years of community-based stewardship effort in Haida Gwaii to rebuild abalone and prospects for recovery over the long-term. Steps taken include forging a community partnership through regular meetings of a core group and development of a Community Action Plan. The Action Plan's goal is to rebuild abalone populations sufficiently to support both Haida traditional and recreational food fisheries. Specific initiatives include public education, curricula development, establishment of two large abalone stewardship areas and a research area, creation of an Abalone Watch (coastal surveillance) program, and research diving to test rebuilding approaches and monitor recovery. The community response has been positive, but it is too soon to confirm whether there have been changes in human attitude and increases in abalone populations. The challenge is to maintain community interest and commitment over the long-term to allow results to be manifested. Much will depend on setting achievable stock rebuilding reference points both for the stewardship areas and Haida Gwaii. As well, the prospect of the return of the sea otter (also listed as a "threatened" species) that is a keystone species in the kelp forest ecosystem and a predator of northern abalone could result in reduced abalone abundance despite stewardship efforts.

THE KITASOO ABALONE STEWARDSHIP PROJECT: SMALL PROJECT, BIG HOPES. Joel Harding, Kitasoo Fisheries Program, 955 Comox Rd., Nanaimo, BC V9R 3J7 Canada.

The Kitasoo Fisheries Program (KFP), operating out of the remote coastal First Nations community at Klemtu, has been working since 1995 to gain an understanding of northern abalone (*Haliotis kamtschatkana*) population demographics within the Kitasoo/Xaixais traditional territory. The traditional territory is large with a one small village of 350 people. The objective of the KFP is to develop capacity within the Kitasoo/Xaixais Nation, to foster active community participation in the conservation and management of the fisheries resources in the region. The program covers many species, including salmon, herring, manila clams, urchin, prawn, sea cucumber *Porphyra*, and abalone. Over exploitation and depletion of northern abalone stocks has brought this species to the forefront of the program. A significant portion of the Kitasoo/Xaixais traditional territory has been surveyed for remnant abalone populations and information shared with Fisheries and Oceans Canada has helped to document the post-closure distribution of abalone. Inventory surveys were initiated in 1995 at sites throughout the area. A study site was established in south Nowish Inlet during 1999. Since then, data on abalone growth from tagging, habitat requirements, predator, and competitor relationships to abalone abundance, have been recorded.

In 2001, the KFP and the Habitat Stewardship Program initiated the Kitasoo Abalone Stewardship Project, with the purpose of expanding the scope and capacity of abalone rebuilding efforts while joining with local stewardship initiatives, such as education and monitoring campaigns. The main objective of this program is to rehabilitate local abalone populations to self-sustaining levels within the Kitasoo/Xaixais traditional territory. The level of community support and participation will determine the success of the program. Community workshops and follow-up meetings, since 2002, have raised awareness and encouraged local participation in project initiatives. Ongoing outreach efforts include project updates on the community radio channel, distribution of material to visitors and tourists, and youth education activities. The project has promoted participation by supporting those able to combine local food fishing activities with voluntary monitoring. The KFP used local knowledge and past survey information to establish two new stewardship areas to provide sites for evaluation of wild stock manipulation as a means to increase abalone densities and reproductive success. In addition, artificial cement habitats (condos) are being evaluated as an index tool to monitor juvenile recruitment and abundance. To date, juvenile abalone have been found in the condos, but whether the abalone density data from the condos are representative of wild resident juvenile densities or are useful as an index tool to monitor changes in juvenile abundance over time is still unclear.

The negative effect of illegal harvesting on recovery efforts is likely substantial. Increased local monitoring and decentralization of enforcement power, from Fisheries and Oceans Canada to community-based programs, would benefit the abalone resource, stewardship programs, enforcement agencies, and communities involved. The KFP is a strong proponent of information exchange on this project and is eager to develop working partnerships with other abalone stewardship groups. The project is taking an ecosystem-based approach to abalone recovery where all work undertaken is within the constraints of the natural environment.

ABSOLUTELY ABALONE: HABITAT STEWARDSHIP PROGRAM FOR THE PINTO ABALONE ON THE WEST COAST OF VANCOUVER ISLAND. Anne Stewart, Bamfield Huu-ay-aht Community Abalone Project, Bamfield Marine Sciences Center, Bamfield, British Columbia, Canada, V0R 1B0

Pinto or northern abalone (*Haliotis kamtschatkana*) were harvested traditionally at low tide, for millennia on the west coast of Canada. After intense diving harvests and an inability of management strategies to control harvests, the pinto abalone was designated as a threatened species in 1999. The Bamfield Huu ay aht Community Abalone Project (BHCAP) was formed in response to a request for proposals, to work on abalone recovery on the west coast of Vancouver Island. The two key elements of the project strategy are to engage the community in abalone recovery and to operate a successful abalone hatchery for out-planting abalone. The objective of the project is the promotion of Pinto abalone recovery through conservation, education, and community engagement. Collaborations have been established with Fisheries and Oceans Canada, Canadian universities, the Nuu chah nulth Tribal Council and the Pacific Rim National Park Reserve.

Members of BHCAP are the Huu ay aht First Nations, Bamfield Community School Association and the Bamfield Marine Sciences Center. The Huu ay aht First Nations have a goal of restoring abalone to the point where they can harvest for food and ceremonial use. The Community School Association is involved in building capacity. The Bamfield Marine Sciences Center, a non-profit society with five western Canadian universities as members, has a mandate for research and education in marine sciences. They provide a base of operation for both the abalone education and research programs and the co-ordination of the dive program for abalone surveys, collections, and out-planting.

Public education is a major component of the program and 3500 students per year, including students from school, college and universities, adult programs, and the Community School learn about abalone conservation biology. Raising the profile of the stewardship project at public events also inspires concern for abalone habitat and the kelp forest ecosystem for thousands of people. The Ocean Link website (www.oceanlink@island.net) provides a wealth of information on abalone and this project and had over 8 million visits during 2001.

To reduce abalone poaching, the Huu ay aht First Nations crews patrol traditional territories and Coast Watch members keep a look out for poaching. Fishers, boaters, crews, lodge operators, and dive operators are also part of the Abalone Coast Watch.

Future plans include out-planting projects in conjunction with Fisheries and Oceans Canada, sourcing funding options and continuation of education, outreach and community engagement to strengthen community involvement. This last aspect is especially important to reduce illegal harvesting. This project is a fine example of First Nations and non-First Nations groups working together. With hard work and co-operation, the future of a healthy and sustainable abalone community and ecosystem is possible.

STATUS OF ENFORCEMENT

HAVE WE GOT PROBLEMS. Bryan Jubinville, Conservation Protection Branch, Fisheries and Oceans Canada, Labieux St., Nanaimo, BC V9R 5K6 Canada

Do we have problems? Yes we do—on 2 fronts: (1) the continued illegal harvesting of northern abalone (*Haliotis kamtschatkana*) and (2) the reduction of reports on illegal activity. Essentially the same core group of fisheries officers has been enforcing the abalone fishery closure and has advocated protection of northern abalone in British Columbia (BC) since the closure started in 1990. We have received considerable support from other jurisdictions both within and outside the province, from stakeholders, the public, and federal Science and Fish Management branches. In the early 1990s, the conservation and protection of abalone started slowly, with the development of public and informant contacts, the creation of an awareness campaign using multi-lingual posters, media contacts, court cases, impact statements, convictions, imaginative sentencing efforts by lawyers and judges, and video clips. Protection has progressed which includes the ability to identify the species DNA footprints, the development of abalone stewardship groups, and the fostering of contacts with the public. Public support in enforcement is critical. When the public observes, records and reports poaching activity to enforcement officers, all reports are examined and the information provided assists in developing a file and an investigation. Although some of the information received may prove to be of little value, some can be of significant value resulting in a conviction.

Six years ago, fisheries officers would receive numerous calls each year, which they would investigate. A file would be created and, if possible, poachers prosecuted with success. I do not know of a file that we have failed on when we have had the accused in possession of northern abalone. However, recently important information being provided has diminished. Is it because the amount and the quality of effort by the fishery officers in the field have increased resulting in an exceptional job of enforcing the closure in BC? Are the illegal harvesters now more reluctant to poach abalone because of the increased deterrence created? I would like to think that these are the answers, but I am realistic enough to know there is a bigger picture in terms of the global problem of poaching abalone. Recently, South Africa reported that the annual seizure of illegal abalone had exceeded the legal harvest. I have to conclude that our northern abalone is under similar pressure. So where are the general public reports of illegal activity? I believe that we need another method of getting the message out to the public to increase the information being sent to us. The Haida Gwaii stewardship emphasizes that "every tip counts" and the guardians will be documenting and sending reports. This should foster credibility for Fisheries and Oceans Canada and the guardians in the communities. Perhaps in addition to the telephone, word of mouth, and other

means of communicating, such as an Internet tip line, would be helpful. Do we have a problem? Yes, we do and one aspect of the problem is the recent reduced information provided on illegal abalone harvesting activity. We need the assistance of the public and from communities throughout coastal BC in observing and reporting poaching incidents.

AQUACULTURE

RECENT PROGRESS IN HATCHERY PRODUCTION OF PINTO ABALONE, *HALIOTIS KAMTSCHATKANA*, IN BRITISH COLUMBIA, CANADA. **Christopher M. Pearce,** Fisheries and Oceans Canada, Pacific Biologic Station, 3190 Hammond Bay Road, Nanaimo, BC V9T 6N7, Canada; **Pelle Ågerup,** Malcolm Island Shellfish Co-operative, 430 First Street, Box 229, Sointula, BC V0N 3E0, Canada; **Abayomi Alabi,** Probiotic Solutions, 7143 Blackjack Drive, Lantzville, BC V0R 2H0, Canada; **Dawn Renfrew,** Bamfield Huu-ay-aht Community Abalone Project, Bamfield Marine Sciences Center, Bamfield, BC V0R 1B0, Canada; **John Rosser,** Malcolm Island Shellfish Co-operative, 430 First Street, Box 229, Sointula, BC V0N 3E0, Canada; **Guy Whyte,** Bamfield Huu-ay-aht Community Abalone Project, Bamfield Marine Sciences Center, Bamfield, BC V0R 1B0, Canada; and **Fu Yuan,** Island Scallops Ltd., 5552 West Island Highway, Qualicum Beach, BC V9K 2C8, Canada.

In July 1999, Fisheries and Oceans Canada issued a Request for Proposals for 18-mo pilot projects that would develop land-based hatchery rearing techniques for the pinto abalone, *Haliotis kamtschatkana.* A percentage of the cultured juveniles produced were to be utilized for wild stock rebuilding. Six projects were initially approved and five proceeded with the collection of wild broodstock for the purpose of developing hatchery techniques. Of these projects, three were successful at rearing substantial numbers of juveniles (ie, Bamfield Huu-ay-aht Community Abalone Project, Island Scallops Ltd., and Malcolm Island Shellfish Cooperative). Their techniques for broodstock conditioning, spawning, larval rearing, larval settlement, and early juvenile grow out are summarized in this review paper. Adult broodstock were conditioned with wild kelp (*Laminaria saccharina, Macrocystis integrifolia, Nereocystis luetkeana*) and spawned using hydrogen peroxide, temperature shock, and/or UV-treated seawater. Larvae were reared in flow-through or static systems at 11°C to 15 C at a density of 1 to 9 larvae ml^{-1} and settled on wavy or flat plastic sheets covered with natural biofilms of various ages. Early juveniles fed on benthic diatoms and were later converted to kelp and/or prepared diets. Grow out time to commercial size is predicted to be 4 to 6 years. To date, these three projects have produced approximately 170,000 juvenile abalone of various sizes.

FIELD RESEARCH

NIGHT AND DAY SURVEYS OF A NORTHERN ABALONE, *HALIOTIS KAMTSCHATKANA*, POPULATION IN EAGLE BAY, BRITISH COLUMBIA. **James P. Mortimor,** Caitlin R. Henderson, Bamfield Marine Sciences Center, Bamfield B.C. V0R1B0 Canada; and **Glen R. D. Elliott,** Bamfield Huu ay aht Community Abalone Project, Bamfield B.C. V0R1B0 Canada.

This study, initiated by the Bamfield Huu ay aht Community Abalone Project, attempted to establish characteristics of behavior and site selection, for possible out-planting of northern abalone (*Haliotis kamtschatkana*). Diurnal and nocturnal surveys were undertaken to determine population estimates of emergent juvenile and adult abalone at one small area in Barkley Sound. While using conventional methodology the survey conducted was intensive and small scale in nature, contrasting with previous studies that established abalone population estimates over broader areas. No clear community association was identified, however, behavioral and physical constraints were established. Recommendations for increasing out-planting effectiveness include out-planting juvenile abalone at night, between 4 and 6 m below chart datum, on structurally complex substrates.

TRENDS IN PINTO ABALONE (*HALIOTIS KAMTSCHATKANA*) ABUNDANCE IN THE SAN JUAN ISLANDS AND MANAGEMENT OF ABALONE IN WASHINGTON STATE. **D. P. Rothaus,** Washington Department of Fish and Wildlife, Marine Resources, 16018 Mill Creek Blvd, Mill Creek, WA 98012-1296 USA; and **C. S. Friedman,** School of Aquatic and Fishery Sciences, University of Washington, Box 355020 Seattle, WA 98195 USA.

Northern abalone are contagiously distributed in shallow, rocky, exposed, and kelp covered habitats from Sitka Alaska to Monterey California. In Washington State, abalone are found in the San Juan Islands, Strait of Juan de Fuca, and northern coastal waters. They are a slow growing species, reproductively mature at 25 to 50 mm shell length (SL) depending on location. In 1984, the sport harvest was estimated at 38,200 abalone annually and by 1991 this had increased to 40,934. Before 1992, regulations allowed a sport fishery for abalone of 90 mm SL or greater with a harvest limit of 5 abalone per day, and an abalone iron (for removal of abalone from rocks) was required. From 1992 to 1994, the allowable harvest was 3 abalone per day, minimum size 102 mm SL, and an abalone iron and calipers were required. A total closure was instituted in August of 1994. Stocks have declined in both British Columbia and Washington State, leading to the listing of northern abalone as a "Threatened Species" in Canada and as a "Species of Concern" in the USA. Common concerns and potential

trans-boundary issues suggest co-operative restoration efforts be-tween BC and Washington State may be valuable.

Surveys conducted throughout the San Juan Islands, 1979 to 1982, with timed 15-min dives, have provided baseline informa-tion on abalone abundance and size for this area. Twenty-three of these sites were again surveyed between 1990 and 1991. Abalone density at 1 site increased, at 4 sites stayed the same, at 9 sites decreased, and at 9 sites no abalone were found. The overall den-sity decrease was approximately 50%. Locating the original 23 sites was problematic and may have been a factor in the dramatic decrease observed. Even with the potential problems with this comparison, the magnitude of the apparent decline, combined with the anecdotal information from sport divers and University of Washington researchers, raised serious concerns about the health of abalone stocks in Washington. Further surveys were required to adequately evaluate the apparent trend in abalone abundance. As with other areas of the world, illegal harvesting is considered to have a major impact on the abalone stocks in Washington State.

In 1992, 10 permanent abalone index dive stations were estab-lished around the San Juan Islands. The sites ranged in size from 50 m^2 to 380 m^2, averaging 220 m^2 with depths between 0 to 30 ft MLLW. Abundance and size of emergent abalone were deter-mined over the whole site (census) with dives of 180 to 340 min bottom time. A declining trend in total abundance for all sites was observed 1992 to 1994 ($n = 351$ to $n = 288$), with no statistical difference. Following the fishery closure in August of 1994, the 1996 survey results were $n = 297$. Additionally, no significant difference in the mean shell length over time was observed. The average density of half the sites surveyed in 1996 was less than 0.15 abalone/m^2. Research indicates that sedentary invertebrates, such as abalone, must be within 1.0 to 2.0 m of one another (0.33–0.15 abalone/m^2) for successful fertilization. Therefore, low population levels can lead to inability for gametes to cross-fertilize resulting in recruitment failure. In Washington State, data shows that half of the index stations have abalone densities below the level for successful recruitment.

Recovery efforts include a captive broodstock project initiated in 2002 for the development of hatchery techniques using 80 aba-lone collected from Lopez Island. Sixteen percent mortality in the broodstock has occurred over the subsequent 5 months of captiv-ity.

Anecdotal information and quantitative survey data suggest a decline in abalone populations in Washington State. Data from index stations show a gradual decrease in abundance at 6 of 10 sites but no overall significant change in abundance from 1992 to 1996. Some of the current index stations report abalone densities below the minimum density levels that are needed for successful recruitment.

Additional stock assessment studies will include the re-evaluation of the 10 index sites in February 2003, more frequent (yearly) assessment of abalone abundance at the 10 index sites, development of a better survey method so that population esti-mates can be obtained, creation of additional index sites in the Strait of Juan de Fuca, and initiation of juvenile abundance sur-veys. Genetics studies, in collaboration with Canadian scientists, will include analysis of relatedness between sites and between individuals within a site. The captive broodstock project will con-tinue to culture abalone using techniques to maximize genetic diversity and to compare behavior of hatchery reared animals in normal versus "natural" tanks.

Should surveys show continued instability in abalone popula-tions, management plans would be developed for abalone stock restoration, potentially including out-planting of hatchery raised juveniles and aggregation of adults. Public information meetings and scientific workshops will be held in co-operation with the Puget Sound Restoration Fund to raise public awareness.

REHABILITATION METHODS

OVERVIEW OF ABALONE STOCK ENHANCEMENT IN NEW ZEALAND AND LESSONS FROM LABORATORY STUDIES OF ABALONE LARVAL SETTLEMENT AND POST-LARVAL FEEDING. R. Roberts, Cawthron Institute, Private Bag 2, Nelson, New Zealand. E-mail:rodney@cawthron.org.nz; and N. Andrew, NIWA. PO Box 14-901, Kilbirnie, Wellington, New Zealand, E-mail:andrew@niwa.cri.nz

In New Zealand, abalone catch from commercial, recreational, traditional, and illegal harvest is approximately 1700 t per year. Fishing effort is controlled by catch limits, minimum size (125 mm shell length, SL), and method restrictions. Since 1999, the com-mercial fishery has been reduced in several main fishing areas through quota cuts and voluntary reduction in catch entitlement. Investigations have begun that may provide alternatives to further quota cuts including temporary closures, larger minimum harvest size, and release of hatchery reared juveniles or larvae. Catch reporting has been modified to provide data at high spatial reso-lution, as population dynamics in abalone can vary over small spatial scales and recruitment may be localized. A project under-way is to determine the reason(s) for the large number of stunted, sub-legal size animals, (<125 mm SL) in certain areas. Abalone less than 110 mm SL in these areas were removed and ongoing surveys will determine if this improves the growth rate of the remaining animals and if the transplanted animals reach minimum legal size.

The commercial fishery is dominated by *Haliotis iris*, a large abalone up to 180 mm SL, which is also the only species currently farmed in New Zealand. A minor fishery exists for *Haliotis aus-tralis*, a small (<110 mm SL) abalone. *Haliotis virginea* is a small (<70 mm SL) cryptic species that is not landed in the commercial harvest.

The most substantial study of abalone reseeding in New

Zealand produced very promising results at some of 8 sites, with the best site showing 54% survival of 10,000 of 7 to 12 mm SL seed, 2 years after release. Apparent survival was higher at 2 y than at 1 y, illustrating the difficulty in obtaining accurate survival estimates for cryptic life-stages.

In New Zealand reseeding studies, a large proportion of juvenile mortality often occurred soon after release. Burial by sand was a major cause of mortality in three of four studies. Lower survival and naive behavior from hatchery seed compared with wild juveniles was observed in each of 3 studies. Predation was considered in only 1 study and found to be minor. Naive behavior of hatchery abalone may be reduced if the hatchery encouraged appropriate abalone behavior, (eg, by providing shelters to maintain cryptic behavior) using strong light cycles to encourage feeding at night, and exposing abalone to predators periodically to maintain defense responses.

Two small trials of larval release have been carried out in New Zealand. In the first trial, 300,000 larvae were released in a 50-m2 gully. Minimum survival was 0.4% after 3 months and the calculated cost of each surviving animal was US$0.14, indicating this method could be economically viable. In a second trial, mesh-tented seafloor areas of 1 m^2 were seeded with 20,000 larvae. Only 10% of larvae settled and minimum survival after 5 months was 0.06% resulting in the cost of each surviving abalone of US $0.80, indicating this method would be uneconomic.

Laboratory studies on larval settlement and post-larval feeding have provided insights into larval reseeding and natural recruitment. Abalone larvae are capable of attaching and crawling prior to metamorphosis. *Haliotis iris* will attach from 4 days of age and metamorphose at 7 to 8 days at 17 °C. Abalone can delay metamorphosis for 2 to 3 weeks at 17°C to 20°C. In cold water, both the pre-competent period, and the ability to delay metamorphosis would be extended. Hence, potential larval dispersal may be wider than previously assumed for abalone.

Larvae become increasingly responsive to metamorphosis cues as they age, so older larvae are more likely to metamorphose close to the point of release. Crustose coralline algae are the most effective settlement-inducer for most abalone species, but larvae often resume swimming after landing on corallines, particularly less preferred species. Resumption of swimming could lead to transport out of the study area with consequences for survival estimates.

Abalone of less than 5 mm SL consume the biofilm on coralline algae. Abalone less than 0.8 mm SL will scoop up loose diatoms, bacteria, and coralline secretions, competing with many generalist grazers. Abalone of 0.8 to 5.0 mm SL develop radula teeth specialized for gouging, increasing their grazing capability and reducing competition. In animals more than 5 mm SL, the radula is further specialized and the diet expands to coralline crusts, macroalgae, sea grasses, and drift particles, further reducing competition. The modest carrying capacity of corallines for young abalone should be taken into account when deciding release densities in

larval reseeding. Visible signs of starvation have been described from laboratory studies and reported in post-larvae from natural habitat.

Areas with good recruitment are not necessarily recruitment saturated. However, reseeding may not be successful if there is strongly density-dependant mortality at some stage of life. Little is known about the prevalence or intensity of density-dependant mortality in abalone—whether it can be strong enough to negate reseeding returns, or how it may vary spatially, temporally, or between species. Sites that previously had good recruitment and a strong fishery but currently suffer from recruitment failure should be ideal for reseeding. Though the results of New Zealand seeding studies are encouraging, more research is needed, especially to determine the factors controlling survival after release.

A REVIEW OF ABALONE ENHANCEMENT AND REHABILITATION IN SOUTH AFRICA.

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The South African coastline is approximately 3,000 km long with very few bays, inlets or sheltered areas. The exposed coastline limits opportunities for mariculture. However, the commercial harvest of abalone is threatened by a high level of illegal poaching and this situation has provided support for a successful farming industry for *Haliotis midae*. Abalone farming has expanded rapidly in South Africa and, by 2004, annual production is expected to exceed 600 tonnes per year. Most farms have hatcheries and this leads to excess production of juveniles that could be used for enhancement or ranching. Wild populations, consisting of six species of abalone, occur on the southwest, south and southeast coasts of South Africa. Although the west coast is a highly productive area with extensive kelp beds and high wave action, abalone occur naturally only in the southernmost sections.

Experiments to determine the feasibility of abalone enhancement and ranching in South Africa were carried out at Port Nolloth, on the northwest coast. This site was chosen due to the presence of abalone fossils, the presence of high densities of urchins—both indicating appropriate environmental conditions for abalone—and the availability of security from a diamond mine in the vicinity. The site was over 300 km from any natural abalone population, assuring that any animals in the area were from the ranching experiments.

Anticipated problems with the sea ranching included release mechanisms that could cause mortalities, predation after release, monitoring success (% survival), and assessing economic viability. To reduce the mortality caused by handing during the transportation and release of abalone, special release mechanisms were devised. These devises consisted of PVC pipes halved lengthwise and glued to a Perspex sheet. Both ends of the PVC covered with mesh after the abalone entered the devices naturally. The devices

were transferred intact without handling the individual animals. The devices were attached to concrete blocks at the experimental site in the late afternoon. Twenty-four hours later, the mesh was removed and the abalone were allowed to exit at will. This procedure also provided protection from predators for the first 24 h while the abalone became acclimatized to the environment.

At four experimental sites, 500 or 800 abalone of approximately 14 mm shell length were released per site. Growth and survival were monitored over 24 months and there was significantly slower growth through the summer compared with the winter months suggesting that the timing of the release is important to both survival and growth. Survival is reported in Table 1.

The maximum mortality occurred during the first twenty-four hours after release and many remained in the transport devices for extended periods. Even at good sites there was little dispersal from the release area. Abalone appeared to seek out urchins for protection and were often found under the urchin spines. However, the presence of urchins did not appear to ensure a high survival rate since at one site no surviving abalone were found in March 1997. Factors affecting survival are complex, variable, and inter-related, displaying a variable hierarchy of importance.

For ranching to be economically viable a survival rate of 5% to 10% for out-planted juveniles is required. Preliminary results suggested that, in certain circumstances, survival rates in excess of 30% could be obtained with seeded animals with particularly good survival being obtained when precautions are taken to reduce handling stress and at sites where sea urchins were present. Later work at similar sites, however, produced contrasting results and it was concluded that an extremely complex interplay between many different factors affected survival.

Of these, the presence of sea urchins was only important at certain sites, whilst, at other sites, the present of optimum-sized boulders seemed to replace that requirement. Overall, the size of seeded animals was the most important factor that influences survival, larger seed having better survival rates. Following the demonstration that, under certain circumstances, ranching could be economically feasible, genetic implications of this operation were investigated. Using mt-DNA markers, it was shown that, not only could animals from different geographic regions be shown to be genetically differentiated but, in addition, distinct genetic differ-

ences between naturally occurring and hatchery reared animals was also apparent.

REBUILDING CALIFORNIA ABALONE POPULATIONS.

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Since 1997, all seven California abalone species have been closed to commercial and recreational fishing south of San Francisco Bay. California abalone occur throughout the coastal marine environment from the intertidal zone to deep offshore reefs, presenting a challenge for the development of an abalone recovery plan. A successful plan needs to address the various characteristics of abalone and include an approach that can be applied as broadly as possible using available resources and a method for evaluation of progress.

Assessment of remaining abalone stocks is of immediate importance. Assessment goals have been established to address and prevent extinction of the abalone, to restore resource sustainability, and to rebuild resources to fishery sustainability levels. Assessment must include the identification of remnant populations, establishment of locations for further evaluation, and determination of locations for enhancement.

The range of abalone is often specific, extensive, and with patterns of distribution. For each species, information on landings from fishing effort is accumulated into catch block areas which are used to determine the most extensive and the best habitat for abalone and used to determine research index sites. Population density is an essential element of the assessment; however, when numbers are low standard density surveys are uninformative. Free-form searches can yield more individuals than transect constrained surveys and data on individuals, such as size, can be collected.

Shell length (SL) is a population indicator, where the occurrence of a broad size range, even at low numbers, is evidence of reproduction and growth. Size surveys are conducted using 3 size categories, 0 to 100 mm SL, 100 mm SL to minimum legal size (MLS), and MLS to maximum size. Since the 0 to 100 mm SL category includes the cryptic population, which is difficult and destructive to assess, it is only used for occasional determination of settlement. When a broad range of sizes is present, quantitative surveys are used to determine emergent abalone densities. A minimum viable population target size is an emergent population of 2,000 abalone per hectare, at all index locations. When an average of 6,600 abalone per hectare at three out of four of the index locations is reached, a fishery could be considered.

Survey criteria and modifications should be made according to species characteristics. For example, surveying intertidal populations with a GPS can provide the position of each abalone. White abalone, currently surveyed using free form dives, could be sur-

TABLE 1.

Survival of released abalone.

Simple Sites	Released	April 1996	September 1996	March 1997	Survival to 6 Months
Site A	500	97	40	0	27.4%
Site B	500	117	70	19	39.2%
Site C	800	–	124	99	27.8%
Site D	800	–	145	60	25.6%
					Average 30%

veyed using an ROV or submarine to provide geographic position. Multi-beam sonar sea floor maps are being generated by Department of Fish and Game and, together with data from a ROV, could be used to construct benthic habitat maps.

Abalone populations are at extremely low levels throughout southern California. Some remaining populations are so dispersed that successful natural reproduction is unlikely and enhancement may be the only remaining method of intervention. Enhancement techniques include aggregation of abalone, translocation of individuals from remote source populations, and out-planting of cultured juveniles and competent larvae. At the present, aggregation is seen as the only viable method of enhancement.

In abalone recovery, there are several challenges that must be addressed as part of the recovery process. Some challenges must be considered before the recovery process can proceed. The presence of sea otters precludes a fishery of abalone and most other marine invertebrates. In the abalone recovery plan, any areas either currently or previously occupied by sea otters are excluded from assessment.

Disease has severely affected abalone stocks. The black abalone was virtually extirpated from southern California by withering syndrome. Warmer seawater temperatures enhance withering syndrome, which is a concern for translocation projects. A parasitic sabellid worm, which causes shell growth disruption and deformity, was introduced into California aquaculture facilities and currently a prohibition exists on the out-planting of abalone from non-certified facilities.

Genetic questions need to be addressed, prior to translocation of abalone from different bio-geographic zones and using cultured animals for enhancement of natural populations.

Poaching of abalone is a serious problem in California. Recovery site criteria should include a low likelihood of illegal activity. Part of recovery is establishing large, dense populations and groups of individuals to facilitate reproduction. It is precisely those conditions that are good for poaching. Optimal locations would include remote islands, and mainland locations within limited access reserves.

Marine Protected Areas, MPAs, offer one of the best opportunities for abalone restoration activities. Recently, California established a number of MPAs at the Channel Islands National Marine Sanctuary and most include ongoing abalone study sites and appropriate abalone habitat.

Each abalone species has specific environmental requirements, which must be addressed to optimize successful recovery. Southern California is at the northern end of the range of pink, green, and white abalone, and the southern end of red abalone range. Red abalone growth and reproduction is depressed during warm water periods, but they can survive until temperatures decline. Pink and green abalone prefer warmer water which may allow their populations to extend farther northward with increasing sea water temperatures. Environmental effects complicate other factors. If seawater temperatures increase farther north, withering syndrome may become infectious in northern populations.

Our major challenge in rebuilding abalone stocks is to return at least part of the abalone population to a natural situation, where bio-diversity and natural selection can be effective.

SIZE AT MATURITY OF FEMALE AMERICAN LOBSTERS FROM AN ESTUARINE AND COASTAL POPULATION

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ABSTRACT   The size at which female lobsters reach sexual maturity was determined for two populations that inhabit waters along the coast of New Hampshire. One group was captured in the Great Bay estuary, where water temperatures in the summer typically average between 17°C and 20°C. The other group of lobsters resided in coastal waters, near the Isles of Shoals, where the water temperature was much colder during the summer (11–15°C). Maturity was assessed using criteria that included the following: ovarian classification; abdominal width/carapace length (CL) ratio; and the size frequency distribution of berried females. All the techniques yielded similar results and consistently demonstrated that female lobsters in the estuary matured at a smaller size than those in colder coastal waters. The smallest mature females from Great Bay were 72 mm in CL, with 50% reaching sexual maturity by 83 mm CL and all becoming mature by 89 mm CL. The smallest mature female from the Isles of Shoals area was 77 mm CL, with 50% mature by 86 mm CL and all mature by 93 mm CL. The difference in the proportion of mature lobsters in the estuarine versus coastal populations was much greater in the smaller size classes than in the larger size classes, suggesting a mixing of the two populations, most likely due to females from Great Bay migrating into coastal waters.

KEY WORDS: estuary, *Homarus americanus*, lobster, sexual maturity

INTRODUCTION

The American lobster, *Homarus americanus* (Milne-Edwards) is the most commercially valuable species harvested in the northwest Atlantic Ocean (NMFS 2002). Although lobsters are most abundant in coastal waters, estuarine populations are common and have been investigated from Canada to Massachusetts (Thomas 1968, Thomas & White 1969, Munro & Therriault 1983, Reynolds & Casterlin 1985, Jury et al. 1995; Howell et al. 1999; Watson et al. 1999). One population that has received considerable attention is located in the Great Bay estuary in New Hampshire. Howell et al. (1999) have demonstrated that, like the lobsters in the Iles-de-l-Madeleine in Canada (Munro & Therriault 1983), the sex ratio is skewed toward males throughout the estuary, with the greatest proportion of male lobsters found in the portions of the estuary furthest from the coast. It has been proposed that the skewed sex ratio in the estuary is the result of the differential seasonal migration of mature female lobsters out of the estuary (Watson et al. 1999).

To ensure that there are enough mature females in a given lobster population, a minimum legal size has been established. This allows a given proportion of the females to reach sexual maturity and reproduce at least once before they are landed. The size at which 50% of the females from an area are mature (50% maturity) is often used as a reference point because most models indicate that when the minimum size is set at this value sufficient recruits will be produced to sustain the fishery. Currently, the minimum size limit in the inshore waters of New Hampshire is 83 mm carapace length (CL).

There is a wide range of sizes over which female lobsters reach maturity. The smallest size at 50% maturity, 70 to 74 mm CL, is found in western Long Island Sound (Briggs & Mushacke 1979), and the largest size, 110 to 120 mm CL, is found in the Bay of Fundy (Templeman 1936, Groom 1977, Campbell 1983). It has been suggested that a number of different factors influence the size

at which female lobsters mature, including nutrient availability (Lawton & Lavalli 1995), fishing pressure (Polovina 1989, Landers et al. 2001), and temperature (Templeman 1936, Templeman 1944, Aiken & Waddy 1980, 1986, Estrella & McKiernan 1989, Fogarty 1995). Increases in all, or any, of these factors results in a decrease in the size at which females reach sexual maturity.

Temperature is thought to be the most influential of these factors because it is known to directly affect the growth rates of lobsters, with development occurring more quickly with increased temperature (Aiken & Waddy 1976). The rate of ovarian development is primarily controlled by summer water temperature, with little development occurring throughout the winter months (Templeman 1936). Thus, in areas with warmer water in the summer, lobsters reach sexual maturity at smaller sizes.

Estuaries, such as the Great Bay estuary in New Hampshire, are characterized by large daily and seasonal fluctuations in temperature and salinity. In the Great Bay estuary, the water temperature in the summer is approximately 10°C higher than in New Hampshire coastal waters (Short 1992). Given the apparent influence of water temperature on the rate of maturation of female lobsters, we hypothesized that female lobsters in the Great Bay estuary would reach sexual maturity at a smaller size than those in coastal waters, such as near the Isles of Shoals, which are located 11 km away from where the Great Bay estuary empties into the Gulf of Maine (Fig. 1).

To test our hypothesis, we determined the size at maturity for 92 lobsters collected in the Great Bay estuary with 106 lobsters collected near the Isles of Shoals. A comparison of the results yielded by analyzing (1) the size distribution of berried females, (2) the size of female abdomens relative to their length, and (3) the stage of eggs removed from the ovaries yielded the same pattern. Female lobsters from the estuarine site matured at a smaller size than those from the coastal site, probably due to the influence of warmer summer water temperatures on their growth and development.

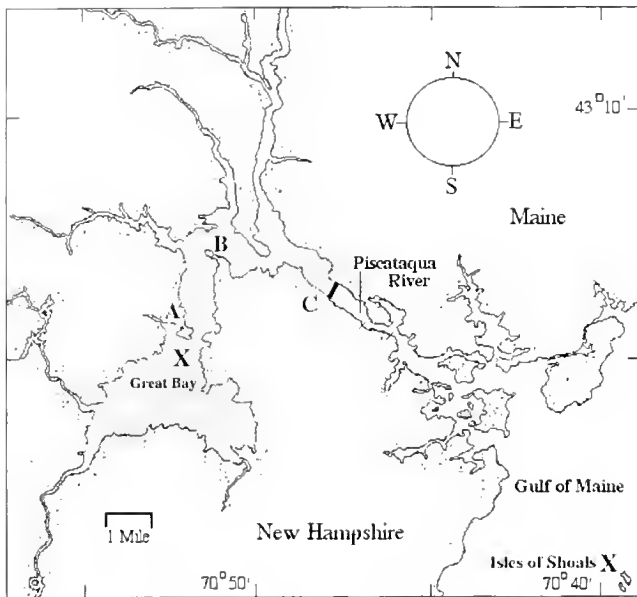
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Figure 1. The two study sites are marked with an X [Great Bay Estuary and Isle of Shoals (11 km off the coast of New Hampshire)]. Sites of temperature data collection for the Great Bay Estuary are: A, Jackson Estuarine Laboratory; B, Fox Point; and C, Upper Piscataqua River. Lobsters were obtained from the Great Bay estuary within the area indicated by shading.

MATERIALS AND METHODS

Temperature

Bottom temperatures were collected in the waters surrounding the Isles of Shoals from 1997 to 2001 at depths of approximately 8 to 10 m using HOBOTemp temperature data loggers (Onset Computer, Falmouth, MA) that recorded water temperature at 2-h intervals for 5 to 6 mo at a time. Bottom temperature data for Great Bay was collected from 1997 to 2001 at three different locations that spanned the area where lobsters were collected (Fig. 1). The most consistent data set were obtained from a location near the University of New Hampshire Jackson Estuarine Laboratory, at a depth of approximately 3 to 5 m, using a YSI multiparameter 6600 datalogger (YSI Inc., Marion, MA) that recorded the water temperature every 30 min. Water temperature also was recorded near Fox Point and along the Piscataqua River in 1990 and 1993, using a YSI meter model 33 attached to a probe that was lowered to a point near the bottom. Data were obtained from these two sites approximately every other day while hauling some of the traps used to collect lobsters for this study. Data from all three sites were averaged from all available years to yield a temperature profile of the area from which lobsters were collected. The mean monthly temperature then was calculated, and the total annual degree-days >8°C were summed for each location by adding together the number of degrees that exceeded 8°C for each day of the year and summing them for the entire year.

Maturity Assessments

Dissections

Lobsters were collected from two areas (Fig. 1) by commercial fishermen and by University of New Hampshire personnel using standard traps. The first site consisted of the upper region of the

Great Bay estuary (i.e., Great Bay, Little Bay, and the upper Piscataqua River), and the second site included waters near the Isles of Shoals.

Lobsters were collected in 1991, 1992, 1994, and 2002. The lobsters from each site were divided into 1-mm size classes ranging from 66 to 110 mm CL. A total of 92 lobsters were dissected from Great Bay, and a total of 106 from Isles of Shoals.

Female, nonovigerous, lobsters were examined, using multiple criteria, to determine whether they were sexually mature. For each animal, the CL and the width of the second abdominal segment were measured in millimeters, and the molt stage was recorded by examining the carapace and pleopods. One pair of pleopods then was removed for examination under a dissecting microscope to determine the cement gland stage (Aiken & Waddy 1982) and whether lobsters were in a premolt condition (Aiken 1973). A small circular incision then was made just behind the eye socket to access the anterior end of one of the ovaries. Several eggs were removed, and their size range and color were recorded. An egg stage was assigned to each lobster based on criteria established by Aiken and Waddy (1980).

Whether a female was sexually mature, or not, was determined using a combination of criteria, with ovarian stage as the primary tool. Any females with resorbed oocytes were considered to be mature, as these are an indication of prior spawning. Of the females without resorbed oocytes, those with ovaries that were at stage 4 and higher were also considered to be mature. The size range for stage 4 ovaries is different in the spring (stage 4b) than in the fall (stage 4a) due to the timing of development, and this was taken into account. Those females with ovaries at stage 2 and below were considered to be immature. To determine the maturity of those with stage 3 ovaries, we considered cement gland stage as well as egg stage. If a female lobster with stage 3 ovaries had cement glands that were at stage 3 or greater, then the lobster was considered to be mature.

To determine the size at which 50% of the females from each area were mature, a nonlinear regression of percent mature for each 1-mm CL size class was carried out using the statistical program, SYSTAT. The following equation was used:

$$p = (1/(1 + \exp(-b0*(L-b1))))$$

where p is the proportion mature, b0 is the curve shape parameter, L is the carapace length, and b1 is the size at 50% maturity (estimated as a starting point for calculations by the user). The program estimated values of b0, based on the data set, until it found the best-fit curve. This resulted in sigmoid curve from which b1 could be calculated with a 95% confidence interval. A statistical comparison of the regression lines that resulted from each population of lobsters was made to determine whether they were significantly different from each other.

Sea Sampling Data

Sea-sampling data were obtained from University of New Hampshire research traps, and during trips on commercial lobster boats in 1990 to 1993 and 2002 at each location. The data collected included CL, width of the second abdominal segment, sex, and whether females were ovigerous. A total of 8199 lobsters were examined during these sea-sampling trips.

Abdominal Width

A ratio of abdomen width to CL (ABD/CL ratio) was calculated for each female, and these were averaged for each 1-mm CL

size class. A plot then was made of CL versus this ratio for each size class. A nonlinear polynomial regression of these data was created for each site using SYSTAT. The following equation was used: ABD/CL = a + bx + cx^2 + dx^3, where x = CL. SYSTAT then estimated the values of a, b, c, and d to most closely fit the curve to the data. To determine the inflection point of the curve, which represents the point at which the rate of change in the ABD/CL ratio is greatest, and therefore approximates the size at which 50% of the females have reached maturity, the second derivative of the original equation, y = 2cx + 6dx, was calculated. That equation was then set to equal zero and was solved for x, yielding the equation x = −2c/6d. Then, the c and d values from SYSTAT were used to solve for x (the CL at 50% maturity) (Landers et al. 2001). The size at 50% maturity that was estimated by this method was compared with that obtained by dissection for the estuarine and coastal lobster populations to determine whether the abdominal width estimates fell within the 95% confidence intervals of the dissection estimates.

Berried Female Size Frequency Distributions

From the sea-sampling data, a size frequency distribution of berried females, as well as a plot of the overall size frequency distribution of the population was made for each area. The plots of overall size frequency were divided into the proportions that were male and female in each size class so that the proportion that was female at a given size class could be compared with the proportion of females that were berried at that same size class. For each plot the average size, the SEM, size range, and sex ratio were calculated for comparison. The size distributions for the overall population and for only berried females were compared between sites using a χ^2 test of independence.

RESULTS

A Comparison of Estuarine Versus Coastal Water Degree-Days

There was a large difference between the number of annual degree-days (>8°C) in the Great Bay estuary (1532) compared to those in the waters near the Isles of Shoals (738) (Fig. 2). The greatest difference in temperature occurred during the summer
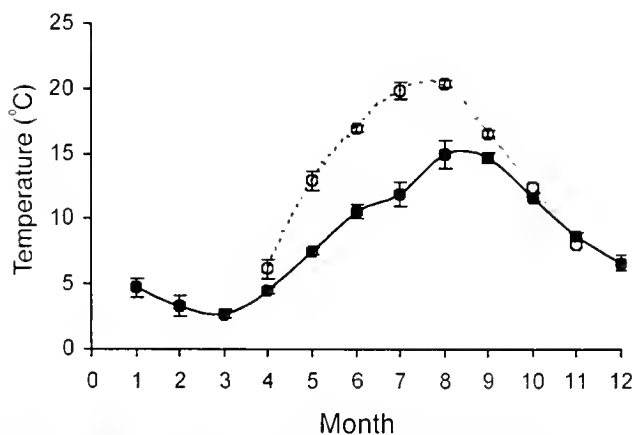
months (June–August; Great Bay 995; Isles of Shoals 404). The difference in degree-days between the two sites for these 3 mo accounted for 75% of the difference in degree-days for the entire year. During this period, the mean water temperature averaged 12.5°C at Isles of Shoals and 19°C in Great Bay.

Maturity Assessments

Dissections

Nonlinear regressions of CL versus percent mature, as determined by dissections, were used to calculate the size at 50% maturity for each site (Fig. 3a). The size at 50% maturity for females obtained from waters near the Isles of Shoals was 85.9 mm CL (95% confidence interval 85.3–86.5; n = 106). Fifty percent of females from Great Bay were mature at 83 mm CL (95% confidence interval 80.6–85.4 mm; n = 92). A comparison of the two regressions showed that they were significantly different from each other (P < 0.001). The smallest mature female captured near
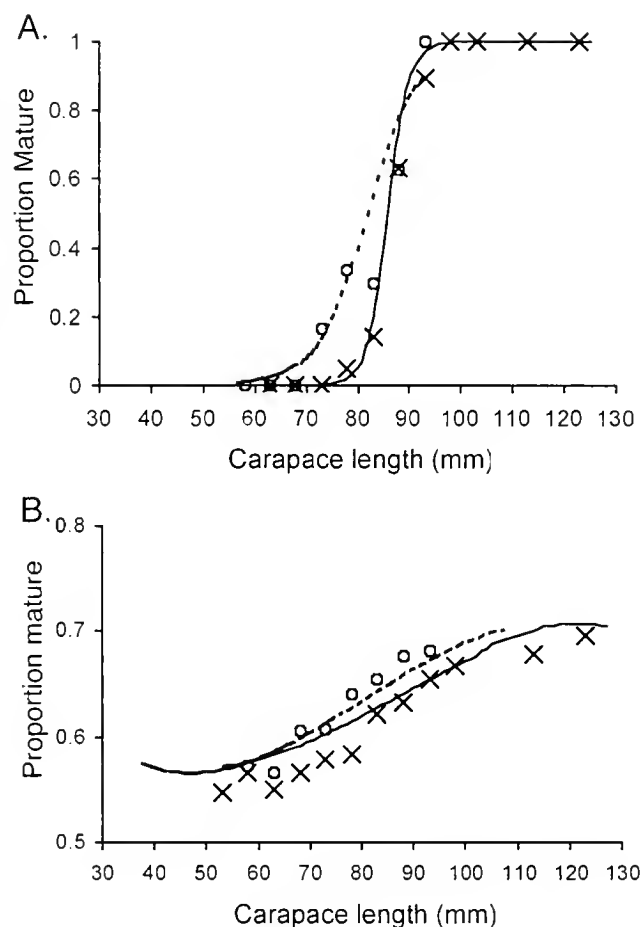
Figure 3. (A) Maturity ogives estimated by nonlinear regressions based on dissection data from 1-mm size classes from Great Bay (dashed line) and Isles of Shoals (solid line): Great Bay 50% maturity = 83 mm CL (95% confidence interval 80.6–85.4; n = 92); Isles of Shoals 50% maturity = 85.9 mm CL (95% confidence interval 85.3–86.5; n = 106). Actual values are plotted for each 5-mm size class. (B) Polynomial regression estimated from abdominal width measurements for 1-mm size classes from Great Bay (dashed line) and Isles of Shoals (solid line): Great Bay 50% maturity = 81.5 mm CL (n = 1613); Isles of Shoals 50% maturity = 86.9 (n = 1699). Actual values are plotted for each 5-mm size class.

Figure 2. Mean monthly bottom temperatures (°C), with SE bars, for water in the Great Bay estuary (open circle) and near the Isles of Shoals (solid circles) (1997–2001). Water temperature for Great Bay is an average of three sites that encompass the area from which lobsters were collected.

the Isles of Shoals was 80 mm CL, while in the estuary a 72-mm CL mature female was captured. All females were mature by 93 mm CL at the Isles of Shoals study site, and by 89 mm CL in the Great Bay estuary.

Abdominal width: CL ratios

Nonlinear regressions of ABD/CL ratios were fitted to the data to calculate size at 50% maturity (Fig. 3b). The resulting curves indicated that half the females from Isles of Shoals were mature by 86.9 mm ($n = 1699$), while the size at 50% mature for lobsters captured in the estuary was 81.5 mm ($n = 1613$). The estimate for the Isles of Shoals lobsters did not fall within the 95% confidence interval generated from the dissection data (85.3–86.5), but was very close. The estimate for the Great Bay estuary lobsters fell within the 95% confidence interval (80.6–85.4).

Size frequency distributions

The size range of berried females collected near the Isles of Shoals was 77 to 138 mm CL, with an average (±SEM) size of 92 ± 1.0 mm CL ($n = 152$; Fig. 4b). The size range of berried females from the Great Bay estuary was 72 to 107 mm CL, with an average size of 85 ± 0.6 mm CL ($n = 98$; Fig. 4a). These means were significantly different from each other ($P < 0.001$ two-tailed t test). Only a small portion (30%) of berried females from near the Isles of Shoals were smaller than 85 mm CL, whereas 50% of the berried females from the estuary were <85 mm CL. In contrast, very few berried females (1%) from the Great Bay estuary were >100 mm CL, while 20% of berried females from waters near the Isles of Shoals were >100 mm CL. Nevertheless, despite these differences, the distribution of sizes of berried females was not significant between the two sites ($P = 0.067$).

Figure 4. Size frequency histograms of berried females from (A) Great Bay and (B) Isles of Shoals ($P = 0.067$ χ^2 test of independence).

The size range of the overall lobster population at the Isles of Shoals site was 48 to 144 mm with a mean size of 81 ± 0.1 mm CL ($n = 3337$; Fig. 5b), while the size range of the population from the Great Bay site was 38 to 113 mm CL, with an average size of 78 ± 0.1 mm CL ($n = 4862$; Fig. 5a). The size frequency distribution of all lobsters was significantly different between the two sites ($P < 0.05$). The Great Bay population includes more small lobsters <65 mm CL (6%) than the Isles of Shoals population (3%), and the Isles of Shoals site has more legal lobsters >83 mm CL (27%) than the Great Bay estuary (18%), particularly those >100 mm CL (2% at Isles of Shoals, <1% at Great Bay). The most striking difference between these sites is the sex ratio, as reported by Howell and Watson (1999). The overall proportion of females at the Isles of Shoals site (64%) was much larger than that in the Great Bay estuary population (35%), and this was increasingly true at larger sizes. The percentage of females in the Great Bay estuary fluctuated between 30% and 40% but dropped to <30% at sizes >82 mm CL, and no females >96 mm CL were captured in the Great Bay estuary. In contrast, the proportion of females near the Isles of Shoals increased with size class, so that 80% of the lobsters >96 mm CL were female.

DISCUSSION

All three methods used to assess the size at maturity of female American lobsters (i.e., egg stage, ABD/CL ratios, and berried female size frequency distributions) indicate that female lobsters from the Isles of Shoals mature at a larger size (50% = 85.9 mm CL) than those from the Great Bay estuary (50% = 83 mm CL), even though the two populations are <14 km apart. One of the major differences between these two locations is water temperature. The Great Bay estuary (1532 annual degree-days) is significantly warmer than the Isles of Shoals study site (738 degree-days), with the greatest difference in temperature (74% of the total difference in degree-days) occurring in the summer months. We conclude that this increased temperature accelerates the rate of development of females in the Great Bay estuary, thereby causing them to reach sexual maturity at a smaller size. This finding once again supports the theory first put forth by Templeman (1936) that summer water temperatures determine size at maturity. The small difference in size at maturity reported is similar to a larger scale pattern observed along the entire range of the American lobster. For example, 50% of female lobsters from Long Island Sound reach maturity at 70 to 74 mm CL (Briggs & Mushacke 1979), while those from the Bay of Fundy do not reach maturity until 110 to 120 mm CL (Templeman 1936, Groom 1977, Campbell 1983).

While the size at 50% maturity for female lobsters from Great Bay is significantly different ($P < 0.001$) than that of females from Isles of Shoals, it is clear from the maturity ogives (Fig. 3) that the greatest difference in the two populations exists in the smaller size classes. This may be due to the mixing of mature females from Great Bay with those from the coast, as mature females migrate out of the estuary. As reported by Howell et al. (1999), the proportion of females in Great Bay (35%) is much smaller than that near the Isles of Shoals (64%), and this difference is most pronounced in the larger size classes. In fact, the proportion of females in Great Bay begins to decline above the 82-mm CL size class (Fig. 4), which is approximately the size at which lobsters are reaching maturity. As proposed by Watson et al. (1999) and Howell et al. (1999), it would be advantageous for females to move out of the estuary for optimal egg development and survival of larvae. While
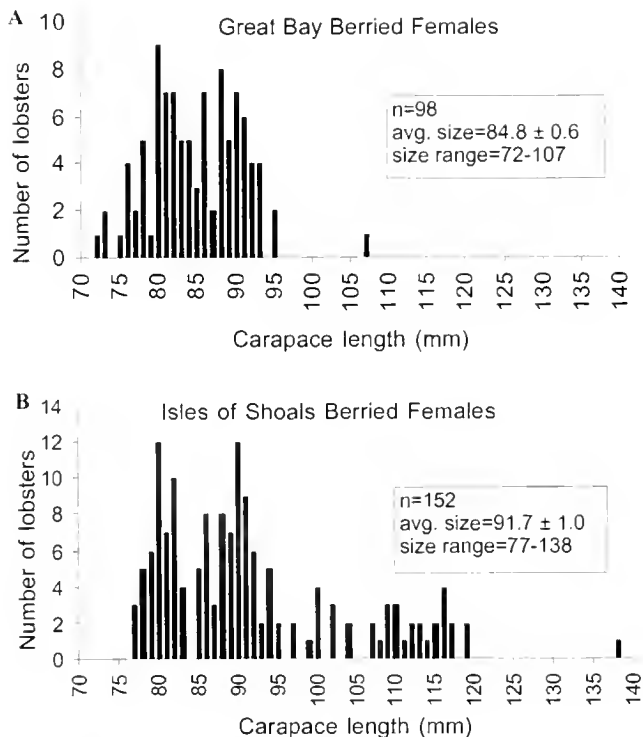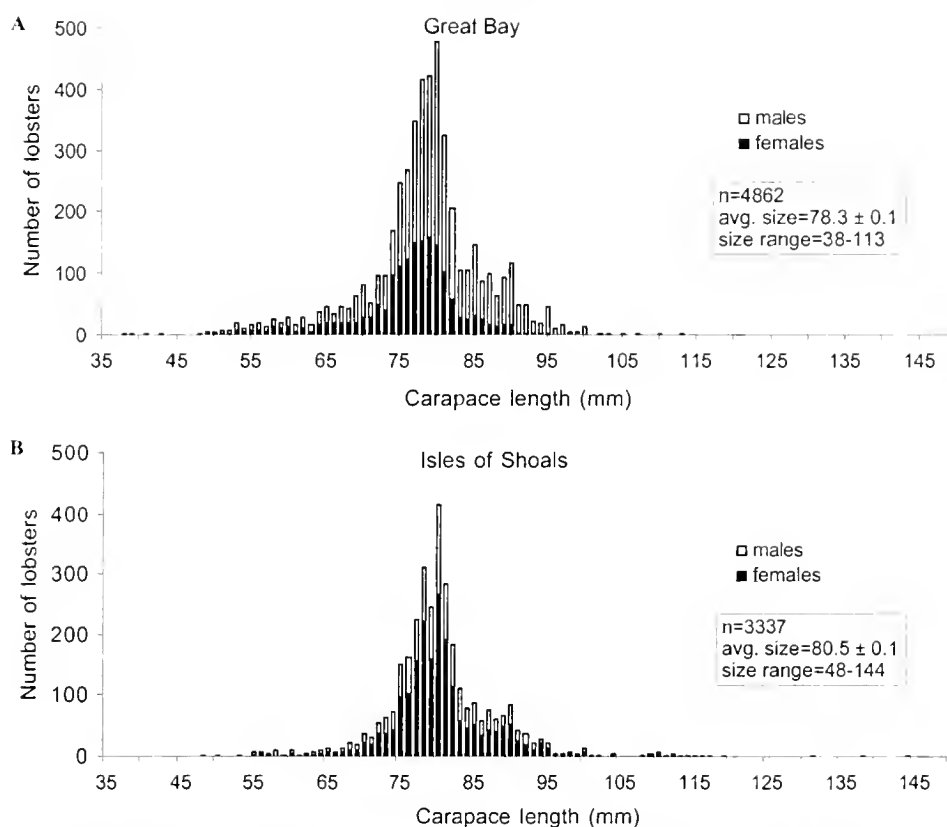
Figure 5. Size frequency histograms of the overall catch from (A) Great Bay and (B) Isles of Shoals, divided into proportions of males and females (*P* < 0.05 χ^2 test of independence).

there is a greater tendency for lobsters to leave the estuary, a number of coastal lobsters also move into the estuary, especially in the summer, presumably to take advantage of the warmer temperatures (Watson et al. 1999). Therefore, while there is a clear difference in the size at maturity of female lobsters from the two populations, the mixing of the coastal and estuarine lobsters due to seasonal migrations may be responsible for making this difference less evident, especially in the larger size classes.

Although warmer summer water temperature appears to be the most likely factor causing lobsters in the estuary to mature at a smaller size than New Hampshire coastal lobsters, another possibility is that berried females from offshore waters migrate inshore to the waters near Isles of Shoals and skew the size frequency of berried females there toward larger sizes. Berried females often migrate inshore to complete their reproductive cycle because the warm temperature inshore speeds their development (Cooper & Uzmann 1971, Uzmann et al. 1977, Cooper & Uzmann 1980, Fogarty et al. 1980, Campbell et al. 1984, Campbell & Stasko 1986). Seasonal concentrations of large berried females in inshore areas off Cape Cod, MA (Estrella & McKiernan 1989), and Long Island, NY (Briggs & Mushacke 1979), are thought to be the result of berried females from offshore migrating shoreward. Berried females from offshore in both of these areas are larger than those inshore, and thus the mixing of offshore berried females with the local inshore populations would distort the apparent size frequencies. This remains a viable explanation for the size at maturity differences that we have observed.

Analyses of both egg stage data and ABD/CL ratios yielded similar results, in terms of size at maturity. Based in egg stages,

50% of females from the waters off the Isles of Shoals were mature at 85.9 mm CL, while, according to ABD/CL ratios, 50% were mature at 86.9 mm CL. In Great Bay, the values were 83 and 81.5 mm CL, respectively. The value based on ABD/CL ratios for the estuarine lobsters fell within the 95% confidence interval generated from egg stage data, and, while the estimate based on ABD/CL ratios from Isles of Shoals lobsters did not fall within the 95% confidence interval (85.3–86.5) generated from dissection data, it was very close. Thus, it seems that ABD/CL ratios provide a reasonably good estimate of size at maturity, as indicated in several previous studies (Skud & Perkins 1969, Krouse 1973, Briggs & Mushacke 1979, 1980, Ennis 1980).

The size ranges of berried females from both sites were very similar to what one would predict from analyses of the egg stages of dissected lobsters. In the population near the Isles of Shoals, the smallest mature female was 80 mm CL, while the smallest berried female captured was 77 mm CL. Likewise, the smallest mature Great Bay female was 72 mm CL, which was the same size as the smallest berried female observed while sea sampling. This suggests that it might be possible to construct a fairly accurate maturity ogive using a combination of two noninvasive methods: the size range of berried females and ABD/CL ratios. Measurements of berried females are useful in defining the size range of mature females in a population and can serve as a good indication of the size at which the smallest females become mature. However, these measurements do not indicate what proportion of the females at a given size are mature, and these data could be derived from measurements of the ABD/CL ratios over a range of relevant size classes.

While the size frequency distributions of berried females from the two sites were not significantly different ($P = 0.067$), there were clearly more large berried females near the Isles of Shoals (20% >100 mm CL at Isles of Shoals vs. 1% >100 mm CL in Great Bay) and more small berried females in Great Bay (50% <85 mm CL in Great Bay vs. 70% >85 mm CL near the Isles of Shoals). Therefore, it is likely that the size frequency distributions of berried females in both study sites were not significantly different due to the low sample size of berried females in the Great Bay estuary ($n = 98$). This assumption is supported, in part, by the fact that the size frequency distributions of the overall populations ($n = 4862$ for the estuary) at the two sites were significantly different ($P < 0.05$). As with the berried female size frequency distributions, the bulk of this difference can be accounted for by the lack of large lobsters in the Great Bay estuary (<1% were >100 mm). As discussed earlier, these data support the hypothesis that as lobsters reach sexual maturity they migrate out of the estuary into deeper water (Watson et al. 1999, Howell et al. 1999). While mature females probably undergo this migration shortly after reaching sexual maturity, giving rise to the skewed sex ratios observed in the estuary in size classes >80 mm CL and the low number of large berried females, male lobsters eventually move into coastal waters as well, as indicated by the scarcity of any lobsters >100 mm CL in the Great Bay estuary.

Our results indicate that while there is a small difference in the size at which females from the two sites reach maturity, that difference is small, suggesting that these are not two distinct populations. There appears to be mixing between the two areas, particularly among the sexually mature lobsters. Thus, despite the small differences in size at maturity, it is probably not necessary to implement different management measures for each area. The size at which half of the females mature from both sites approximates the minimum size limit, and thus it appears to be appropriate to maintain adequate egg production and recruitment to satisfy the F10 requirement.

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GREEN CRAB (*CARCINUS MAENAS* LINNAEUS) CONSUMPTION RATES ON AND PREY PREFERENCES AMONG FOUR BIVALVE PREY SPECIES

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ABSTRACT Laboratory experiments were conducted to determine green crab, *Carcinus maenas*, consumption rates on and prey preferences among four bivalve species: Olympia oysters (*Ostrea conchaphila* Carpenter), Japanese littleneck clams (*Venerupis philippinarum* A. Adams and Reeve), bent-nosed macoma clams (*Macoma nasuta* Conrad), and California softshell clams (*Cryptomya californica* Conrad) of different sizes. The bivalve size classes tested ranged in length from 10–14 mm to 33–37 mm. Consumption rate and prey preference experiments were conducted by allowing one starved (48 h) green crab (55–75 mm carapace width) to feed *ad libitum* on bivalve prey for 16 h. All tests were conducted in 38-L aquaria containing sand substrate 13 cm deep. A total of either 60 or 30 individuals of each prey species were offered without replacement in each test. Mean green crab consumption rates varied depending upon the prey species and size class. For bivalve prey of similar size, Olympia oysters were consumed at a higher rate than bent-nosed macoma clams and Japanese littleneck clams, while Olympia oysters and California softshell clams were consumed at about the same rate. Green crabs preferred Olympia oysters to both bent-nosed macoma clams and Japanese littleneck clams by ratios ranging from 2:1 to 28:1, depending upon the prey size. Small California softshell clams were preferred to small bent-nosed macoma clams by a ratio of 8:1. The mean total biomass of Olympia oysters and bent-nosed macoma clams eaten was 2.31 g · d⁻¹. Our results show that green crabs are capable of consuming large quantities of all four bivalve species tested, and that on bare sand substrate Olympia oysters are at greater risk of green crab predation than bent-nosed macoma clams and Japanese littleneck clams, and California softshell clams are at greater risk than bent-nosed macoma clams.

KEY WORDS: *Carcinus maenas*; consumption rates; *Cryptomya californica*; *Macoma nasuta*; *Ostrea conchaphila*; prey preference; *Venerupis philippinarum*.

INTRODUCTION

The green crab, *Carcinus maenas*, a species native to Europe, has recently invaded Pacific Northwest (PNW) estuaries (Dumbauld & Kauffman 1998, Hunt et al. 1998, Yamada 2001). Green crabs prey heavily upon bivalves (Ropes 1968, Davies et al. 1980, Parache 1980, Dare & Edwards 1981, Elner 1981, Dare et al. 1983, Grosholz & Ruiz 1995, Mascaró & Seed 2000). Ropes (1968) and Elner (1981) attributed the decline in the softshell clam (*Mya arenaria* Linnaeus) fishery along the northeastern coast of the United States to invasive green crabs. Studies by Grosholz and Ruiz (1995, 1996) suggest and Jamieson et al. (1998) have predicted that invasive green crabs could impact bivalve populations in PNW estuaries.

The objectives of this study were to estimate green crab consumption rates on four bivalve prey species inhabiting PNW estuaries and to determine green crab prey preferences among these prey species under controlled laboratory conditions. Consumption rate experiments were conducted on one to three size classes of the four bivalve species to determine the effect of prey species and prey size on consumption rates. Prey preference experiments were conducted with two or three bivalve species of similar size. The bivalve species tested were the Olympia oyster (*Ostrea conchaphila*), the Japanese littleneck clam (*Venerupis philippinarum*), the bent-nosed macoma clam (*Macoma nasuta*), and the California softshell clam (*Cryptomya californica*). Olympia oysters are native to and were once widely distributed throughout PNW estuaries but now, probably primarily because of overharvesting (Baker 1995, Robinson 1997, Cook et al. 2000), only remnant natural and

culture populations remain. Bent-nosed macoma clams and California softshell clams are common native PNW bivalves. The Japanese littleneck clam is a nonindigenous species that has been naturalized and is cultured in PNW estuaries for its commercial value.

MATERIALS AND METHODS

Green crabs used in our experiments were collected from Yaquina Bay, OR (44 °37 ′N, 124 °02 ′W) with crab traps deployed subtidally and baited with salmon scraps. Prior to their use in an experiment, the crabs were fed a standardized diet of squid while being held submerged in individual containers in flow-through water tables in the U.S. Environmental Protection Agency Laboratory at the Hatfield Marine Science Center, Newport, OR. The flow-through water system supplies fresh filtered or unfiltered seawater from Yaquina Bay. Only intermolt crabs were used as experimental subjects to avoid possible behavioral differences associated with molting. The size range of green crabs in our experiments (55- to 75-mm carapace width, CW) reflected the size range of crabs collected in the field.

Olympia oysters and Japanese littleneck clams used in our experiments were obtained from the Olympia Oyster Company, Shelton, WA. Bent-nosed macoma clams and California softshell clams used in our experiments were collected from Yaquina Bay. Experimental bivalves were measured and divided into size classes (Table 1). Shell length was measured as the distance from the hinge (umbo) to the furthest edge of the shell. Bivalves were held in the laboratory in water tables supplied with unfiltered, flow-through seawater prior to their use in our experiments. The bivalves appeared healthy and did not lose weight or die while being held.

TABLE 1.

Bivalve species and prey size classes used in the consumption rate[a] and prey preference[b] experiments

Size (Class)	Olympia Oyster	California Softshell Clam	Bent-Nosed Macoma Clam	Japanese Littleneck Clam
Small (I)	19–23 mm[ab]	10–14 mm[ab]	12–15 mm[ab]	14–18 mm[b]
Medium (II)	26–30 mm[ab]		18–21 mm[ab]	
Large (III)	33–37 mm[ab]			22–26 mm[ab]

Consumption Rate and Prey Preference Experimental Protocol and Data Analysis

Both consumption rate and prey preference laboratory experiments were performed in 38-L (50 cm × 25 cm × 30 cm) glass aquaria placed in flow-through water tables. Sand, that had been air-dried for at least five days and sieved through a 1.0-mm mesh screen, was placed in each aquarium providing 13 cm of substrate depth. Each aquarium was continuously supplied with fresh, filtered Hatfield Marine Science Center seawater with out flow near the top through a mesh cover. At the short (25 cm) end of each aquarium a clear plastic partition was installed about 10 cm into the aquaria to separate the green grab predator from the bivalve prey during a 24-h pre-experimental acclimation period. This setup created staging and feeding areas in each aquarium. Semiopaque visual barriers were placed on the vertical sides of each aquarium to minimize external influences on predator and prey behavior. Seawater temperature (range, 12–16°C) and salinity (range, 32–33 ppt) were monitored during every experiment. The light regimen was fixed using a timer and matched the natural daylight regimen (14L:10D). The consumption rate and prey preference tests were each of 16-h duration, beginning with 10 h of darkness followed by 6 h of light. Prior to each experiment, each experimental crab was starved for a total of 48 h: 24 h in its holding container plus 24 h in the staging area. The bivalve prey were measured and placed in the feeding area of the aquaria and allowed to acclimate to the test conditions at least 18 h before the beginning of each test.

The same basic protocol was used in both the consumption rate and prey preference experiments. One experimental crab was used in each test, and each crab was used only once. After 24 h in the staging area, partitions between the staging and feeding area were removed and crabs were allowed to feed *ad libitum* on one bivalve prey species (consumption experiments) or two or three bivalve prey species (preference experiments) without replacement for 16 h. At the end of each test all bivalves were removed from each aquarium and whole, live bivalves were counted and remeasured. The number of individuals of each species eaten was determined as the number originally available minus the number of whole, live individuals remaining at the end of each test. In the consumption rate tests, the feeding area originally contained 60 bivalves (prey) of the same species and the same size class. In the prey preference tests, the feeding area originally contained either 60 or 30 bivalves of similar size of each of two or three species. The total number of tests performed was constrained by bivalve prey availability. Due to laboratory space limitations or prey availability, a maximum of twelve tests could be run at the same time. Tests were randomly assigned among aquaria, and each experiment was completed within one month. Seven replicated ($n = 4$–8) consumption rate experiments and five replicated ($n = 3$–8) pairwise and one replicated ($n = 4$) three-way prey preference experiments were conducted (Table 1).

Differences in mean consumption rates (number bivalves eaten in 16 h) between two prey species or size classes were tested by t-tests after confirming the parametric assumptions of normality and homogeneity of variances (Sokal & Rohlf 1995). Differences in mean consumption rates among three prey species or three size classes were tested by analysis of variance and Tukey's test, or, when the data failed to meet the parametric assumptions, by an approximate test of the equality of means using the Games and Howell method (Sokal & Rohlf 1995). Prey preference was inferred using single classification G-tests with Williams' correction (Sokal & Rohlf 1995) by determining if the observed proportion of prey species eaten differed from the expected ratio (1:1 and 1:1:1 for two and three prey species, respectively) if there was no preference.

Bivalve Biomass Estimates

Meat weight–length relationship models for Olympia oysters and bent-nosed macoma clams were developed by regressing the logarithms of the biomass (g, flesh dry wt) of 50 individual Olympia oysters (18–38 mm shell length) and 30 individual bent-nosed macoma clams (12–22 mm shell length) on shell length (mm). We did not have a sufficient number of individuals of different shell lengths to generate biomass-length relationships for Japanese littleneck clams and California softshell clams. The flesh of each bivalve was removed from the shell, placed in a pre-weighed drying tin, and dried in an oven for 48 h at 70°C. Upon removal from the oven, the tins were kept in a dessicator, allowed to cool, and re-weighed. Flesh dry weight was determined by subtracting the weight of the drying tin from the total weight (dried flesh + drying tin).

Biomass-length regression models were used to convert the known length of individual Olympia oysters and bent-nosed macoma clams eaten in our consumption rate and prey preference experiments to biomass. The individual biomass estimates were summed to estimate the total bivalve biomass of each species consumed in each test. ANOVA was used to test for differences among the mean total bivalve biomass eaten in our Olympia oyster and bent-nosed macoma clam consumption rate and prey preference experiments.

RESULTS

Consumption Rate Experiments

The number of bivalve prey eaten in our consumption rate tests ranged from zero large Japanese littleneck clams to fifty-four small California softshell clams. Mean (SE) green crab consumption rates and results of analysis of variance comparing mean consumption rates across prey species within a size class and across different size classes within prey species are presented in Table 2. The rank order of green crab mean consumption rates for bivalve

TABLE 2.

Prey species,[a] prey size class,[b] sample size (n), and mean (SE) number of bivalves eaten by one 48-h starved green crab in 16 h and ANOVA results in tests of five hypotheses of no significant differences between/among mean consumption rates for different prey species of similar size [H_0 (1a–1c)] and for different size classes of the same prey species [H_0 (2a and 2b)]

Prey Species	Size Class	n	No. Consumed Mean (SE)	Consumption Rate (per day)	H_0 (1a)	H_0 (1b)	H_0 (1c)	H_0 (2a)	H_0 (2b)
OO	I	4	41.5 (5.24)	62.3	A				A
OO	II	8	17.4 (1.74)	26.1		A			A
OO	III	8	10.3 (0.82)	15.5			A		B
BN	I	8	17.7 (2.61)	26.6	B				A
BN	II	8	7.4 (1.56)	11.1		B			B
CS	I	8	43.4 (2.99)	65.1	A				
JL	III	8	1.8 (0.49)	2.7			B		

Different letters (A, B) in the columns indicate statistically significant different ($P < 0.05$) means.
[a] OO = Olympia oyster; BN = bent-nosed macoma clam; CS = California softshell clam; JL = Japanese littleneck clam.
[b] See Table 1.

species by size class (I, II, III; see Table 1) was Olympia oyster (I) = California softshell clam (I) > bent-nosed macoma clam (I), Olympia oyster (II) > bent-nosed macoma clam (II), and Olympia oyster (III) > Japanese littleneck clam (III). The rank order of green crab mean consumption rates for different size classes of the same bivalve species were Olympia oyster (I) = Olympia oyster (II) > Olympia oyster (III), and bent-nosed macoma clam (I) > bent-nosed macoma clam (II).

Prey Preference Experiments

When two bivalve prey species were present, green crabs ate, on average, 16× more small Olympia oysters than small bent-nosed macoma clams, 2× more small Olympia oysters than small Japanese littleneck clams, 8× more small California softshell clams than small bent-nosed macoma clams, 3× more medium Olympia oysters than medium bent-nosed macoma clams, and 28× more large Olympia oysters than large Japanese littleneck clams (Table 3). When three bivalve prey species were present, green crabs ate, on average, small California softshell clams, small Olympia oysters, and small bent-nosed macoma clams in a 6:4:1 ratio (Table 3). The proportions of the prey species eaten were all significantly different from 1:1 or 1:1:1 (Table 3), indicating strong green crab prey preferences among the bivalve species tested.

Bivalve Biomass Estimates

Regressions of the logarithm of Olympia oyster and bent-nosed macoma clam flesh dry weight on their shell lengths (mm) were: log (Olympia oyster dry wt, g) = −2.40 + 0.048 Olympia oyster shell length, $r^2 = 0.79$, $P < 0.001$, and log (bent-nosed macoma clam dry wt, g) = −2.26 + 0.077 bent-nosed macoma clam shell length, $r^2 = 0.95$, $P < 0.001$.

Using the regression equations above, we converted the shell lengths of the Olympia oysters and bent-nosed macoma clams eaten in our consumption rate and prey preference tests to individual oyster or clam biomass. Individual biomass estimates of consumed prey were then summed to estimate the total biomass of Olympia oysters and bent-nosed macoma clams eaten in each test. There were no significant differences (ANOVA, $P > 0.05$) among the mean total biomass of bivalves eaten in our Olympia oyster and bent-nosed macoma clam consumption rate and prey preference experiments (Table 4). The grand mean total biomass of Olympia

oysters and bent-nosed macoma clams eaten in these experiments was 1.54 (±0.10) g · 16 h^{-1}, which extrapolates to 2.31 g · d^{-1}.

DISCUSSION

Consumption Rates

This is the first published report of green crab consumption rates on Olympia oysters, bent-nosed macoma clams, and California softshell clams. Parache (1980) previously reported green crab consumption rates on Japanese littleneck clams. For a given bivalve prey size, green crab (55–75 mm CW) consumption rates were highest for California softshell clams and Olympia oysters, intermediate for bent-nosed macoma clams, and lowest for Japanese littleneck clams (Table 2), smaller individuals of each prey species were consumed at a faster rate than larger individuals (Table 2), and the mean total biomass of Olympia oysters and bent-nosed macoma clams consumed was 2.31 g · d^{-1} (Table 4).

Crab consumption rates on bivalves can vary depending on the crab (species, size, hunger level, and health), the bivalve (species,

TABLE 3.

Prey preference ratios of green crabs for two or three bivalve prey species[a] of comparable size and results of G-tests comparing the observed versus the expected ratios if there was no prey preference

Size Class[b]	Observed Preference Ratio	n	Expected Ratio If No Preference	G Statistic	P
I	16 OO:1 BN	3	1:1	94.5	<0.001
I	2 OO:1 JL	5[c]	1:1	29.7	<0.001
I	8 CS:1 BN	6	1:1	118	<0.001
II	3 OO:1 BN	4	1:1	34.9	<0.001
III	28 OO:1 JL	8	1:1	82.4	<0.001
I	6 CS:4 OO:1 BN	4	1:1:1	93.2	<0.001

All tests were replicated (n) and 16-h duration.
[a] OO = Olympia oyster; BN = bent-nosed macoma clam; CS = California softshell clam; JL = Japanese littleneck clam.
[b] See Table 1.
[c] Thirty individuals of each bivalve prey species were originally available in each replicate test. In all other experiments, 60 individuals of each bivalve prey species were originally available in each replicate test.

TABLE 4.

Mean total bivalve biomass (g) consumed by one 48-h starved green crab in 16 h in consumption rate and prey preference experiments with Olympia oysters (OO) and bent-nose macoma clams (BN)

Prey Species	Size Class[a]	Experiment[b]	n	Biomass Consumed (g) Mean (SE)
OO	I	C	4	1.70 (0.183)
OO	II	C	8	1.54 (0.350)
OO	III	C	8	2.01 (0.235)
BN	I	C	8	1.10 (0.116)
BN	II	C	8	1.35 (0.759)
OO + BN	I	P	3	1.55 (0.124)
OO + BN	II	P	4	1.63 (1.067)

[a] See Table 1.

[b] C = Consumption; P = Preference.

size, density, shell strength, and morphology), and the experimental conditions (water temperature, duration, with or without prey refuge, with or without prey replacement, etc) under which the rates are measured (Jubb et al. 1983, Arnold 1984, Sanchez-Salazar et al. 1987, Juanes 1992, Ebersole & Kennedy 1995, Mascaró & Seed 2000, Yamada 2001). Our experiments were conducted in aquaria with sand substrate to approximate prime oyster and clam culture habitat in the field. Further research is needed to estimate green crab consumption rates and prey preferences on bivalves in other PNW estuarine habitats (e.g., salt marsh, eelgrass, burrowing shrimp). We attempted to minimize potential confounding variables in our experiments, and to obtain near maximum estimates of average green crab consumption rates under environmental conditions as similar as possible to those in the field. We only experimentally varied the bivalve prey species and size (Table 1). The predator crab species, size (55–75 mm CW), number (one), and initial hunger level (48 h starved) were constant, and environmental conditions (water temperature, salinity, photoperiod, etc.) were held constant at levels matching local field conditions. The bivalve prey were placed on sand substrate (13 cm deep) and given time (18 h) to acclimate and orient themselves naturally on and in the sediment. Thus relative differences in predator foraging times for the different prey species are subsumed in our consumption rates. Sixty bivalve prey were available at the beginning of each consumption rate test, and, on average, twenty whole, live bivalve prey remained at the end. Mean bivalve prey densities, therefore, decreased but remained high (446–131 m^{-2}) throughout the tests, thus minimizing the effect of decreasing prey density on crab consumption rates. Bivalves eaten during the experiments were not replaced as newly introduced bivalves would tend to be more vulnerable to predation than the original bivalves that had time to acclimate and bury. Since starved green crabs consume prey more rapidly in the first three feeding hours (Jubb et al. 1983), our tests were run for 16 h to better reflect longer term, average rates. Our experimental light regimen (10D:6L) approximates the green crab's natural foraging cycle (Klein Breteler 1976, Elner 1981). In Table 5 we summarize the experimental conditions and results of this and other green crab consumption rate studies.

In Parache's (1980) laboratory experiments, green crabs (50–69 mm CW) consumed 0.2–0.7 Japanese littleneck clams (23.5 mm) · d^{-1}, whereas in our experiments green crabs (55–75 mm CW) consumed Japanese littleneck clams (22–26 mm) at an aver-

age rate of 2.1 clams · d^{-1}. We used one crab in each test as compared with Parache's three, and our prey densities were higher (Table 5). Aggressive competition is high among green crabs, especially in the presence of food (Kaiser et al. 1990; Sneddon et al. 1997), and green crab consumption rates decrease with decreasing prey density (Walne & Dean 1972). Our estimates of green crab consumption rates on Japanese littleneck clams, therefore, better reflect rates when green crab densities are low and clam densities are high, whereas Parache's (1980) estimates may reflect rates when green crab densities are higher and clam densities are somewhat lower.

Green crabs consumed 19–37 mm Olympia oysters in our experiments at an average rate of 15–62 d^{-1} (Tables 2 and 5). This consumption rate is much higher than the ≤ 2.75 d^{-1} reported by Dare et al. (1983) for 19–37 mm Pacific oysters (*Crassostrea gigas* Thunberg) and the 1.1 d^{-1} reported by Mascaró and Seed (2000) for 5–40 mm edible oysters (*Ostrea edulis* Linnaeus) (Table 5). Differences in the experimental conditions (Table 5) preclude direct comparisons of these results. Nevertheless, such large differences in consumption rates suggests that green crabs can eat Olympia oysters at a faster rate than other oysters, perhaps due to differences in shell strength or morphology (Mascaró & Seed 2000).

Green crabs consumed bent-nosed macoma clams and Japanese littleneck clams at a slower rate than similar sized Olympia oysters and California softshell clams (Table 2). Bent-nosed macoma clams and Japanese littleneck clams in our experiments buried into the sediment, some along the sides of the aquaria where they were observed at the maximum sediment depth of 13 cm. California softshell clams buried just below the surface, and Olympia oysters remained on the surface. Slower green crab consumption of deeper burying bivalve species supports the premise that burying provides greater refuge from predation. Blue crab (*Callinectes sapidus* Rathbun) consumption rates were also less on deeper-burying bivalves (Blundon & Kennedy 1982, Ebersole & Kennedy 1995).

Green crab consumption rates on bent-nosed macoma clams were less than those on similar size Olympia oysters on a numerical basis (Table 2), but not significantly different on a total biomass basis (Table 4). These results suggest that crab consumption rates, measured as number of prey eaten per hour, may have been largely a function of the crab's hunger level. Initial hunger levels of our experimental crabs were the same (48-h starved). But as crabs ate prey, their hunger levels must have decreased, and, logically, the rate of decrease would be more closely related to biomass of prey than number of prey consumed. Our length-biomass regressions (see Results, *Bivalve Biomass Estimates*) show that bent-nosed macoma clams have a greater flesh biomass than Olympia oysters of the same length. The hunger level of a starved green crab feeding on bent-nosed macoma clams, therefore, would decrease at a faster rate than if the same crab fed on the same number of similar size Olympia oysters. The total biomass of Olympia oysters and bent-nosed macoma clams eaten in our experiments (Table 4) exceeded the approx. 0.8 g of dry blue mussel (*Mytilus edulis* Linnaeus) flesh required to satiate green crabs (70–75 cm CW) (Jubb et al. 1983). It, therefore, appears that the crabs in our consumption rate experiments ate to satiation, but that more individual Olympia oysters than bent-nosed macoma clams of the same size had to be eaten to reach satiation and to maintain approximately the same hunger level thereafter.

Green crab consumption rates on larger Olympia oysters were less than those on smaller Olympia oysters, and green crab consumption rates on larger bent-nosed macoma clams were less than

TABLE 5.

Green crab consumption rate studies on bivalve prey with rates standardized to mean number consumed in 24 h

Citation	Crab Size (mm)	Prey Species[a]	Prey Size (mm)	Consumption Rate (24 h^{-1})	Tank Size (cm)	Number Prey Offered	Prey Replaced	Time (d)	Sediment Depth (cm)
Palacios & Ferraro	55–75	Oc	19–23	62.3	50 × 25	60	No	0.7	13
(This study)	55–75	Oc	26–30	26.1	50 × 25	60	No	0.7	13
	55–75	Oc	33–37	15.5	50 × 25	60	No	0.7	13
	55–75	Mn	12–15	26.6	50 × 25	60	No	0.7	13
	55–75	Mn	18–21	11.1	50 × 25	60	No	0.7	13
	55–75	Cc	10–14	65.1	50 × 25	60	No	0.7	13
	55–75	Vp	22–26	2.7	50 × 25	60	No	0.7	13
Walne & Dean (1972)	60–69	Mm	14–20	3.22	27 × 18	15	Yes	7	0
	60–69	Me	24–32	4.83	27 × 18	15	Yes	7	0
Elner & Hughes (1978)	60–65	Me	5–35	13	43 × 23	90	Yes	11	0
Parache (1980)[b]	50–59	Vp	8	1.71	50 × 50	50	No	8	8–10
	50–59	Vp	14	2.78	50 × 50	50	No	8	8–10
	50–59	Vp	23.5	0.17	50 × 50	30	No	8	8–10
	60–69	Vp	8	2.88	50 × 50	50	No	8	8–10
	60–69	Vp	14	5.88	50 × 50	50	No	8	8–10
	60–69	Vp	23.5	0.71	50 × 50	30	No	8	8–10
Dare et al. (1983)	65	Cg	19–23	2.75	28 × 18	2–10	Yes	4–10	0
	65	Cg	26–30	1.75	28 × 18	2–10	Yes	4–10	0
	65	Cg	34–37	1.00	28 × 18	2–10	Yes	4–10	0
Jensen & Jensen (1985)	6	Ce	2–6	7.00	7 × 7	30	No	1	3
Sanchez-Salazar et al. (1987)	65–70	Ce	13	3 @ 9°C 9 @ 15 C	50 × 30	40	Yes	5–10	5
Mascaró and Seed	55–70	Me	5–40	12.0	30 × 20	35	Yes	~10	0
(2000)	55–70	Oe	5–40	1.1	30 × 20	35	Yes	~10	0
	55–70	Cg	5–40	2.1	30 × 20	35	Yes	~10	0
	55–70	Ce	5–40	10.1	30 × 20	35	Yes	~10	0

[a] Oc = *Ostrea conchaphila*; Mn = *Macoma nasuta*; Cc = *Cryptomya califonica*; Vp = *Venerupis* (previously, *Ruditapes*) *philippinarum*); Mm = *Mercenaria mercenaria* (Linnaeus); Me = *Mytilus edulis*; Cg = *Crassostrea gigas*; Ce = *Cerastoderma edule*; Oe = *Ostra edulis*.
[b] Parache's experiments had three crab predators per tank. All other reported experiments had one crab per tank.

those on smaller bent-nosed macoma clams (Table 2). There was no significant difference, however, in the mean total biomass of larger and smaller prey consumed (Table 4). Dare et al. (1983), Jubb et al. (1983), and Sanchez-Salazar et al. (1987) also found prey species specific, inverse relationships between green crab consumption rates and bivalve prey size within the range of consumable prey sizes. Such relationships probably hold generally because more time, on average, is needed to handle and eat larger bivalve prey (Jubb et al. 1983; Sanchez-Salazar et al. 1987), while crabs need to eat fewer individuals to reach satiation when consuming larger prey.

Green Crab Prey Preferences

Green crabs exhibited prey species preferences based on the proportional number of similar size individuals eaten in our experiments. In tests with two prey species, Olympia oysters were preferred to bent-nosed macoma clams and Japanese littleneck clams of similar size, and small California softshell clams were preferred to small bent-nosed macoma clams (Table 3). In a three-way test, small California softshell clams, Olympia oysters, and bent-nosed macoma clams were preferred in a ratio of 6:4:1 (Table 3). These results indicate that, on bare sand substrate, Olympia oysters are more susceptible to green crab predation than bent-nosed macoma clams and Japanese littleneck clams, and California softshell clams are more susceptible to green crab predation than bent-nosed macoma clams.

Factors that influence crab prey preferences include the prey encounter rate, the time and energy the crabs expend to handle and eat the prey, and the nutrient and energetic value of the prey (Elner & Hughes 1978; Ebersole & Kennedy 1994). Our study was not designed to determine the relative importance of these factors. However, because green crabs are tactile and chemosensory hunters (Cohen et al. 1995), and their prey preference ratios (Table 3) were almost always consistent with the bivalve prey burial depths observed in our experiments (Olympia oysters < California softshell clams < bent-nosed macoma clams = Japanese littleneck clams), prey encounter rates were probably an important factor.

A summary of the results and experimental conditions under which our study and other studies on green crab prey preferences on bivalves is presented in Table 6. Jensen and Jensen (1985) found that juvenile green crabs preferred small cockles (*Cerastoderma edule* Linnaeus) to small Baltic macoma clams (*Macoma balthica* Linnaeus), and they concluded that juvenile green crabs could be responsible for the decline of small cockles and changes in benthic macrofaunal diversity in the Wadden Sea. Cohen et al. (1995) found that green crabs preferred brackish-water corbula clams (*Potamocorbula amurensis* Schrenck) to Japanese littleneck clams and mussels (*Mytilus* spp.) of similar size. Cohen et al. (1995) speculated that green crab predation might lead to a decrease in brackish-water corbula clams and an increase in benthic diversity in San Francisco Bay. Grosholz and Ruiz (1995) showed that green crabs preferred larger individuals of two *Nutricola* (previously, *Transennella*) clam species, and they predicted that green

TABLE 6.

Green crab prey preference studies with bivalve prey and results presented as ratios

Citation	Crab Size (mm)	Prey Species[a]	Prey Size (mm)	Preference Ratio	Tank Size (cm or L)	Number Prey Offered[b]	Time (days)	Sediment Depth (cm)
Palacios & Ferraro	55–75	Oc:Mn	12–23	16:1	50 × 25	120	0.7	13
(This study)	55–75	Oc:Mn	18–30	3:1	50 × 25	120	0.7	13
	55–75	Oc:Vp	14–23	2:1	50 × 25	60	0.7	13
	55–75	Oc:Vp	22–37	28:1	50 × 25	120	0.7	13
	55–75	Cc:Mn	10–15	8:1	50 × 25	120	0.7	13
	55–75	Cc:Oc:Mn	10–23	6:4:1	50 × 25	180	0.7	13
Jensen & Jensen (1985)	11	Ce:Mb	2–6	7:1	7 × 7	30	1	3
Cohen et al. (1995)	55–60	Pa:Ms	10–20	1:1	25 × 25	30	0.08	0
	55–60	Pa:Vp	10–20	3:1	25 × 25	30	0.08	0
	55–60	Ms:Vp	10–20	16:1	25 × 25	30	0.08	0
	55–60	Pa:Vp	10–20	8:1	25 × 25	30	0.08	6
	55–60	Pa:Ms	10–20	5:1	25 × 25	30	0.08	6
Grosholz & Ruiz (1995)	44–61	Nc (<1 & >3) Nt (<1 & >3)	<1 & >3	52 (Nc & Nt >3); 1 (Nc & Nt <1)	40L	40	2	0

[a] Oc = *Ostrea conchaphila*; Mn = *Macoma nasuta*; Vp = *Venerupis* (previously, *Ruditapes*) *philippinarum*; Cc = *Cryptomya califonica*; Ce = *Cerastoderma edule*; Mb = *Macoma balthica*; Pa = *Potamocorbula amurensis*; Ms = *Mytilus* spp.; Nc = *Nutricola* (previously *Transenella*) *confusa* (S. Gray), Nt = *Nutricola* (previously, *Transenella*) *tantilla* (Gould).

[b] Numbers are totals for all bivalve prey species, and all studies were conducted without prey replacement.

crabs will impact *Nutricola* clam populations and benthic communities in West Coast embayments.

The impact of green crabs on bivalve populations in PNW estuaries will depend upon many factors, including green crab abundance, distribution, predators, competitors, and recruitment success. Currently, poor recruitment appears to be the main factor limiting green crab abundances in PNW estuaries (Yamada 2001). Intra- and interspecific predation and competition for food and/or shelter could also limit their abundance and spatial distribution (McDonald et al. 1998, Moksnes et al. 1998, Yamada 2001, Jensen et al. 2002). If green crab populations increase, however, their potential direct impact is high, as they are capable of consuming large quantities of ecologically and economically important PNW bivalve species (Tables 2 and 4). In bare sand habitat, at or near surface dwelling bivalve species (e.g., Olympia oysters, California softshell clams) are probably at greater risk of green crab predation than deeper dwelling species (e.g., bent-nosed macoma clams,

Japanese littleneck clams; Table 3). Heavy green crab predation on bivalves could also have substantial indirect effects on benthic macrofaunal community structure and composition (Grosholz et al. 2000).

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HISTOPATHOLOGY AND PREVALENCE OF THE PARASITIC DINOFLAGELLATE, *HEMATODINIUM* SP, IN CRABS (*CALLINECTES SAPIDUS, CALLINECTES SIMILIS, NEOPANOPE SAYI, LIBINIA EMARGINATA, MENIPPE MERCENARIA*) FROM A GEORGIA ESTUARY

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ABSTRACT This study reports on seasonal variations in the prevalence and intensity of *Hematodinium* sp. infection in the blue crab (*Callinectes sapidus*), spider crab (*Libinia emarginata*), a xanthid crab (*Neopanope sayi*), stone crab (*Menippe mercenaria*), and lesser blue crab (*Callinectes similis*) collected from Wassaw Sound on the Georgia (USA) coast. During the fall of each year there has been a peak in the prevalence of *Hematodinium* in *L. emarginata* and *N. sayi*, while in *C. sapidus* there have been infection peaks in both fall and spring. There was a much lower frequency of infection in *M. mercenaria* and *C. similis*. Based on comparisons of 18S rRNA gene sequences of *Hematodinium* sp., it appears that the *Hematodinium* sp. found in spider and stone crabs are the same or very closely related to the *Hematodinium* isolated earlier from the blue crab. Morphologically, most parasites were in the mononuclear trophont form, although occasional binucleated and multinucleated forms were observed. The highest numbers of *Hematodinium* sp. were found in the gills where parasites were present extracellularly within vascular spaces. The parasite infiltrated cardiac and skeletal muscle in an interstitial pattern, but did not invade individual myofibers. Our findings suggest that *Hematodinium* sp. is impacting the blue crab population in Wassaw Sound and is responsible for the disappearance of *C. sapidus* in the summer months, allowing other opportunistic crab species to invade the niche vacated by *C. sapidus*.

KEY WORDS: prevalence, disease, *Hematodinium*, crab, intensity, estuary, Georgia

INTRODUCTION

Hematodinium perezi is a parasitic dinoflagellate that was first reported in 1931 in two crab species, the green shore crab, *Carcinus maenas*, and the harbor crab, *Liocarcinus depurator*, along the French coast (Chatton & Poisson, 1931). Infection with this parasite has since been shown to produce a spectrum of disease ranging from asymptomatic carriage to death. The parasite proliferates in crustacean hemolymph, consuming hemocyanin, along with other hemolymph proteins and possibly hemocytes (Love et al. 1993, Field & Appleton 1995, Field et al. 1992). Hemolymph taken from heavily infected animals subsequently does not clot. The parasite also infiltrates other tissues, including cardiac and skeletal muscle (Hudson & Shields 1994, Shields & Squyars 2000). Morbidity appears to depend on the burden of organisms. Heavily infected crabs become lethargic, possibly due to hypoxemia and compromise of cardiac and skeletal muscle. If not preyed upon, they often succumb to the overwhelming infection.

Since the work of Chatton and Poisson (1931) on diseased crabs in France, there have been reports of crustaceans infected with *Hematodinium* sp. in Australia (Australian blue crab, *Portunus pelagicus*; sand crab, *Portunus pelagicus*; mud crab, *Scylla serrata*; coral crab, *Trapezia aerolata* [Hudson & Lester 1994, Hudson & Shields 1994, Shields 1992, Hudson et al. 1993]), Alaska (Tanner crab, *Chionoecetes baird* [Meyers et al. 1987, 1994]), Scotland (Norway lobster, *Nephrops norvegicus* [Field et al. 1992]) eastern Canada (snow crab, *Chionoecetes opilio* [Taylor & Khan 1995]) and the eastern United States (blue crab, *Callinectes sapidus*; rock crab, *Cancer irroratus*; Jonah crab, *Cancer borealis*; lady crab, *Ovalipes ocellatus*; amphipods, *Leptocheirus pinguis, Ampelisca vadorum* [Johnson 1986, MacLean & Rudell

1978, Messick 1994, Newman & Johnson 1975]). The life cycle of *Hematodinium* sp. in blue crabs is complex and involves several different stages, including dinospores, prespores, trophonts, and plasmodia (Messick 1994, Shields 1994).

While *Hematodinium* sp. has been found in blue crabs, *C. sapidus*, collected on both the Atlantic and Gulf coasts of the United States (Messick 1994, Messick & Shields 2000, Messick et al. 1999, Newman & Johnson 1975, Shields & Squyars 2000), there have been few reports of this parasite in other crab species from the south Atlantic coast of the United States. The present study reports on seasonal variations in the prevalence and intensity of *Hematodinium* sp. infection among the blue crab (*Callinectes sapidus*), spider crab (*Libinia emarginata*), xanthid crab (*Neopanope sayi*), stone crab (*Menippe mercenaria*), and lesser blue crab (*Callinectes similis*) collected from a coastal Georgia estuary (Wassaw Sound, Fig. 1). Histologic examination of tissues from diseased blue, spider and stone crabs was performed to study the pattern of the infection and immune response of the different hosts. The parasites from each of the three crab species were morphologically very similar. The genetic similarity of the parasites in the three crab species was confirmed by sequencing the 18S rRNA gene.

MATERIALS AND METHODS

Collection, Preparation, Fixing, and Staining of Hemolymph

Crabs were collected in the spring and fall from the Wassaw Sound estuary by trawling or with traps baited with menhaden. Crabs were bled at the hemal sinus with a 1-ml syringe. Hemolymph samples were applied to poly-L-lysine-coated microscope slides as described by Messick (1995), fixed in Bouin's fluid, and stained with Mayer's hematoxylin and eosin (Luna 1968). Fixed and stained slides were examined at ×1000 with a Nikon Eclipse 6400 microscope equipped with a Nikon ×100 1.3NA oil objective. *Hematodinium* sp. was identified based on

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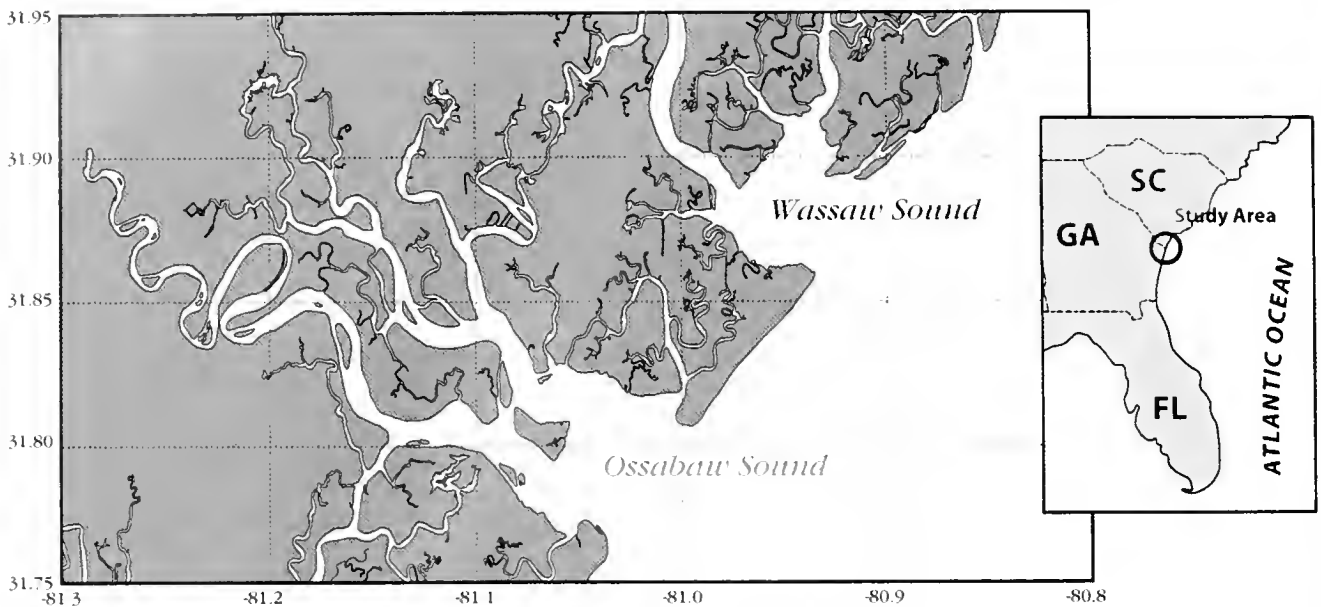
Figure 1. Study site, Wassaw Sound in coastal Georgia.

morphologic similarities to blue crab *Hematodinium* sp. on slides authenticated by G. Messick (NOAA, Oxford, MD). Prevalence. expressed as a percentage, using the definition for this term given by Margolis et al. (1982), was the number of crabs infected with *Hematodinium* sp. divided by the number of crabs examined times one hundred. Infection intensity was the percentage of *Hematodinium* sp. cells counted among a total of 300 cells from the hemolymph from an individual crab. Average intensity for a sampling period was the sum of the intensities of infected crabs divided by the number of infected crabs.

Fixing and Staining of Tissues

Representative portions of tissues were dissected for histologic examination from 10 infected blue crabs, 3 spider crabs, and 1 stone crab. Tissues were fixed in zinc formalin, processed for routine light microscopy and embedded in paraffin. Five-micrometer sections were cut, mounted on glass slides, stained with hematoxylin and eosin, coverslipped and examined by one of us (ANW).

Stages of Hematodinium SP.

Identification of the different forms of *Hematodinium* sp. was based on our own observations and the observations of others, including Appleton and Vickerman (1998). Hudson and Shields (1984), and Shields and Squyars (2000).

The trophont or vegetative form of *Hematodinium* sp. is 8 to 12 µm in diameter. It has a fairly high nuclear cytoplasmic ratio with the nucleus 7 to 9 µm in diameter. Nuclear chromatin varies from appearing rather homogenously dispersed throughout the nucleus to being condensed into structures that resemble chromosomes at metaphase. Trophonts generally possess a single nucleus, but occasional, otherwise typical forms appeared to have two nuclei.

The plasmodium is larger than the trophont form ranging in size from 20 to 50 µm in its longest dimension. Plasmodia are characteristically multinucleated. An elongated, slipper-shape form is referred to as a vermiform plasmodium; the nuclei in this form are usually arranged in a single file along the long axis of the

parasite. There are also more rounded forms that resemble trophonts, but have much greater cytoplasmic volumes and are multinucleated.

Dinospores are notably smaller than trophont forms, 3 to 6 µm in diameter, and are uninucleate.

Molecular Identification and Detection of Hematodinium in Crab Hemolymph

The specific diagnosis of *Hematodinium* sp. in crabs was routinely made using a recently developed Polymerase Chain Reaction (PCR) assay (Gruebl et al. 2002). Hemolymph (0.5–1.0 mL) was collected as described above using a sterile chilled syringe and transferred to sterile 1.5-ml microfuge tubes. Anticoagulant was not required if the hemolymph was kept cool. Total DNA was extracted and purified from hemolymph samples as previously described by Gruebl et al. (2002) using the DNeasy™ Tissue Kit (Qiagen) and the *Hematodinium*-specific primers Hemat-F-1487 (5'-cct ggc tcg ata gag ttg) and Hemat-R-1654 (5'-ggc tgc cgt ccg aat tat tca c) to detect *Hematodinium*. These primers specifically amplify a 195 bp fragment of the 18S rRNA gene from *Hematodinium*. PCR was performed using GenAMP 9700 or 2400 PCR thermal cycler systems (Perkin Elmer). Amplified gene fragments were visualized and sized by agarose gel electrophoresis in 1.2% gels stained with GelStar® nucleic acid stain (Cambrex). The presence of the correct sized amplicon was routinely taken as evidence of *Hematodinium* infection.

To confirm the identity of the parasites detected in each crab species, representative 195 bp PCR amplicons were sequenced. In addition, nearly the complete 18S rRNA gene sequence (1682 bp) from the parasite detected in the spider crab was sequenced and compared with the known *Hematodinium* 18S rDNA fragment that was amplified from DNA purified from a highly infected spider crab (95–98% intensity) using the previously described primers Univ-F-15 (5'-ctg cca gta gtc ata tgc) and Hemat-R-1654 (5'-ggc tgc cgt ccg aat tat tca c) (Gruebl et al. 2002). Sequencing was facilitated by cloning the amplified 18S rRNA gene fragments into the PCR 2.1-TOPO cloning vector using a TOPO™ Cloning Kit.

Version J (Invitrogen) following the manufacturer's instructions. The plasmid was isolated and purified from *E. coli* using the High Pure Plasmid Isolation Kit (Boehringer Mannheim) following the manufacturer's instructions. Plasmid concentrations were estimated by fluorometry after staining with PicoGreen® (Molecular Probes) using a TD-700 fluorometer (Turner Designs). Sequencing was accomplished by automated sequencing using the sequencing primers described in Gruebl et al. (2002) with a Beckman CEQ 2000XL DNA Analysis System. Sequencing reactions were facilitated by using a CEQ DTCS dye terminator cycle sequencing quick start kit, following the protocols recommended by the manufacturer (Beckman Coulter). Sequence analysis was accomplished using the Beckman CEQ 2000XL Sequence Analysis software, version 4.3.9.

RESULTS

Prevalence and Intensity of Hematodinium sp. in Crabs from Wassaw Sound

The prevalence and intensity of *Hematodinium* sp. infection were determined in five crab species collected in Wassaw Sound during different seasons over several years (Table 1, Figs. 2, 3). Prevalence at a time period, expressed as a percentage, is defined as the number of crabs infected with *Hematodinium* sp. divided by the number of crabs examined times 100. Intensity in a crab was the percentage of *Hematodinium* sp. cells in the hemolymph. Average intensity for a sampling period was the sum of the intensities of infected crabs divided by the number of infected crabs. The

TABLE 1.

Prevalence and intensity of *Hematodinium* sp. in *Callinectes similis*, *Neopanope sayi*, and *Menippe mercenaria*.

Species	Collection Data	Number of Crabs	Prevalence (%)	Average Intensity[a] (%)
C. similis	May, 2000	15	0	—
	Aug., 2000	12	0	—
	Oct., 2000	17	0	—
	June, 2001	12	0	—
	Oct., 2001	14	7	11
	June, 2002	18	0	—
N. sayi	March, 2000	5	0	—
	Aug, 2000	4	0	—
	Sept., 2000	8	63	32
	Oct., 2000	5	40	22
	March, 2001	3	0	—
	Oct., 2001	7	43	12
	March, 2002	4	0	—
	Oct., 2002	6	33	26
M. mercenaria	March, 2000	4	0	—
	Aug., 2000	5	0	—
	Oct., 2000	10	0	—
	May, 2001	4	0	—
	June, 2001	8	13	29
	Oct., 2001	7	0	—
	June, 2002	16	0	—

[a] Average intensity for sampling period was the sum of the intensities of infected crabs divided by the number of infected crabs. Infection intensity was the percentage of *Hematodinium* sp. cells counted among a total of 300 cells from the hemolymph from an individual crab.
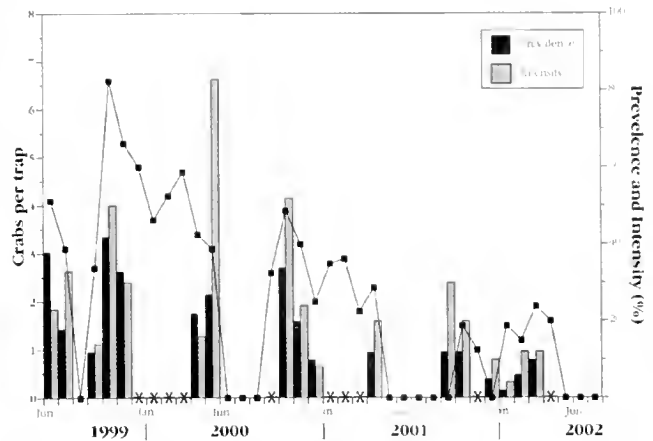
Figure 2. Monthly crab catches and the prevalence and average intensity of *Hematodinium* infection in blue crabs, *Callinectes sapidus*, collected during 1999–2002 in Wassaw Sound. Asterisks indicate that infected crabs were not detected in that sampling period.

average intensities of *Callinectes similis*, *Neopanope sayi*, and *Menippe mercenaria* are reported in Table 1, for *Callinectes sapidus* in Figure 2 and for *Libinia emarginata* in Figure 3. Among the crab species collected, highest prevalences were found in *C. sapidus*, *L. emarginata* and *N. sayi*.

In *C. sapidus*, infection peaks occurred in late spring and fall of each year; moreover, there was an almost complete disappearance of crabs during the summer (Fig. 2). Crabs collected in the winter months of 1999 to 2001 were not infected, but the disease was found in crabs collected during the unusually warm winter of 2001 to 2002. During peak infection periods, prevalence reached 40% with average intensity as high as 80%.

Heavily infected *L. emarginata* were collected each fall for 3 years, but only during one spring (spring 2002) were infected crabs found (Fig. 3). *L. emarginata* normally enter Wassaw Sound in the fall and are common throughout the winter and early spring, and then retreat into cooler, deeper waters in the late spring and sum-

Prevalance and intensity of hematodinium infection in spider crabs collected during 2000 - 2002 in Wassaw Sound.
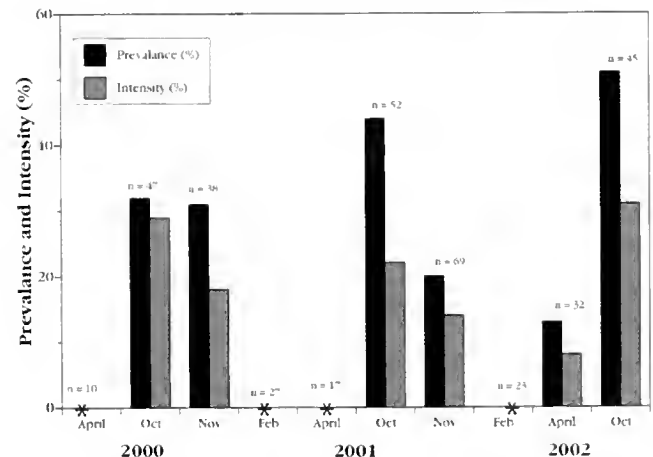
Figure 3. Prevalence and average intensity of *Hematodinium* infection in spider crabs, *Libinia emarginata*, collected during 2000–2002 in Wassaw Sound. Asterisks indicate that infected crabs were not detected in that sampling period.

mer. Infected *N. sayi* were only found in the fall even though this species is a year round resident of Wassaw Sound (Table 1).

In contrast to the high prevalences and intensities of *Hematodinium* sp. found in *C. sapidus, L. emarginata,* and *N. sayi,* only one infected *Menippe mercenaria* and one infected *Callinectes similis* were found during the study (Table 1). The trophont form was the only form observed in the hemolymph from infected *L. emarginata* and *M. mercenaria.* While the trophont was the most common form in *C. sapidus,* the plasmodia form was regularly seen in *C. sapidus* during peak infection periods. Dinospores were observed in three infected *C. sapidus* and one infected *N. sayi.*

Molecular Identification of Hematodinium

Representative 195 bp 18S rRNA gene fragments amplified from both *L. emarginata* and *M. mercenaria* had a 100% sequence similarity to comparable gene fragment of the *Hematodinium* sp. found in *C. sapidus.* Based on these comparisons, the parasite identified in these species was confirmed to be *Hematodinium* sp. and is likely the same species that occurs in the blue crab. To confirm to the species level the identity of the *Hematodinium* sp. found in the spider crab, a larger 18S rRNA gene fragment (1682 bp) was amplified, cloned, and sequenced. This resulting sequence exhibited a 99.6% base pair similarity to the previously sequenced *Hematodinium* sp. (Genbank Accession #AF286023) isolated from the blue crab. By convention, sequence similarities in the 18S rRNA gene greater than 98% are indicative of the same species (Hillis & Dixon 1991). Therefore it can be concluded from these observations that the same species of *Hematodinium* occurs in *M. mercenaria, L. emarginata,* and *C. sapidus.*

Pathologic Findings in Infected Crabs

Libinia emarginata

The three crabs examined varied in their burden of organisms from light to heavy. In the lightly infected crab, the gills contained occasional trophont forms intermixed with equal numbers of granulocytes. There were occasional mononuclear and multinucle-

ated trophont forms on the abluminal side of the hepatopancreas. In the heart there were rare mononuclear trophonts and multinucleated forms. The skeletal muscle was largely spared. In the moderately infected crab, the gill tissues demonstrated mononuclear trophonts in the larger vascular spaces at the base of the gills. There were scattered granular and agranular hemocytes present, but these were considerably outnumbered by parasites. In the heavily infected spider crab, there were numerous mononuclear and multinucleated trophont forms dispersed along the vascular spaces of the gills; few hemocytes were present (Fig. 4). Some skeletal muscle fibers appeared fragmented; there were also interstitial clusters and infiltrates of parasites and foci of myofiber necrosis (Fig. 5A). The hepatopancreas was heavily infected with the trophont forms on the abluminal side of the tubules and in vascular spaces (Fig. 5B). There were no parasites within the hepatopancreatic cells or within the tubular lumina.

Menippe mercenaria

The crab examined was heavily infected. Most of the parasites were in the mononuclear trophont form, although occasional bi-
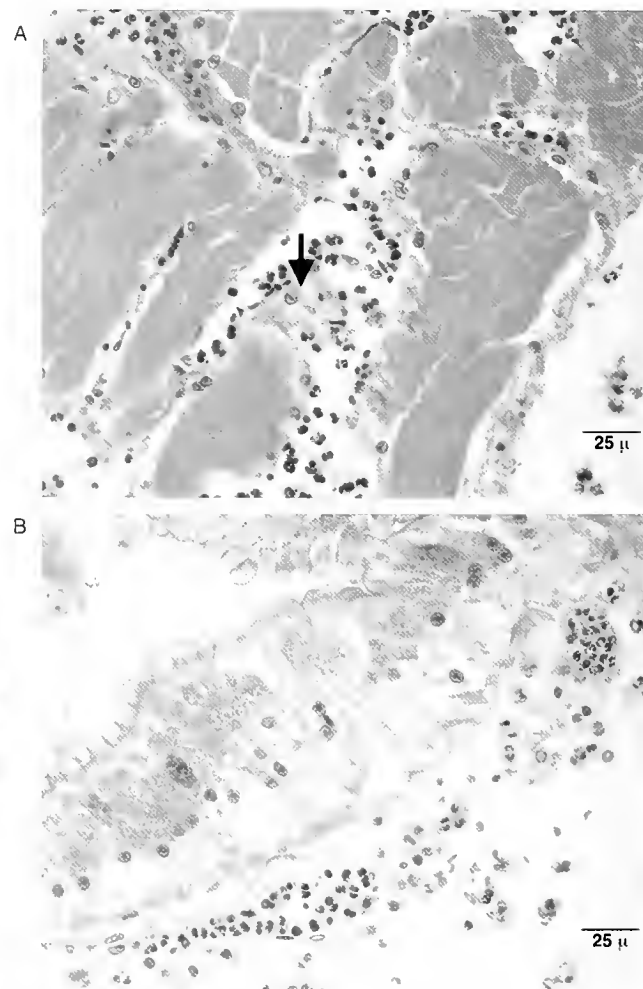
Figure 5. Heavily infected spider crab, *Libinia emarginata.* (A) Interstitial infiltrates of the parasite in the skeletal muscle. The hemocytic response is minimal. Some muscle fibers lack nuclei; there are foci of apparent destruction in association with the parasites (arrow). (B) Hepatopancreatic vascular spaces are filled with parasites, but no infiltration of the glandular epithelium is seen. (Hematoxylin and eosin: original magnifications: ×400).
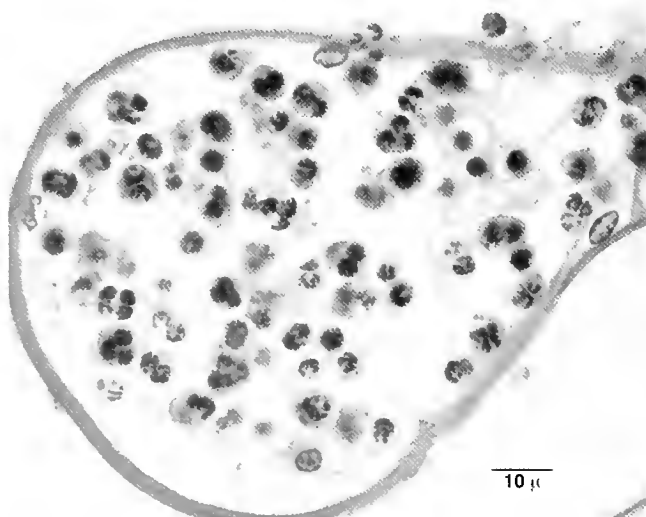
Figure 4. Gill from a heavily infected spider crab, *Libinia emarginata.* The vascular spaces of the gills contain many trophont forms of the parasite and a few host hemocytes. (Hematoxylin and eosin: original magnification: ×1000).

nucleated and multinucleated forms were observed. The highest concentration of *Hematodinium* sp. was in the gills where the parasites were dispersed along the vascular spaces (Fig. 6A). Crab hemocytes, primarily granulocytes, were present in these vascular spaces although they were far outnumbered by the parasite. *Hematodinium* sp. were concentrated on the abluminal sides of the hepatopancreas and in its vascular spaces. Both granular and agranular hemocytes were present in the heart; some had infiltrated the cardiac muscle along with *Hematodinium* sp. (Fig. 6B). There was, in addition, a single focal plaque-like aggregate of parasites and granulocytes on the surface of cardiac muscle. Skeletal muscle contained only a few *Hematodinium* sp. in connective tissue external to muscle fibers. Gonadal tissue appeared to be free of the parasite.

Callinectes sapidus

In lightly infected crabs (less than 2% of hemolymph cells were parasites) there was a strong cellular response to *Hematodinium* sp., as evidenced by scattered aggregates of granulocytes, which formed encapsulating nodules in gill, hepatopancreas, and cardiac muscle (Fig. 7A,B). The nodules in the hepatopancreas were found

Figure 7. Lightly infected blue crab, *Callinectes sapidus*. (A) The vascular spaces of the gills contain abundant granular and agranular hemocytes and occasional hemocytic nodules. A few trophont forms are present (arrow). (B) A cluster of hemocytic nodules on the abluminal side of the hepatopancreas. (Hematoxylin and eosin; original magnifications: A, ×400; B, ×1000)

Figure 6. (A) Gill from an infected stone crab, *Menippe mercenaria*. Trophont forms of the parasite and host hemocytes are present within the vascular space. (B) Cardiac tissue from the same crab. Interstitial infiltrate of trophonts and host hemocytes. (Hematoxylin and eosin; original magnifications: ×1000).

on the abluminal side and there was no invasion by parasites of the hepatopancreatic glandular epithelium or tubular lumina. Only mononuclear trophont forms were observed.

In heavily infected crabs, several parasites but few hemocytes were found in vascular spaces and within tissues (Figs. 8, 9). Plasmodia (Fig. 8A), mononuclear and binuclear trophonts were noted in both the hemolymph and cardiac muscle. Dense infiltrates of parasites were noted on the abluminal side of the hepatopancreas (Fig. 8B), but even with heavy infection there was no evidence of hepatopancreatic epithelial or tubular luminal invasion by the parasite. In the hepatopancreatic region of one moribund animal, there were large numbers of a smaller parasite form that possessed a polymorphic nucleus; these may have been dinospores (Fig. 9A).

In addition to high concentrations of parasites in bivascular spaces, parasites infiltrated cardiac and skeletal muscle. Focal muscle necrosis was present (Fig. 9B); hemocyte nodules were rare or absent. Parasites were present in the tissues adjacent to the gonads, but not within gonadal tissues.
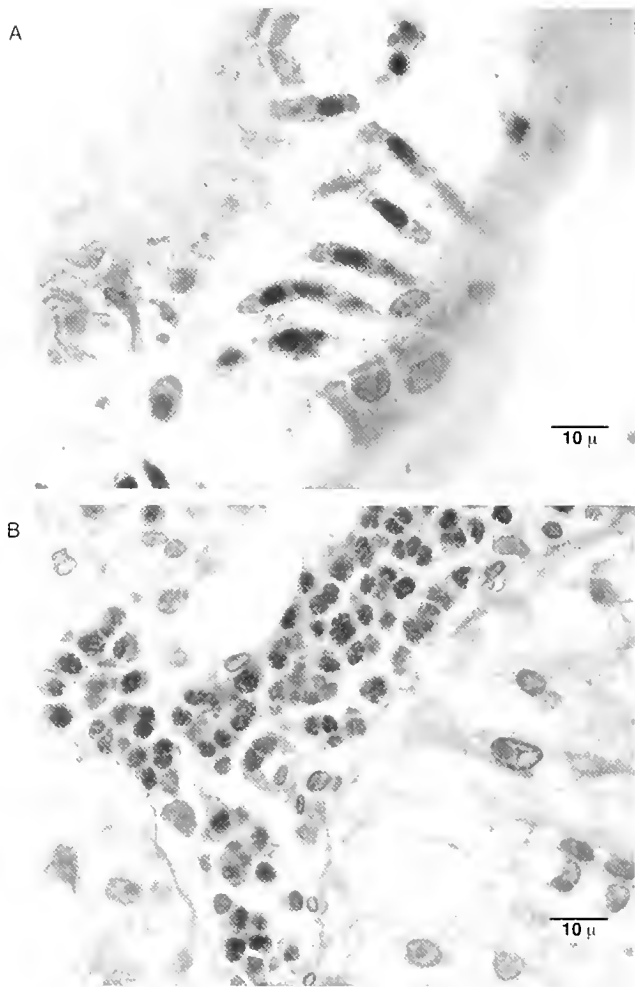
Figure 8. Heavily infected blue crab, *Callinectes sapidus*. (A) The vascular spaces of the gills of this crab contain many plasmodia; few hemocytes are present. (B) Hepatopancreatic tissue from another heavily infected animal. This vascular space is filled with parasites, mainly in the trophont form. (Hematoxylin and eosin; original magnifications: A, ×400; B, ×1000)

Figure 9. Heavily infected blue crab, *Callinectes sapidus*. (A) Hemocytic nodules were rare in most of the heavily infected animals. This one is on the abluminal side of the hepatopancreas. Also present are thousands of small forms of the parasite, possibly dinospores. (B) Infiltrates of the parasite in cardiac muscle. Both trophont and plasmodia forms are present. Focal coagulative muscle necrosis (arrows) has occurred. (Hematoxylin and eosin; original magnifications: ×400)

DISCUSSION

Prior to 1999, Wassaw Sound on the Georgia coast supported a robust, year-round commercial blue crab fishery. Since the studies began in 1999, there have been high *Hematodinium* sp. prevalences in *Callinectes sapidus*, *Libinia emarginata*, and *Neopanope sayi*. In addition to a peak each fall, a peak in *Hematodinium* sp. prevalence in *C. sapidus* also occurred during the spring months. Associated with the increased prevalence of *Hematodinium* sp. in the spring was the disappearance of *C. sapidus* from Wassaw Sound during the summers of 3 successive years (Fig. 2). These observations suggest that high mortality secondary to *Hematodinium* infection in the spring led to the near absence of *C. sapidus* in the summer. During the summer months, blue crabs were abundant in low salinity areas near freshwater rivers in coastal Georgia (Lee, unpubl.). We hypothesize that female blue crabs found each fall for the past 4 years in Wassaw Sound were returning through Wassaw Sound from low salinity areas to spawn in the ocean.

Other seasonal studies on prevalence of *Hematodinium* sp. have been conducted on crabs in different coastal areas. A seasonal study of *Hematodinium* sp. infection in *C. sapidus* collected from coastal bays of Maryland showed a peak of infection each fall. Prevalences reached 80% at this time, while the disease was almost undetectable from March thru May (Messick & Shields 2000). Seasonal studies of *Hematodinium* sp. were conducted in the Norway lobster (*Nephrops norvegicus*) off Scotland (Field et al. 1998) and the tanner crab (*Chionoecetes bairdi*) off Alaska (Eaton et al. 1991, Love et al. 1993). The peaks of *Hematodinium* sp. infection in both species occurred in the late spring and summer, with declines in infection noted during the fall and winter. These studies, along with our own findings, indicate that the seasonality of *Hematodinium* infection can vary among different crustacean species in the same area and among species from different areas.

We have shown that *Hematodinium* sp. can be transmitted when an uninfected crab feeds on an infected crab (Lee et al. unpubl.). Both *C. sapidus* and *L. emarginata* are aggressively cannibalistic. We noted a much lower frequency of infection in *Menippe mercenaria* and *Callinectes similis*. We speculate that the indolent feeding behavior of *M. mercenaria* and *C. similis*

account for their low *Hematodinium* sp. prevalence during periods when there is both high prevalence and intensity of *Hematodinium* sp. among other crab species. Other explanations for the varying prevalence of *Hematodinium* sp. in different crab species include the possibility that *Hematodinium* sp is more virulent for certain species, possesses tropism for particular crab species, or that the immune systems of *C. similis* and *M. mercenaria* are more effective in limiting *Hematodinium* sp. infection. Another important factor may be crab densities, since we find that *Hematodinium* epidemics occur in areas where there are high densities of either *C. sapidus* or *L. emarginata* (Sheppard, Lee, and Fischer, unpubl.). Some marine diseases are well correlated with host densities (Richardson et al. 1998), but in other diseases there is no relationship (Powell et al. 1999).

Only two *Hematodinium* spp., *H. perezi* (Chatton & Poisson 1931), and *H. australis* (Hudson & Shields 1994), have been characterized. While the parasite in *C. sapidus* has been referred to as *Hematodinium perezi* (Messick 1994, Shields & Squyars 2000), Messick and Shields (2000) suggest that the parasite in *C. sapidus* be referred to as *Hematodinium* sp. until more comparisons have been made with the type species. Based on the sequence of fragments of the 18S rRNA gene, it appears that the *Hematodinium* sp. found in *L. emarginata* and *M. mercenaria* are the same or very closely related to *Hematodinium* sp. isolated from *C. sapidus* (Gruebl et al. 2002). It thus appears likely that the infection can be readily transmitted among various crab species in our study area.

Histopathologic studies of *Hematodinium* sp. infections include *C. sapidus* from coastal bays of Maryland (Messick 1994), *Portunus pelagicus* from the eastern seaboard of Australia (Hudson & Shields 1994) and *Chionoecetes bairdi* from southeast Alaska (Meyers et al. 1987). The histologic changes described in infected gill and muscle tissues of the animals in those studies are similar to those seen in the tissues of the infected crabs in our studies. *Hematodinium* sp. was present extracellularly within the vascular spaces of gills. The parasite produced interstitial infiltrates in cardiac and skeletal muscle but did not invade individual myofibers (Figs. 4–5, Hudson & Shields 1994, Meyers et al. 1987). Focal muscle necrosis was apparent in some of our infected crabs. Myers et al. (1987) noted pathologic changes in muscle cells of heavily infected Tanner crabs, including loss of cross striations and cytoplasmic eosinophilia. Parasitic infiltrates and muscle necrosis would likely compromise the structure and function of these organs and thereby contribute, along with the hemocyanin depletion, to the lethargic behavior exhibited by heavily infected animals.

The presence of encapsulating nodules in lightly infected *C. sapidus* and their absence in non-infected crabs is of interest since the response of crustaceans to large foreign bodies is encapsulation by circulating hemocytes (Galloway & Depledge 2001, Holmblad & Söderhäll 1999). In heavily infected animals, the hemocyte population appeared depleted, suggesting that large numbers of parasites can overwhelm the host's ability to contain the infection. Whether such animals are immunocompromised by pre-existing conditions or the parasites gain a proliferative advantage due to environmental circumstances awaits further study. In addition, we have found that bacteria often colonize the hemolymph of heavily parasitized animals (Sheppard, unpublished data). Such secondary invaders may hasten the demise of these impaired hosts, since they cannot mount an adequate hemocyte response.

Our results suggest that *Hematodinium* sp. is impacting the blue crab populations in Wassaw Sound and is largely responsible for the disappearance of *C. sapidus* during the summer months. As the population of *C. sapidus* in Wassaw Sound has decreased there have been increases in the populations of other crab species, such as *C. similis, Ovalipes ocellatus, Petrolisthes armatus,* and *Arenaeus cribrarius* (Sheppard, unpubl.).

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THE ROLE OF MACROALGAL BEDS AS NURSERY HABITAT FOR JUVENILE BLUE CRABS, *CALLINECTES SAPIDUS*

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ABSTRACT We investigated the role of macroalgal beds as juvenile habitat for the blue crab *Callinectes sapidus*. A 2-year study was conducted in Rehoboth Bay, a lagoonal estuary in the Middle Atlantic Bight along the east coast of North America. Sea grass meadows do not occur in Rehoboth Bay, and submersed aquatic vegetation consists entirely of macroalgae. Quantitative samples were collected from both vegetated and open (unvegetated) habitat with a throw trap. Results indicate that macroalgal beds provide important habitat for juvenile blue crabs, beginning at settlement and continuing until the crabs reach a carapace width of about 30 mm. Average abundance of juveniles in macroalgal beds was 7 times greater than in adjacent open habitat, and maximum abundance in the beds reached weekly mean values >90 crabs m^{-2} during periods of high recruitment in early autumn. Mean size of individual crabs was 15 mm carapace width when sampling began in May. These crabs had settled the previous autumn and had over-wintered in the bay. Mean size continued to increase through early summer, and the crabs had reached a mean carapace width >30 mm by August. These 30-mm crabs disappeared from the beds in mid-August and were replaced by newly metamorphosed juveniles <10 mm in carapace width. Very small crabs were common in the beds throughout September and October. Results of gut-content analysis imply a direct trophic linkage between indigenous macroalgal production and juvenile crabs collected from the beds. This putative linkage involves various species of amphipods that graze directly on the macroalgae and constitute over 25% (by volume) of the gut contents of juvenile crabs collected from macroalgal habitat.

KEY WORDS: juvenile, blue crab, *Callinectes sapidus*, macroalgae, nursery habitat

INTRODUCTION

The preservation of plant-based habitats such as sea grass meadows has become a lynchpin of international marine conservation policy, but regardless of conservation efforts, there has been a general decline in the extent of this habitat worldwide (e.g., Giesen et al. 1990, Dennison et al. 1993, Heyman & Kjerfve 1999). This problem has been studied intensively in estuaries along the east coast of North America, where sea grass provides nursery area for many species of fish and invertebrates (Orth & Moore 1983, Shepherd et al. 1989, Moore et al. 2000). The value of sea grass beds as nursery grounds has been attributed to the provision of complex bottom topography that reduces the extent of predation on juvenile stages (Orth & van Montfrans 1987, Wilson et al. 1990). However, sea grass meadows are also the sites of high indigenous primary production (e.g., Duarte & Chiscano 1999), and the role of this production in supporting the growth and development of juveniles is less clear (Fry & Parker 1979, Hughes & Sherr 1983).

In areas where sea grass is in decline, newly available bottom often has been colonized by benthic macroalgae (Valiela et al. 1997). But unlike sea grass meadows, the nursery role of these macroalgal beds has not been well studied. For example, there have been only a few experimental investigations of the role of macroalgal beds as refugia from predation (e.g., Wilson et al. 1990, Dittel et al. 1996), and the number field surveys of juvenile forms of fish and invertebrates occupying this habitat is commensurately low (Sogard & Able 1991, Sogard 1992, Szedlmayer & Able 1996). Moreover, the role of indigenous primary production in supporting the growth of juveniles within macroalgal nurseries is virtually unknown.

Growth of macroalgae is maximized under eutrophic conditions typical of poorly flushed lagoonal estuaries (Lavery et al. 1991, Duarte 1995). One such estuary is Rehoboth Bay, which is located in the Middle Atlantic Bight along the east coast of the USA (ca. 38.5°N, 77.1°W). Although historical accounts indicate that areas of sea grass meadow occurred in Rehoboth Bay as recently as the 1960s, submersed aquatic vegetation now consists entirely of macroalgae (Price 1998). The dominant macroalgae occurring in Rehoboth Bay are the green alga *Ulva lactuca* and the red algae *Agardhiella tenera* and *Gracilaria* spp. (Timmons & Price 1996). Macroalgal beds are patchily distributed on sandy bottom throughout the bay, and typical patches are on the order of 10^3 to 10^4 m^2. Macroalgal beds in estuaries like Rehoboth Bay often occur as drift algae (i.e., not attached to the bottom). Thus, the location of patches changes as a function of winds and currents.

We have used Rehoboth Bay as a case study in which we investigated the extent to which one of the dominant invertebrate species in the region (the blue crab, *Callinectes sapidus*) uses macroalgal beds as nursery habitat. Although several types of bottom have been identified as nurseries for blue crabs (Szedlmayer & Able 1996), maximum abundance of juveniles typically occurs in vegetated areas, and sea grass meadows are generally considered critical nursery habitat for the species (Pardieck et al. 1999). The utility of macroalgal beds as surrogates for sea grass has been generally established (e.g., Sogard & Able 1991), but details of the association between macroalgae and juvenile blue crabs (including possible trophic linkages) have not been determined. The study described in this paper addresses this gap and provides data on seasonal changes in the abundance of different life history stages in macroalgal beds and the relationships between the abundance of juveniles and the standing crop of macroalgae. The investigation involved extensive field collections and included comparative analysis of gut contents of juvenile blue crabs collected from macroalgal beds and from two alternative nursery habitats.

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METHODS

Study Location

Rehoboth Bay is a small lagoonal estuary located in the Middle Atlantic Bight (Fig. 1). Mean depth is about 1.7 m with a tidal range < 0.5 m. Rehoboth Bay has no direct connection to the coastal ocean. At its northward end, Rehoboth Bay adjoins the Lewes & Rehoboth Canal, which eventually reaches Delaware Bay, approximately 12 km to the north. At its opposite end, Rehoboth Bay connects through several shallow channels to Indian River Bay, immediately to the south. Indian River Bay communicates with the coastal ocean through an inlet at its eastern terminus. Total surface area of the Rehoboth-Indian River system is approximately 75 km².

Comparison of Vegetated and Open Habitat

In the first year of the investigation (1998), we compared the abundance of juvenile blue crabs in macroalgal beds to abundance at open bottom sites. Crabs were collected using a throw trap that allowed quantitative sampling of a confined area of bottom (see Sogard & Able 1991). The base of the throw trap was an open aluminum box (1 m × 1 m × 0.3 m) with a band of fine-mesh (0.5 cm) nylon netting (1.5 m high) attached around the entire perimeter. The upper edge of the netting was lashed to a buoyant, frame (1 m × 1 m), and the remaining seam was sewn together to complete the trap. The device was deployed from a small boat in water <1.5 m deep. Upon deployment, the base of the trap penetrated several cm into the sediment, and the upper section extended all the way to the surface. Thus, a 1-m² quadrate of bottom was segregated from the surrounding environment and could be sampled quantitatively.

Sampling was conducted every 2 wk, starting in late June and continuing through the end of September. Collections were generally made at three stations during each sampling week. However, inclement weather occasionally restricted effort, resulting in a total of 18 stations sampled over the entire period. Stations were located in shallow water around the periphery of the bay (Fig. 1). The exact location of the stations varied from week to week, depending on the availability of macroalgal beds and adjacent open habitat. Throw trap sampling was performed in conjunction with a beam-trawl survey of the deeper (>1.5 m) parts of Rehoboth Bay. Abundance of juvenile crabs was considerably lower in deep water than in the shallow water sampled with throw traps (Targett et al. 1999).

At each station the trap was deployed once in a macroalgal bed and once on the adjacent open bottom. The two sampling locations were always within 50 m of each other, and the exact site within each habitat was chosen haphazardly. Temperature, salinity, and dissolved oxygen were measured in conjunction with each deployment. Crabs and macroalgae were removed from the trap with a 3-mm mesh dip net. The rectangular frame of the dip net was designed to allow maximum coverage of the area within the throw trap with each sweep of the net. Dip-net sweeps were made along the bottom until 3 consecutive sweeps produced no organisms. Earlier work with similar gear has shown that efficiency of sampling approaches 100% with this technique (Kushlan 1981, Pihl & Rosenberg 1982).

Juvenile blue crabs were returned to the laboratory where carapace width was determined to the nearest mm. Volume of macroalgae in each sample was determined in the field. This involved removal of extraneous water by blotting the sample on paper tow-
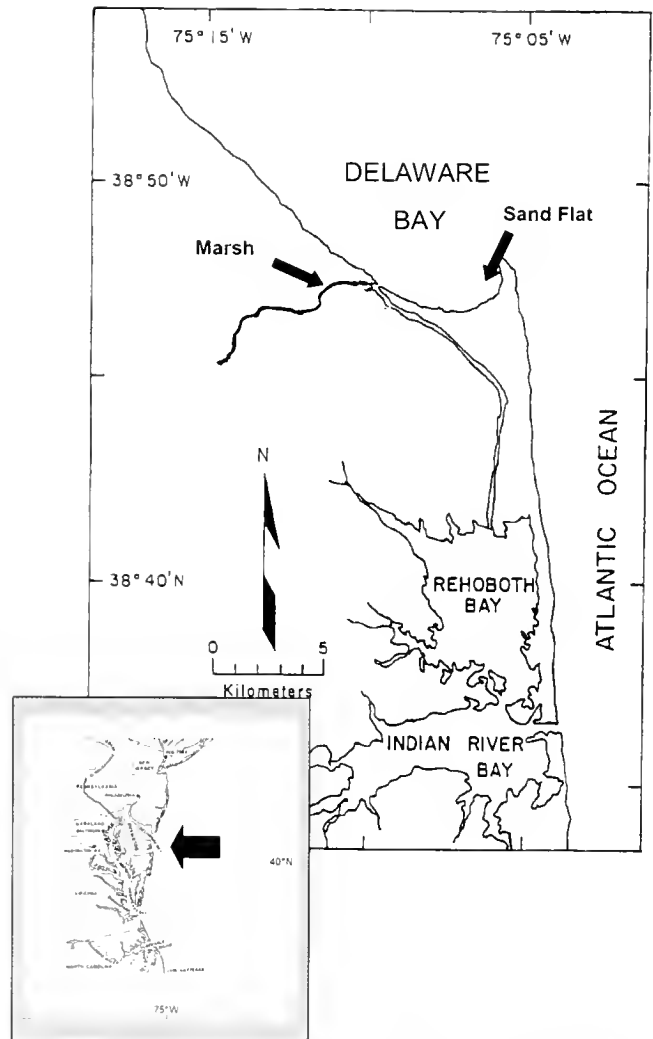
Figure 1. Map of study area. Insert shows location of Rehoboth Bay in Middle Atlantic Bight.

els, followed by measurement of the respective volumes of green and red algae in a large graduated beaker. These values were converted to their gravimetric equivalents using regression equations relating volume to dry weight (green algae: $r^2 = 0.95$, $P < 0.001$, $n = 26$; red algae: $r^2 = 0.97$, $P < 0.001$, $n = 29$). The regression equations were based on volume measurements and dry-weight determinations (60°C, 48 h) for representative macroalgal samples collected from Rehoboth Bay.

Seasonality of Habitat Use

In the second year of the study (1999), we investigated detailed seasonal patterns in use of macroalgal beds by juvenile crabs. Early season sampling (mid-May through early August) was conducted every two weeks and targeted crabs that had settled during the previous autumn and had over-wintered in the bay (i.e., the 1998 y-class). Late season sampling (mid-August to early November) occurred weekly and concentrated on newly settled juveniles (i.e., the 1999 y class). As in the first year of the investigation, stations were located in shallow water around the periphery of the bay, but in this case sampling was restricted entirely to vegetated areas. Again, the exact location of stations varied from week to week, depending on the availability of macroalgal beds.

Sampling was generally conducted at five stations during each sampling week. However, inclement weather occasionally restricted effort, resulting in a total of 81 stations sampled from May through October. Deployment of the throw trap and analysis of samples were the same as in year 1.

Analysis of Gut Contents

Crabs for gut-content analysis were collected as part of routine throw trap sampling in 1999. Sampling areas were always located in macroalgal beds and typically encompassed stands of both red and green macroalgae. A total of eight throw trap samples were collected for gut-content analysis between July and September, resulting in 52 individual crabs (<30 mm carapace width). Comparative samples were collected from two alternative blue crab nursery areas (one a marsh tidal creek and the other an open water tide flat) in nearby Delaware Bay. Marsh-fringe samples were collected with a dip net from a tidal creek within an extensive salt marsh; tide flat samples were obtained with a beach seine from an open water site a few km away (Fig. 1). Sediment at the tide flat site consisted of coarse sand, cobble, and shell fragments—thus providing a modicum of structured nursery habitat for juvenile crabs. Sampling was conducted a total of 12 times at the marsh site between July and October, yielding 73 individual crabs for subsequent analysis. An additional nine sampling efforts at the tide flat site between August and October resulted in 47 individual crabs for analysis.

In each case, crabs were placed on ice while still in the field to minimize digestion of food. Upon return to the laboratory, the crabs were frozen (−20°C) for later analysis. Gut contents of thawed specimens were determined using standard dissection and microscopy techniques (e.g., Dittel 1993). Separate analysis was conducted for each of the 172 crabs in the samples. The occurrence of each food item was represented as a proportion of the total volume of food in the stomach, and mean values were determined for each of the 3 sample groups (Hines et al. 1987).

Statistical Analysis

Mean abundance of crabs in sea grass beds was compared with abundance on open bottom by a two tailed t-test ($\alpha = 0.05$). Data were log-transformed to meet assumptions of the t-test model. Relationships between crab abundance and standing crop of macroalgae were determined by Pearson product-moment correlation analysis. All correlations were done using pooled data from both years. Separate analyses were conducted for the entire data set, for early-season data, and for late-season data. A similar approach was used to examine correlations between crab abundance and the respective proportions of green and red algae in each sample. Significance of all correlations was determined at $\alpha = 0.05$.

RESULTS

Hydrographic conditions were similar during the two years of the study, and any differences in the overall range of values were attributable to the broader seasonal coverage during 1999. Salinity ranged from approximately 23–32 ‰ over the period of the investigation, with a median value around 29‰. Temperature reached maximum values in August (>25°C), and was minimum (<15°C) in early November 1999. Water was typically well oxygenated, and levels rarely fell below 5 mg L^{-1}. Supersaturated values as high as 15 mg L^{-1} were occasionally measured in macroalgal beds on calm, sunny days.

Abundance of juvenile blue crabs was significantly greater in macroalgal beds than in adjacent open bottom (t-test, $t_{34} = 3.1$, $P < 0.001$). When calculated for all stations in 1998, mean abundance in macroalgae was 7.3 m^{-2} (±10.1), while open areas had an abundance of 1.0 m^{-2} (± 1.9). The large standard deviations resulted from seasonal differences wherein the abundance of crabs in macroalgal beds increased markedly in September (Fig. 2).

Results from 1999 showed strong seasonal variation in both numbers and size of juveniles in macroalgal beds. Mean abundance from May through early August was always <10 crabs m^{-2}, but increased to levels >20 crabs m^{-2} by mid-August and reached values >90 m^{-2} in September and October (Fig. 3). The mean carapace width of juveniles was somewhat less than 15 mm when sampling began in May and approached 30 mm by late July (Fig. 4). The large juveniles disappeared from the beds in mid-August and were replaced much smaller crabs (<10 mm). These small individuals dominated the population throughout the remainder of the study period and were still abundant when sampling ended in early November.

Macroalgal beds were well developed during both years of the investigation, with a median standing crop of macroalgae of approximately 150 g m^{-2}. This is within the range of values typical for macroalgae at high nutrient levels (e.g., Schneider & Searles 1977, De Busk et al. 1986). Of the 99 vegetated stations sampled over the two years of the study, 40 had a greater proportion of green algae and 59 a greater proportion of red. Among these, 18 stations were pure stands of red species, while only 3 stations were pure stands of green forms.

Analysis of all stations pooled over the 2 y of the investigation showed a significant positive correlation between abundance of crabs and total standing crop of macroalgae (Table 1). However, there was no correlation between abundance of crabs and the dry-weight ratio of green to red algae at the respective stations. Separate analysis of early-season data and late-season data gave results that were similar to those for the entire data set.

As expected, the gut contents of crabs collected from all three habitats showed a wide variety of prey items (Table 2). These included a number of crustacean groups, bivalve and gastropod mollusks, polychaetes, vascular and macroalgal plant material, and considerable amounts of highly digested tissue that we were unable to assign to any particular taxonomic group. Regardless of this
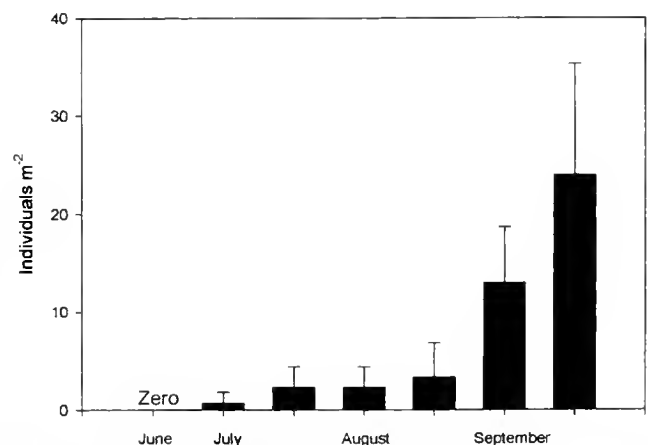

Figure 2. Abundance of juvenile blue crabs *Callinectes sapidus* in macroalgal beds in Rehoboth Bay, Delaware. Solid bars are weekly mean abundance in 1998. Error bars = one standard deviation.
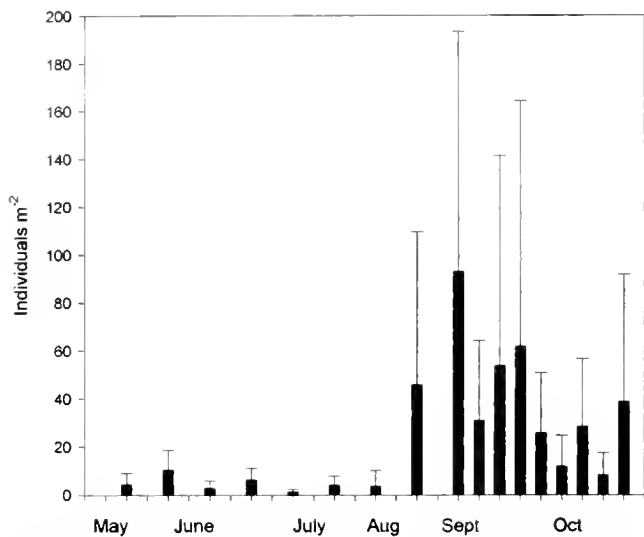
Figure 3. Abundance of juvenile blue crabs *Callinectes sapidus* in macroalgal beds in Rehoboth Bay, Delaware. Solid bars are weekly mean abundance in 1999. Error bars = one standard deviation.

taxonomic diversity, crustaceans were the dominant stomach component in crabs from each of the three sampling sites. However, the taxonomic groups comprising this crustacean component varied greatly among crabs from the three respective habitats. For example, crab body parts accounted for nearly 30% (by volume) of the stomach contents of juvenile *C. sapidus* collected from marsh habitat adjacent to Delaware Bay, but never exceeded 13% in either of the other two habitats. In contrast, a miscellaneous group that we called "other crustaceans" composed almost 50% of the stomach contents of crabs collected from tide flat habitat in Delaware Bay. This group consisted of harpacticoid copepods, palaemonid and crangonid shrimp, and crustacean body parts that could not be assigned to any particular taxon. Crabs collected from macroalgal beds in Rehoboth Bay differed most notably from the other two habitats in the very low proportion of crab body parts in their gut contents and in the high proportion of amphipods in their

Figure 4. Size of juvenile blue crabs (*Callinectes sapidus*) in seaweed beds in Rehoboth Bay. Solid bars are weekly mean carapace width in 1999. Error bars = one standard deviation.

stomachs (>30%). This was remarkable because amphipods were entirely absent from the identifiable gut contents of crabs from the other two sampling sites.

DISCUSSION

Results of our investigation indicate that macroalgal beds provide important habitat for juvenile blue crabs, beginning at settlement and continuing until the crabs reach a carapace width >30 mm. Average abundance of juveniles in macroalgal beds was approximately 7 times greater than on adjacent open bottom, and maximum abundance in the beds reached weekly mean values >90 crabs m^{-2} during periods of high recruitment in early autumn. Mean size of individual crabs was about 15 mm in carapace width when sampling began in May. Because settlement of blue crabs in this region occurs almost exclusively in late summer and autumn (Jones & Epifanio 1995), the crabs collected in May apparently had settled during the previous autumn and had over-wintered in Rehoboth Bay. Mean size continued to increase through early summer, and the crabs had reached a mean carapace width >30 mm by mid-summer. The 30-mm crabs disappeared from the beds in mid-August and were replaced by newly metamorphosed juveniles <10 mm in carapace width. These small crabs had probably settled in the beds as megalopae and had undergone metamorphosis soon thereafter (Orth & van Montfrans 1987, Jones & Epifanio 1995). Very small crabs were common in the beds throughout September and were still abundant when sampling was completed at the end of October. Mean size of the crabs did not increase during this period, probably a result of overlapping cohorts of new recruits. However, there was considerable variation in abundance among stations (note the high standard deviations in Fig. 4), which undoubtedly reflects the patchy nature of settlement in the bay. This was probably a result of the patchy distribution of megalopae in the water column (Natunewicz & Epifanio 2001), rather than some difference in the attractiveness among beds (Brumbaugh & McConnaugha 1995).

Earlier work in the Little Egg Harbor–Great Bay system along the coast of New Jersey (100 km north of Rehoboth Bay) also addressed the importance of macroalgal beds as juvenile habitat (Wilson et al. 1990). This system is similar to our study site, but has ample sea grass meadow in addition to macroalgal beds (Sogard & Able 1991). As in our investigation, early-season abundance at the New Jersey site was on the order of 5–10 crabs m^{-2} in vegetated habitat and considerably lower on open bottom. Moreover, the general pattern of seasonal abundance of different size classes was similar to that in Rehoboth Bay. However, late-season sampling in New Jersey did not find the extremely high abundance of newly settled crabs seen at our study site, perhaps reflecting a greater distance from the very large spawning stock of blue crabs in Delaware Bay (Garvine et al. 1997).

This difference aside, it appears that macroalgal beds generally provide nursery habitat for blue crabs that is comparable to that of sea grass meadows. For example, there was little difference in mean abundance of juveniles in macroalgal and sea grass habitats in the Little Egg Harbor–Great Bay system; in fact, the abundance of crabs was slightly higher in macroalgal habitat (Sogard & Able 1991). Likewise, mean abundance in macroalgal beds at our study site in Rehoboth Bay was similar to that in sea grass meadows in Chesapeake Bay, and general patterns in seasonal occurrence were nearly identical (e.g., Orth & van Montfrans 1987).

The present investigation has provided a much more detailed description of the utilization of macroalgal habitat than was previously

TABLE 1.

Correlations between abundance of juvenile blue crabs (*Callinectes sapidus*) and two properties (algal standing crop and the ratio of green to red macroalgae) of macroalgal beds in Rehoboth Bay, Delaware, USA.

	Full Season			Early Season			Late Season		
	r	P	n	r	P	n	r	P	n
[C] × A	0.379	<0.001	99	0.443	0.002	45	0.575	<0.001	54
[C] × R	−0.103	0.313	99	−0.034	0.824	45	−0.184	0.183	54

Data were analyzed separately for full season, early season, and late season (see text). Correlation statistics: r = Pearson product-moment correlation coefficient. P = probability of rejecting a correct null hypothesis. n = number of coordinate observations. Variables: $[C]$ = crab abundance, A = algal standing crop. R = ratio of green to red algae (dry weight).

available. For example, our results imply that macroalgal beds are important settlement sites blue crab megalopae in autumn and further demonstrate the consequent role of the beds as nurseries for the earliest juvenile stages. In addition, our analysis shows that juvenile blue crabs use beds of red and green macroalgae with equal propensity and that abundance of crabs in a bed increases in proportion to the standing crop of macroalgae. Moreover, these relationships are equally valid during early season when the population is dominated by over-wintered crabs or later in the season when beds are populated entirely by newly settled juveniles.

Supplemental to their provision of complex benthic structure, macroalgal beds may also be important as a source of primary production that supports growth of juvenile blue crabs. Results of our analysis indicate that crustacean body parts dominated the gut contents of crabs collected from all three nursery habitats considered in this investigation. However, the taxonomic groups represented within this dietary category varied considerably among habitats. Of primary relevance is the fact that amphipods were the dominant component in the stomach contents of crabs collected from macroalgal beds, but were entirely absent from the identifiable gut contents of crabs from marsh or tide flat environments. Available evidence in the present investigation is restricted en-

TABLE 2.

Mean percentage by volume of prey items in gut contents of juvenile blue crabs (*Callinectes sapidus*) collected from three different nursery habitats.

Gut Contents	Macroalgae	Marsh	Tide Flat
Amphipods	31.5	0.0	0.0
Crab body parts	3.3	27.4	12.9
Other crustaceans	13.0	19.0	46.1
Bivalves mollusks	2.8	3.5	0.0
Gastropod mollusks	9.7	1.5	15.7
Polychaetes	1.1	6.1	4.2
Foraminiferans	0.8	0.0	0.0
Macroalgal material	4.3	3.7	1.5
Vascular plant material	0.8	8.3	1.0
Highly digested material	32.3	30.2	16.8
Sand grains	0.4	0.3	1.8

Macroalgal beds were located in Rehoboth Bay, Delaware, USA. Marsh and tide flat habitats were located in nearby Delaware Bay. Explanation of selected gut-content categories: *Crab Body Parts* = items identified to the infraorder Brachyura; *Other Crustaceans* = items identified as harpacticoid copepods, palaemonid and crangonid shrimp, or simply to the subphylum Crustacea; *Highly Digested Material* = organic material unidentifiable to a taxonomic group.

tirely to gut-content analysis, which has an inherent bias in favor of material that is refractory to digestion. Nevertheless, the high proportion of amphipods in the guts of crabs from macroalgal beds is in striking opposition to the complete lack of this taxon in the gut contents of juveniles from the other two environments and strongly suggests a major difference in diet between crabs from macroalgal habitat and either of the other habitats.

Because of the very high abundance of amphipods in macroalgal beds in Rehoboth Bay (Timmons & Price 1996), it is reasonable to conclude that the amphipods found in crab stomachs analyzed in our study originated in the beds themselves. Moreover, the common taxa of amphipods found within these macroalgal beds (various species in the families Gammaridae, Amphitoidae, and Bateidae) graze directly on macroalgae, which constitute the main portion of their diets (Watling & Maurer 1972, Macko et al. 1983, Parker et al. 1993, Lotze & Worm 2000, Kamermans et al. 2002). Thus, it is likely that macroalgal production is an important component of the food web supporting juvenile blue crabs in estuarine systems like Rehoboth Bay.

This is in contradiction to results of earlier work with juvenile shrimp in mangrove nurseries, which has shown a link with primary production originating in benthic diatoms, rather than with production emanating from the mangroves (Stoner & Zimmerman 1988, Newell et al. 1995, Dittel et al. 1997). Likewise, previous work with juvenile blue crabs in salt marsh environments has demonstrated at least partial dependence on benthic diatom production and only indirect linkage to production by emergent marsh plants (Dittel et al. 2000). Investigations of sea grass systems have come to varying conclusions concerning the role of indigenous primary production in supporting growth of juvenile crabs (e.g., Fry & Parker 1979, Hughes & Sherr 1983), but a recent review finds little evidence that sea grass production *per se* is a major contributor (France 1996).

When considered as a whole, the results of our investigation provide credible evidence that macroalgae beds constitute critical nursery habitat for juvenile blue crabs in areas where seagrass beds are lacking. Moreover, the value of this habitat may include a direct trophic linkage between primary production originating in the macroalgae; this has not been demonstrated in other plant-based nursery habitats used by juvenile blue crabs.

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ISOLATION AND MOLECULAR CHARACTERIZATION OF VITELLIN FROM THE MATURE OVARIES OF THE PRAWN *LITOPENAEUS VANNAMEI*

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ABSTRACT Vitellins from ovaries in shrimp *Litopenaeus vannamei* were examined by polyacrylamide gel electrophoresis, sodium dodecyl sulfate polyacrylamide gel electrophoresis, crossed-immunoelectrophoresis, chromatography (Sepharose CL 2B and hydroxylapatite columns), and high-performance liquid chromatography. Using these methods, two forms of vitellin (Vt1 and Vt2) were observed in ovaries (oocyte 110 μm). The vitellins identified appear to be lipoglycoproteins. Similar vitellin polypeptide composition was observed in the two forms of vitellin, with molecular weights of approximately 60, 90, 95, 100, 140, and 160 kDa. Policlonal antibodies against the two forms of purified protein were prepared, and their specificity was demonstrated by radial immunoprecipitation and Western blotting analysis. The P1 and P2 peptides from N-terminal 100 kDa and 60 kDa polypeptides were highly similar to regions of proline 20 and glycine 635 residues of crustacean vitellogenins.

KEY WORDS: ovary, shrimp, vitellin, lipovitellin, vitellogenesis, *L. vannamei*

INTRODUCTION

Vitellin is the major yolk protein accumulated in developing oocytes of a female crustacean. Yolk protein is the source of nutrition for development of embryos and larvae. The vitellin from ovaries and vitellogenin from the hemolymph have been characterized for several species of penaeids (*Penaeus japonicus*, Vazquez Boucard et al. 1986; *P. monodon*, Quinitio et al. 1990; *P. semisulcatus*, Browdy et al. 1990; Tom et al. 1992 and Lubzens et al. 1997; *P. monodon*, Chen & Chen 1993; Chang et al. 1993 and Chang et al. 1994; *P. chinensis*, Chang & Jeng 1995; Chang et al. 1996).

In vertebrates and several invertebrates, vitellogenin transported into the blood or hemolymph is considered the precursor of vitellin. In the Crustacea, it is still uncertain whether vitellogenin is the precursor of vitellin, even though intraovarian synthesis has been demonstrated by Yano and Chinzei (1987), Rankin et al. (1989), Fainzilber et al. (1992), and Khayat et al. (1994). Recent molecular studies showed that specific vitellogenin mRNA was expressed in both the ovary follicle cells and the hepatopancreas parenchymal cells of penaeid shrimp *P. japonicus* (Tsutsui et al. 2000) and *Macrobrachium rosenbergii* (Soroka et al. 2000). Vitellin has been found in subepidermal adipose tissue of penaeids *P. japonicus* (Vazquez Boucard 1985), *P. longirostris* (Tom et al. 1987a), and *P. semisulcatus* (Fainzilber et al. 1992), but their role in active synthesis of these compounds is not confirmed. Fainzilbert et al. (1992) confirmed the double synthesis of vitellin, in the hepatopancreas and the ovary, but in different proportions depending on ovarian maturity. Khayat et al. (1994) suggested that ovarian vitellin and hepatopancreas vitellogenin are the products of one gene.

Litopenaeus vannamei is an important commercial species in Mexico and other countries. The failure of ovarian maturation is an obstacle for reproduction control. Accordingly, purification and characterization of vitellin from mature ovaries of *L. vannamei* were the objectives of this study.

Using this information, we will be able to undertake molecular studies in vitellogenin gene expression by several tissues of *Litopenaeus vannamei*. The complete primary structure of vitellogenin has been elucidated for several crustaceans. The vitellogenin amino acid sequences of *Marsupenaeus japonicus* (Tsutsui et al. 2000), *Metapenaeus ensis* (Tsang et al. 2003), *Penaeus semisulcatus* (GenBank accession number AY051318), *Cherax quadricarinatus* (Abdu et al. 2002), and *Macrobrachium rosenbergii* (Yang et al. 2002) share several conserved regions. These are more than 2,500 residues long, and vitellins are derived from each vitellogenin.

MATERIALS AND METHODS

Preparation of Ovarian Homogenate

Mature (110 μm oocyte) and immature female prawns (35 μm oocyte) were obtained from Acuacultura Mar, La Paz, B.C.S., Mexico. Ovaries were rinsed and homogenized in glassware at 4°C with 0.05 M Tris, 0.5 M NaCl, and 5 mM EDTA (pH 7.0). Protease inhibitor cocktail (Sigma P-2714) was added (0.005%) to the extraction buffer, just before use. The homogenate was centrifuged at 10,000 g for 15 min at 4 C (Beckman ultracentrifuge, Pasadena, CA). The supernatant was frozen at −70°C until analysis.

Electrophoresis

For identification of vitellins in the vitellogenic female, the ovary homogenates were separated by native PAGE on 6% polyacrylamide gel in TRIS-glycine buffer (pH 8.8). The vitellin fraction was characterized by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE: 7.5% polyacrylamide gel). A solution of 0.5 M TRIS-HCl (pH 6.8), 1% SDS, 1% 2-mercaptoethanol, 10% glycerol, and 0.05% bromophenol blue was used as dissociation buffer. Molecular masses of native proteins and dissociated subunit polypeptides were determined by comparison of the relative mobility of molecules to those of molecular mass markers. The molecular masses of polypeptides were determined by native PAGE (precast gel gradient polyacrylamide 4–20% Bio-Rad) with a kit containing midrange protein molecular mass standards: β–thyroglobulin (669 kDa), ferritin (440 kDa), catalase (232 kDa), lactate dehydrogenase (140 kDa), and albumin (67 kDa; Pharmacia Fine Chemical, Uppsala, Sweden). The molecular

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masses of standard proteins on SDS-PAGE were myosin (200 kDa), β-galactosidase (116 kDa), phosphorylase (97 kDa), serum albumin (66 kDa), and ovalbumin (45 kDa; Bio-Rad, Richmond, CA). The gel was stained with Coomassie brilliant blue R-250 and Silver Stain Plus kit (Bio-Rad) for proteins, Sudan black B for lipids, and periodic acid-Schiff's reagent for carbohydrates.

Preparation of Antisera

Rabbits were immunized with 6% page purified *L. vannamei* specific ovarian yolk polypeptides. Small gel portions were cut vertically from both extremes and stained with Coomassie brilliant blue R-250 to reveal the migration distance of proteins. These portions were placed next to the rest of the gel without stain at the same level, and the gel vitellin band was cut horizontally. The two proteins (50 µg) separated from the polyacrylamide gel were homogenized with NaCl (0.9%), emulsified with complete Freund's adjuvant, and injected at multiple sites on the backs of rabbits. Boosters of 120 µg of antigen emulsified with incomplete Freund's adjuvant were injected at intervals of two weeks.

Purification of Vitellin

Litopenaeus vannamei vitellin was purified according to Chang et al. (1996, 1993). The ovarian extracts were gel filtered in a Sepharose CL-2B column (Pharmacia Fine Chemicals, Uppsala, Sweden; 100 cm × 1.8 cm i.d.) equilibrated in 0.01 M TRIS buffer with 2 mM phenylmethylsulphonyl fluoride (pH 7.0), and eluted in the same buffer at flow rate 18 mL/h. Effluent was collected in 2.4-mL fractions, and the absorbance of each fraction was measured at 280 nm. Each concentrated peak (PM 10 membrane, Amicon, Danvers, MA) was analyzed by immunodiffusion precipitation and PAGE (5% gel). The vitellin peak was applied to a hydroxylapatite column (Bio-Rad, Richmond, CA, #732-0085) using a 0.01 M potassium phosphate buffer (PPB), pH 7.0, with 2 mM phenylmethylsulphonyl fluoride with stepwise gradients of 0.01 M, 0.10 M, 0.20 M, and 0.35 M. The flow rate was 18 mL/h and the fraction size was 2.4 mL. Immunoprecipitation and PAGE of concentrated peaks (PM 10 membrane, Amicon, Danvers, MA) were also analyzed. The concentrated vitellin peak was further separated by high-performance liquid chromatography (HPLC, Beckman Spherisorb) equilibrated with 0.2 M sodium sulfate in 0.1 M sodium phosphate pH 6.5. The flow rate was 1 mL/min.

Immunologic Procedures

Immunodiffusion precipitation proceeded according to Outchterlony (1948). Agar gel (1%, 2 mm thick) was prepared on a glass slide. Vitellin antisera and samples were put in separate wells 0.9 cm apart and put in a humid chamber (4°C) for 48 h. After washing (3 × 6 h) in 0.9% NaCl, the gel was stained with Coomassie blue R250.

For crossed immunoelectrophoresis analysis of vitellins in the vitellogenic female, the homogenates of ovaries were separated by agarose gel (1%) in 0.02 M veronal buffer (pH 8.6).The gel portion enclosing the antigen was cut and placed on a glass slide (6.5 × 10 × 0.1 cm). The slide was then covered with 6.5 ml of 1% agarose in veronal buffer and 1% anti-vitellin antibodies of *L. vannamei*. After 18 h of migration at 2 volts/cm, the slide was washed with 9% NaCl and colored with Coomassie blue.

The immunoreactivity of the subunits of vitellin with vitellin antisera was examined by Western blotting. After purification of vitellin in a hydroxylapatite column, effluent containing the fourth peak was analyzed by SDS-PAGE (7.5% polyacrylamide gel in

TRIS-glycine buffer, pH 7.2, 1% SDS). Proteins in the polyacrylamide gel were transferred to polyvinylidene diflouride (PVDF, Immobilon transfer membranes, Bio-Rad, Richmond, CA) with a mini transblot electrophoretic transfer cell (Bio-Rad #170-3930) using 25 mM TRIS, 192 mM glycine, 20% methanol buffer. Nitrocellulose paper was immersed in the following solutions: 5% blotting grade blocker (Bio-Rad # 170-6404) in TBS buffer (0.15 M NaCl, 10 mM, TRIS), antisera against vitellin (1/3000), and goat anti-rabbit IgG-alkaline phosphatase conjugate (1/3000). Color was developed using diaminobenzidine in TBS buffer.

N-Terminal Amino Acid Sequence

After purification, the vitellin was analyzed by SDS-PAGE: 7.5% polyacrylamide gel in Tris-glycine buffer, 1.5 M, thioglycolate 0.1 M (pH 7.2), 10% SDS. A solution of Tris-Hcl, 312.5 mM; Na2 EDTA, 10 mM (pH 6.9); 15% SDS; and 0.5 M sucrose was used as dissociation buffer at 37°C for 10 min. The proteins in the polyacrylamide gel were transferred to Sequi Blot PVDF (polyvinylidene diflouride) membrane (Bio-Rad, Richmond, CA) with a mini transblot electrophoretic transfer cell (Bio-Rad #170-3930) using a 25 mM TRIS, 192 M glycine, 20% methanol buffer. The membrane was stained for 5 min with PVDF Coomassie blue R-250, and destained for 10-15 min with PVDF destain solution. The 100 and 60 kDa bands were cut and N-terminal sequenced in a protein/peptide sequencer at the Molecular Medicine Laboratory Biotechnology Institute UNAM, Mexico (DF).

RESULTS

The native gel electrophoresis patterns of ovarian extracts (precast gel gradient polyacrylamide 4-20%) of mature and nonvitellogenic *L. vannamei* females showed a specific protein from mature females with an apparent molecular mass of 500 kDa. The protein contained carbohydrates and lipids, based on staining by Sudan black B (Fig. 1) and periodic acid Schiff's reagent, respectively. However, when the sample was centrifuged before electrophoresis, and the gel was stained with Sudan black B, we observed two female-specific lipoproteins of nearly the same molecular size (Fig. 1). The crossed immunoelectrophoretic pattern of the ovarian extract of mature *L. vannamei* females with antiserum against ovarian extract of the same species is shown in Fig. 2. There were two precipitation lines in the ovarian extracts of vitellogenic shrimp (Vt1 and Vt2).

Two proteins peaks in ovarian homogenate of mature females were obtained from gel filtration chromatography in a Sepharose CL-2B column (Fig. 3). The concentrated second peak showed a single band considered vitellin (500 kDa) and another more negatively charged non-vitellogenic band. The second peak from gel filtration chromatography had a specific immunodiffusion precipitation line that reacted with antisera against vitellin of *L. vannamei*, but the first peak did not (results not shown). The concentrated second peak was separated into four peak fractions, by hydroxylapatite column chromatography. Electrophoretic analysis revealed two proteins with very approximate migration coefficient (Fig. 4). The third (Vt1) and fourth (Vt2) peaks separated by hydroxylapatite column chromatography, the mature female hemolymph, and vitellogenic ovarian extracts were recognized by polyclonal anti-Vt antibodies raised against *L. vannamei* (Fig. 5). To confirm these results, the third and fourth peaks were combined, concentrated, and separated by reverse-phase chromatogra-
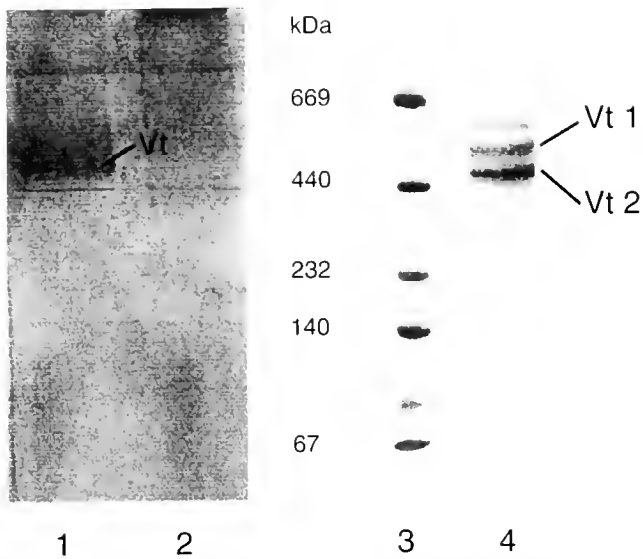
Figure 1. Native-PAGE of *Litopenaeus vannamei* ovaries. Precast gel gradient 4–20%. (B) Lane 1: silver stain ovarian extract mature females; Lane 2: silver stain ovarian extract nonvitellogenic females; Lane 3: molecular marker; Lane 4: Sudan black B stained ovarian extract mature females after centrifugation.

Figure 3. Elution profiles of ovarian homogenates from a Sepharose CL-2B gel filtration column equilibrated and eluted with 0.01 M Tris buffer. Flow rate: 18 mL/h. Fraction size: 2.4 ml. PAGE (5% gel). Blue Coomassie stain. Peak 1: High molecular weight proteins. Peak 2: Vitellin (Vt) and contaminate proteins.

phy (HPLC), revealing two peaks with retention times of 14.53 and 19.55 min (Fig. 6).

Characterization of Purified Vitellin

The third (Vt1) and fourth (Vt2) peaks from the hydroxylapatite column, analyzed by SDS-PAGE, both showed six polypeptide subunits. The molecular weights of the subunits were estimated at 60, 90, 95, 100, 140, and 160 kDa. To confirm the subunits corresponding to each vitellin, Western-blotting was conducted with the anti-Vt antibodies raised against *L. vannamei*, thus confirming that they were molecules composed of six similar polypeptide subunits (Fig. 7).

Protein Sequencing

We sequenced the N-terminal ends of the 100 kDa and 60 kDa subunits, and two amino acid residue sequences, P1 (GQVSLA-

PEFALGXTVE) and P2 (APXGADVPSKG) respectively, were obtained. P1 and P2 were aligned to each vitellogenin reported (Fig. 8), and similarity specific to two regions was observed. The conserved residues [20]P and [29]S aligned with P2, and the conserved residues [635]G and [639]E aligned with P1. This suggests that P1 and P2 are derived from a vitellogenin, as in *M. japonicus* (Tsutsui et al. 2000) and *Metapenaeus ensis* (Tsang et al. 2003).

DISCUSSION

One specific protein, with an approximate molecular weight of 500 kDa, was identified by electrophoresis (PAGE 6% and gradient gel 4–20%) in the ovaries of *Litopenaeus vannamei* females in vitellogenesis. The characteristics of this fraction (lipo-glycoprotein) were similar to those of penaeid vitellins, but did not exist

Figure 2. Crossed immunoelectrophoretic pattern of ovarian extract *L. vannamei* using vitellin specific polyclonal antibodies. (Vt1 and Vt2 vitellins)
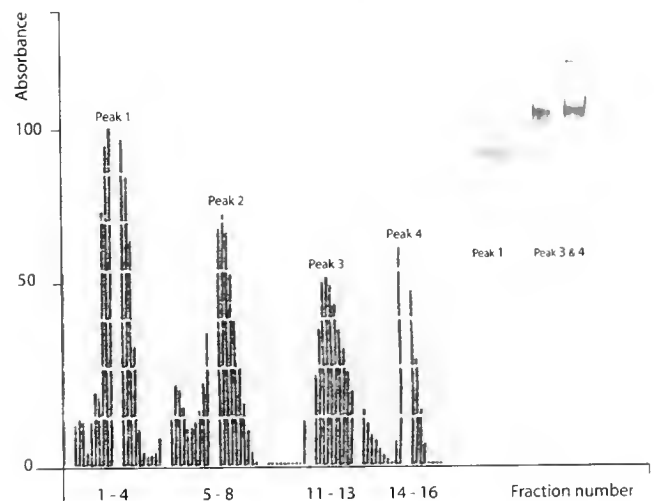
Figure 4. The second peak fraction from Sepharose column was fractioned in a hydroxylapatite column equilibrated with 0.01 M PPB buffer. Step-wise gradients of 0.01 M, 0.10M, 0.20M, and 0.35M PPB buffer. Flow rate: 18 mL/h. Fraction size: 2.4 ml. Peak 1 and 2: contaminate proteins. Peak 3 (Vt1) and 4 (Vt2).
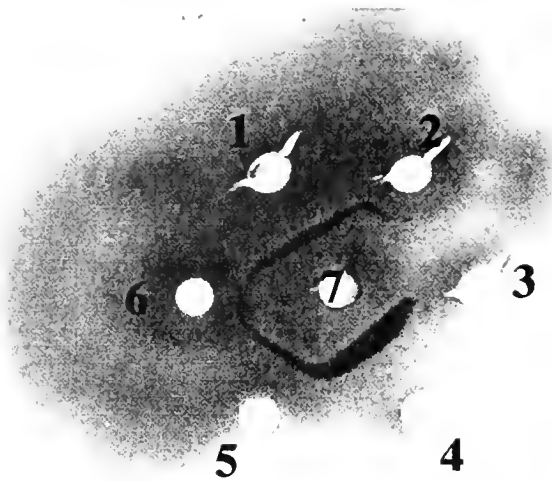
Figure 5. Immunoprecipitation of mature female ovary (1) mature female hemolymph (2, 6) hemolymph male (3) third peak hydroxylapatite column (4) fourth peak hydroxylapatite column (5) reacted to specific antiserum against vitellin (7) from *L. vannamei*.

in the ovaries of immature females. Only one form of vitellin has been detected in *Parapenaeus longirostris* (Tom et al. 1987b), *P. monodon* (Quinitio et al. 1990, Chang et al. 1993), *P. semisulcatus* (Tom et al. 1992, Lubsenz et al. 1997), *Metapenaeus ensis* (Qiu et al. 1997), *P. Japonicus* (Vazquez Boucard 1986, Kawazoe et al. 2000), and *P. vannamei* (Tom et al. 1992, Garcia Orozco et al. 2002).

However, when we centrifuged the mature female ovary sample before loading the electrophoretic gel (gradient 4/30%), and stained it with Sudan black B, we confirmed two lipoproteins.
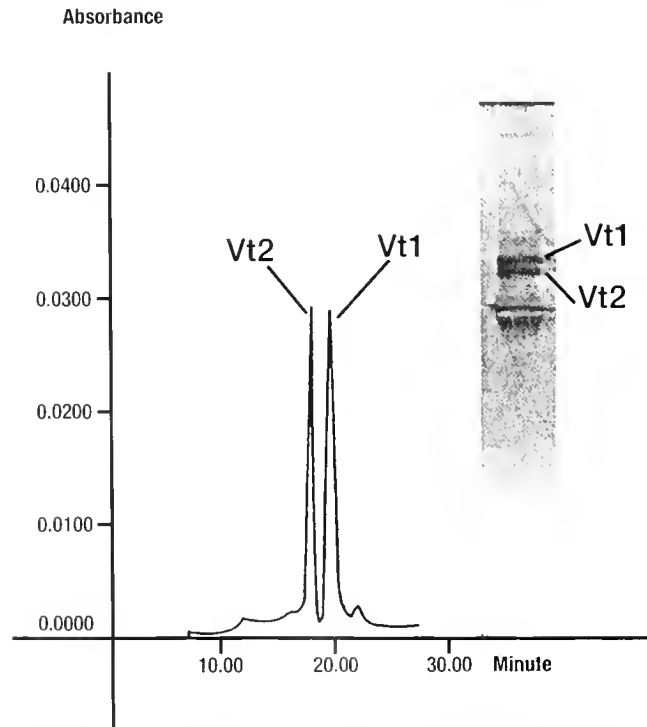
Figure 6. Analytical HPLC of combined third Vt1 and fourth Vt2 peak obtained from a hydroxylapatite column. Flow rate, absorbance at 280 nm.
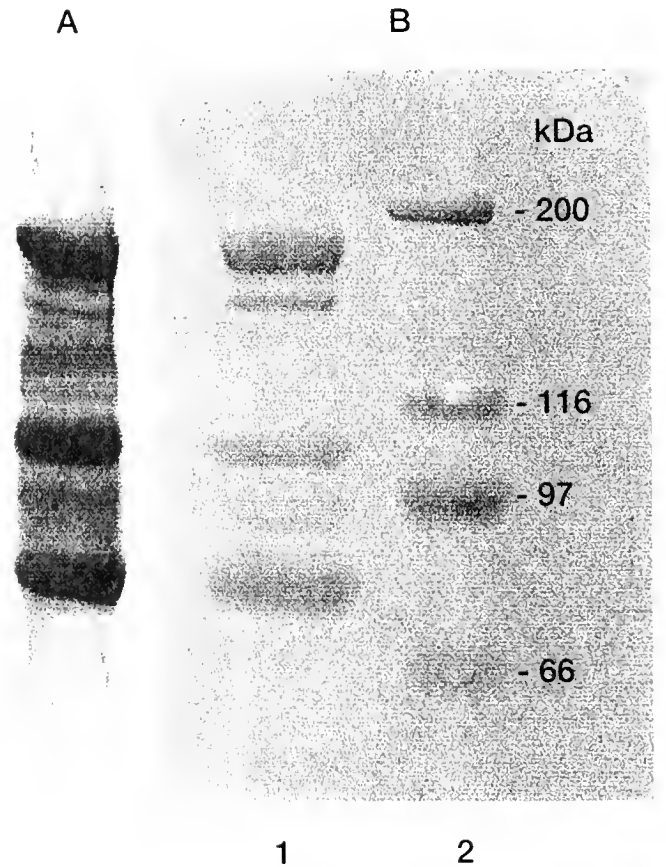
Figure 7. A, Western blotting analysis of vitellins purified by hydroxylapatite column (third and fourth peak); B, SDS-PAGE (7.5%) of (1) third and fourth peak of hydroxylapatite column, (2) molecular marker

Similarly, the crossed immunoelectrophoresis showed two lines of precipitation with *L. vannamei* anti-vitellin antibodies. After further separation by hydroxylapatite column and HPLC, two main vitellin peaks were seen also, which might correspond to the vitellins detected by native gradient gel electrophoresis and crossed-immunoelectrophoresis. Denatured SDS gel electrophoresis of the native vitellin isolated (Vt1 and Vt2) showed six subunits with
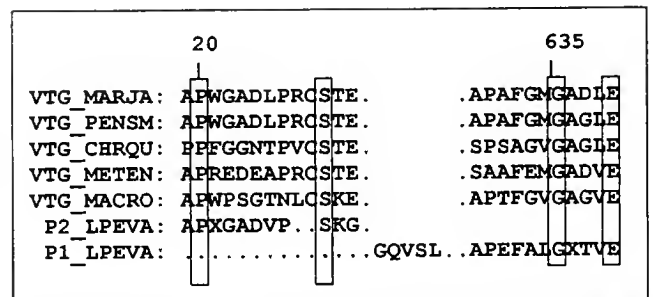
Figure 8. Alignment of crustacean vitellogenins. VTG_MARJA: *Marsupenaeus japonicus*, GB AB033719 (BAB01568) (Tsutsui et al., 2000); VTG_PENSM: *Penaeus semisulcatus* ,GB AY051318 (AAL12620); VTG_CHRQU: *Cherax quadricarinatus*, GB AF306784 (AAG17936) (Abdu et al., 2002); VTG_METEN: *Metapenaeus ensis*, GB AF548364 (AAN40701) (Tsang et al., 2003); VTG_MACRO: *Macrabrachium rosenbergii*, GB AB056458 (BAB69831) (Yang et al., 2000);. P1 and P2 LPEVA: peptides 1 and 2 of *Litopenaeus vannamei*

molecular weights of 60, 90, 95, 100, 140, and 160 kDa. The polypeptide subunits of each vitellin presented similar molecular masses. Chang et al. (1996), using our purification methods, detected two forms of native vitellin in *P. chinensis* also. The molecular masses they reported were 380 and 500 kDa. However, the authors miscalculated the molecular masses because they used native molecular weight markers designed for gradient gel electrophoresis in an 8% native PAGE gel, where proteins are separated by differences in charge, not by weight. Serrano Pinto et al. (2003) observed two forms of vitellin in ovaries and eggs at different stages of development in freshwater crayfish *Cherax quadricarinatus* with a similar vitellin polypeptide composition.

Electrophoresis, analytical ultracentrifugation, and chromatography have been used for the isolation and purification of vitellins of penaeid shrimp. The common goal was to increase vitellin purity, and prevent or retard its degradation. Vitellins are very susceptible to dissociation during and after isolation, so it is important to treat sample with protease inhibitors, and keep them at low temperature during extraction, purification, and storage to delay degradation. In this study, besides adding protease inhibitors to the tissue extracts and chromatography buffers, we purified and concentrated the vitellin under refrigeration (4°C).

Six major vitellin subunits of 60, 90, 95, 100, 140, and 160 kDa were observed in *L. vannamei* during the present study. Tom et al. (1992) separated ovary proteins in same species by filtration gel and ion exchange chromatography, and concluded that native vitellin is present in this species in one form with a molecular weight of 289 kDa. Only two subunits were detected in this study, and their molecular weights were not determined. Tom et al. used proteolytic inhibitors neither in the preparation of extracts, nor during separation of proteins by chromatography, and this could have caused protein hydrolysis. Garcia Orozco et al. (2002) detected one native vitellin in *L. vannamei* with an apparent molecular weight of 388 kDa. The method used by these authors to homogenize the tissues (30,000 rpm for 25 s, three times), and to concentrate the elutions separated by chromatography (ultrafiltration, PM 100 membrane, Amicon) could have caused degradation.

Using western blotting, we were able to identify the immunoreactivity of the six subunits using the antivitellin antibodies of *L. vannamei* ovaries. The P1 and P2 peptides from N-terminal 60 kDa and 100 kDa polypeptides were highly similar to regions of proline 20 and glycine 635 residues of crustacean vitellogenins. Both regions were highly conserved, which suggests specific processing sites to produce vitellin subunits. More molecular analysis is needed to corroborate this hypothesis.

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COVER PHOTO: Fouling of scallop (*Placopecten magellanicus*) cages in Bayport, Nova Scotia. Growth of scallops in those cages was reduced to 60% of normal. See paper by Carver and Mallet (p. 619). Photo courtesy of Carver and Mallett.

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